Root nodule bacteria and leguminous plants. 1932

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ROOT NODULE BACTERIA AND LEGUMINOUS PLANTS

BY

EDWIN BROUN FRED
IRA LAWRENCE BALDWIN
ELIZABETH McCOY

MADISON
1932

Dedicated to

WILLIAM HARMON WRIGHT

1885-1929

In appreciation of his teaching and research at the University of Wisconsin

"Consider a plant—its life—how a seed faln to ground
sucketh in moisture for its germinating cells,
and as it sucketh swelleth, til it burst its case
and thrusting its roots downward and spreading them wide
taketh tenure of the soil, and from ev'ry raindrop
on its dribbling passage to replenish the springs
plundereth the freighted salt, while it pricketh upright
with its flagstaff o'erhead for a place in the sun,
anon to disengage buds that in tender leaves
unfolding may inhale provender of the ambient air: . . ."

—Robert Bridges

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PREFACE

"Many investigations are lost for years, if not forever, in the jungle of journals and tangle of tongues."

—W. J. HUMPHREY.

The last half century of science has seen the curiosity of the botanist, the bacteriologist, the chemist, the agriculturalist, and the agronomist ever seeking to uncover the secrets of that vast family, the Leguminosae. The thousands of papers which have been written upon their habits and characteristics surely indicate that the Leguminosae are felt to be of prime significance; of significance, indeed, not only to the scientist but to those who feed the world and to those who are fed as well; for it is no exaggeration to say that a comprehension of the root nodule bacteria and their association with the Leguminosae would disclose to the world the basic principle of soil fertility. Surely an economic problem of such lasting importance invites research; it is likewise easy to see how workers have been led on in their study of the peculiar advantage which leguminous plants gain from their partnership with root nodule bacteria, by the tantalizing persistence with which the partnership keeps its secret of nitrogen fixation. There is a constant motive, too, for experimentation with symbiosis between forms of life as dissimilar as the leguminous plant and the bacteria living in its nodules, since the relationship is so delicately balanced that a slight change in environment may transform symbiosis into parasitism. For this reason, the French investigator, Fernbach, spoke aptly of the "plastic physiological properties" of organisms in symbiosis.

Fifty years of probing have brought forth reports in technical journals, bulletins, and whole volumes in English, German, French, Italian, Dutch, and the Scandinavian languages, to mention only the most common sources of material upon the Leguminosae. So much study in a comparatively narrow, though vital and perplexing field has often quite naturally " . . . resulted in unconsciously going over the ground already known and recorded by different investigators." (Buckout, 1889).

There have been three major attempts to review the more important papers on root nodule bacteria. In 1889 and 1890b, Frank prepared a paper with the title *Ueber die Pilzsymbiose der Leguminosen.* In a series of three papers, Mac Dougal, 1894, and Schneider, 1897 and 1903, presented an outline for a "fairly complete history of the study of leguminous root nodules and rhizobia." In 1911 Erwin F. Smith published a book, *Bacteria in Relation to Plant Diseases,* in which he reviewed comprehensively the studies which had appeared upon the root nodules of leguminous plants. Many scientific papers have appeared since then, treating in detail various phases of the root nodule problem; yet not one of them fully covers the subject.

Omission and repetition are not the only defects of the literature. The symbiotic relationship of the bacteria to the plant is by no means a simple one; it is to be expected that, in an effort really to master the problem, investigators should have published a variety of conflicting statements.

The need, then, for a complete review of the subject in the light of our present knowledge is clearly indicated. The authors of this book are eager to do all they can to fulfill that need in so far as they are able from the material available. Where investigators have differed in their conclusions, the authors have examined the conflicting reports and judged them according to current knowledge, realizing of course that these judgments will need to be reconsidered and perhaps modified as more is known of the subject. In many instances the opposing views are not supported by conclusive evidence and consequently the reviewers are not able to decide between the different hypotheses. Such instances have been presented with the comment that additional work is needed before an opinion can be formed.

The last two chapters of the monograph are in subject and treatment somewhat unlike the rest of the material presented. They are of interest primarily to the agronomist and the practical agriculturist and, in the case of Chapter 13, to those responsible for the production and testing of cultures. The latter group particularly is small, but of increasing importance. For convenience and ready reference the subject matter of Chapters 12 and 13 is made complete in itself, even at the expense of repetition in a few instances. The discussion of distribution and testing of cultures, which is of interest only to those directly concerned in the production and inspection of cultures, is set forth separately in a short appendix.

Although the material of *Root Nodule Bacteria and Leguminous Plants* has been assembled first of all as an aid to that branch of research in which its authors are engaged, it is hoped that the book may be useful to others with similar interests. A study of the nature and function of the microörganisms present in the Leguminosae is, in some measure, the work of the agronomist, botanist, plant physiologist, and chemist, and it is the coöperation of workers in all these fields that has made possible the diverse studies carried out at the University of Wisconsin.

The authors are greatly indebted to various workers in Soil Microbiology for the opportunity of discussing with them questions which occurred in the preparation of the text and for their many helpful suggestions. They are especially grateful for the criticism and assistance of W. A. Albrecht, Columbia, Missouri; of L. T. Leonard of the United States Department of Agriculture, Washington, D. C.; of H. G. Thornton, Rothamsted Experimental Station, Harpenden, England; of S. A. Waksman, New Brunswick, New Jersey; of J. K. Wilson, Ithaca, New York; and of O. N. Allen, Lois Almon, E. W. Hopkins, and P. W. Wilson, of the University of Wisconsin.

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CHAPTER 1

THE HISTORY OF THE LEGUMINOSAE IN AGRICULTURE

"The march of the human mind is slow."
—EDMUND BURKE.

Preliminary to consideration of the main topic of this book, it seems worth while to examine the place of the Leguminosae in agricultural history. This preliminary survey falls naturally into two parts: the Leguminosae in early agriculture and the scientific discoveries in the 19th century leading up to Hellriegel's important contribution in 1886a. The first part furnishes the setting in general agricultural practice. We marvel that leguminous crops should have been valued for their soil-improving powers so early in the history of man's cultivation of plants. The latter part of the chapter will illustrate how great discoveries are made by slow building and finally by ingenious fitting together of seemingly unrelated facts. We are too prone to give all credit to him who places the last piece in the puzzle and to forget that all his predecessors had prepared his way.

THE LEGUMINOSAE IN AGRICULTURE FROM ANCIENT TIMES UNTIL THE NINETEENTH CENTURY

We do not know when it was that man made his first crude attempts at raising plants for his own use. Certainly it was long before recorded history. It would be interesting to know how and why the first crops were planted—whether by accident some seeds were dropped, or whether man consciously imitated nature in order to raise more of what he wanted. We can only imagine how he must have given up his wandering life and settled down to tend his crop and protect it from his enemies, wild beasts and alien men. Let us hope that that first crop was a success. At any rate, he must have tried again and again until he came to appreciate the value of settled agriculture. It is significant that the regions from which we have the most ancient records of agricultural achievement are the regions where the first great civilizations arose. In these places the beginnings of agriculture must have long preceded recorded history, for the earliest remains tell of a variety of crops and of fairly complex systems of culture. Excepting China, about whose early agricultural history we know very little, there are three great regions to be considered: the Tigris-Euphrates valley in southwestern Asia, Egypt, and the Americas. Somewhat later than the Mediterranean and Mesopotamian developments, there is the case of central Europe to be judged from the remains of

the Swiss Lake Dwellers and finally from medieval history. At a still later date in New World history, there is also the agricultural situation from the time of Columbus through the colonial period of the United States.

From a climatic point of view there was little similarity in these regions. The temperatures varied from moderate to hot; the rainfall was so light in some places that the principles of irrigation were discovered and put to work in very early times. Fertility was not a vital problem as long as the land was new and the population of any region not great. River valleys were especially attractive to the early farmers because of their natural fertility. In some valleys, those of the Nile, Tigris, and Euphrates for example, yearly floods were instrumental in maintaining the fertility of the fields; and the permanent fertility, in turn, made possible the extensive agricultural development which had to accompany great civilizations in those regions. It was not until Roman times that farmers generally paid very serious attention to maintaining and improving the fertility of their land. It is said that one of the greatest contributions of Roman agriculture to civilization was the introduction of systematic crop rotations, in which leguminous crops played an important rôle.

Egyptian agriculture. In Egypt there is well-founded evidence that agriculture has been practiced for more than six thousand years (Hartmann, 1923). It is reasonably certain that at the time of the Old Kingdom, which began at an uncertain date in the 5th millenium B. C., Egyptian farmers were already well versed in their art. They kept domestic animals and raised a variety of crops with the help of irrigation. From the earliest historic times, the seeds of the Leguminosae have formed an important part of the food of the Egyptians and have entered into many of their religious observances. Beans, presumably *Vicia Faba,* were offered to the dead during the earliest dynasties. They were also placed in necropolises of the XII dynasty and in the Theban tombs (also XII dynasty). Ramses III had quantities of them stored in the temple of Amon. Lentils, *Ervum lens,* were also of ceremonial significance. According to ancient tradition, they were sacred to Harpocrates, the personification of silence, and so it is altogether fitting that they should be the food of the dead. The living also enjoyed lentils, for according to Herodotus, the builders of the pyramids of Giseh ate lentils; and Plato mentioned an old fable that the piles of stone chips in the fields near the royal tombs are in reality petrified lentils left over from the lunches of the workmen! The chick pea, *Cicer arietinum,* was known in Egypt, but its use was prohibited, as Diodorus said, perhaps to teach men to deprive themselves of something. A species of lupine, *Lupinus ternis,* and probably also the bitter vetch, *Lathyrus sativus,* originated in Egypt.

Agriculture in Southwestern Asia. Southwestern Asia was the second (or perhaps the first) great cradle of civilization. In many respects the land and climatic conditions were like those of the Nile valley, but the country was more exposed to attack from warlike neighbors, and consequently it passed through a series of political vicissitudes. It was controlled in succession by the Sumerians, Babylonians, Assyrians, Chaldeans, various Semitic tribes, and Iranians. The agriculture of the region evidently developed along the same lines as that in Egypt, though less is known from actual remains. There are today traces of the irrigation canals

used in very early times in Babylonia. It appears that the main crops were the grains, barley, wheat, and millet, but vetches, alfalfa, and peas were also raised. Somewhat later, in early Biblical times, leguminous crops for human food came into greater prominence, as indicated by the numerous references to them in our Bible. Two of the earliest of these may be mentioned. Jacob in 1773 B. C. (Usher's date) prepared the famous pottage of red lentils with which he treacherously bought his brother's birthright. On a military expedition in 1023 B. C. King David and his men carried provisions of beans (*Vicia Faba*), lentils (*Ervum lens*), and parched pulse (probably peas).

It is difficult to place chronologically the developments of agriculture in the centuries following those early civilizations in Egypt and Babylon; the Lake Dwellers of northern and central Europe surely deserve mention before the Greeks and Romans; and yet the agriculture of the Mediterranean region should be treated as a continuous development from its very beginning in prehistoric times to the present day. It is equally difficult to determine what was happening simultaneously in the various parts of the world. "Prehistoric times" in the Mediterranean region were distinctly earlier than "prehistoric times" in northern Europe and hundreds, even thousands of years earlier than in the New World. For these reasons, it is perhaps as well to consider the later agricultural history arbitrarily in the following order: Achievements of the European Lake Dwellers; of the Greeks and Romans; of the Europeans of the Middle Ages; of the Aborigines of Mexico, south-western United States, and Peru; of the North American Indians before the European colonists arrived; and of the Colonial Americans. No attempt will be made to give a complete picture of their agricultural status and degree of development. Rather there will be picked out, from such records as we have, references to their knowledge of leguminous crops. In some cases, it is certain that they understood the important part which the Leguminosae play in maintaining and improving soil fertility. Such cases will be emphasized as illustrating the practical background in farm practice which preceded scientific agriculture by thousands of years.

Agriculture among the Lake Dwellers. The Swiss Lake Dwellers of the late Stone and Bronze Ages have fortunately left us remains of kitchen refuse as a clue to their agricultural habits. It is believed that they raised barley, wheat, millet, and peas in very early times, and in the Bronze Age (about 2000-1800 B.C.) added oats and the dwarf field bean (*Faba vulgaris*) to their food resources.

Greek agriculture. The Greeks, though not essentially an agricultural people, have left a few agricultural references in the writings of their philosophers and poets. The philosopher, Theophrastus, 370-285 B.C., in his *Enquiry into Plants* spoke of the leguminous plants as "reinvigorating" the soil. He said, "Of the other leguminous plants the bean best reinvigorates the ground, even if it is sown thick and produces much fruit" and in another place "Beans . . . are not a burdensome crop to the ground; they even seem to manure it, because the plant is of loose growth and rots easily; wherefore the people of Macedonia and Thessaly turn over the ground when it is in flower." It is probable that the beans were commonly used as food by the Greeks, for the Athenians celebrated the Kyampsia or Feast of Beans in honor of Apollo. There was a special God of Beans, Kyanites,

whose temple stood on the road to Eleusis. It was thought by both Greeks and Romans that the beans had some connection with the mind or soul. Diogenes Laertius said, "Beans are the substance which contains the largest portion of that animated matter of which our souls are particles." (Hedrick, 1919).

Roman agriculture. The Roman writers, being more agriculturally inclined than the Greeks (several were farmers and wrote of their own experiences) gave us a great deal more insight into the agricultural practice of their time, and it is particularly from their several treatises on agriculture that we know of the beliefs and practices of Roman farmers (Harrison, 1913). They laid particular emphasis on the value of leguminous crops for green manuring.[1] Cato, the earliest of the great Roman agriculturists, has given us *De re rustica,* written in the second century B.C. In it he said, "Lupines, field beans, and vetches manure corn land," and again said, "Sow, for feed for cattle, clover, vetch, fenugreek, field beans, and pulse. Sow these crops a second and a third time." The lupine which Cato mentioned is probably *Lupinus albus,* the species also grown by the Greeks. There is a curious inconsistency in the derivation of the word, lupine. It is believed to come from *lupus,* the Latin word for wolf, and to indicate that at one time the plant was thought to devour the fertility of the soil in a wolfish manner. This misjudgment of the plant must have been corrected long before Cato's time, for he definitely listed it with other leguminous plants as a soil improver. Varro in his *Rerum rusticarum* of 37 B.C. went further than Cato in advocating the Leguminosae, and he said in substance, "Legumes should be planted in light soils not so much for their own crops as for the good they do to subsequent crops." In his writings of about 100 years after Varro, Columella mentioned the raising of alfalfa. Alfalfa, according to Pliny, was introduced into Italy from Greece and into Greece from central Asia during the Persian wars. Its very name, *Medica,* in Greek and Latin is a reminder of its origin in Media. In the time of Columella it was extensively grown in Italy. Columella said of it, "Of all the legumes, alfalfa is the best, because when once it is sown, it lasts ten years; because it can be mowed four times, or even six times a year; because it improves the soil." In the *Georgics* of Virgil, 30 B.C., there is further emphasis on the use of leguminous crops. Like Varro, Pliny, and Columella, Virgil extolled the value of crop rotation, particularly recommending in series fallow, grain, and leguminous crops. Thus he said, "After the harvest, let the fallow fields lie at rest in succeeding years (*alternia*) . . . Then where you have reaped the legume with shaking pod, the vetch and the lupine, sow your wheat or spelt (*far*)." In 79 A.D. Pliny argued that "Lupines enrich the soil of a field or vineyard as well as any manure," and he agreed with Theophrastus that "For this purpose the bean[2] ranks first among legumes."

Medieval agriculture. After the decline of Rome, the practice of crop rotation including leguminous crops was lost for a time, at least during the chaotic early

[1]We do not know where the practice of green manuring originated. Ts'i Min Yao Shu of Chia Szu Hsieh, a Chinese writer of the fifth century B. C., was familiar with it, for he wrote, "For manuring the field, lu tou (*Phaseolus mungo*) is best" (Pieters, 1927). The Greeks evidently practiced it also, according to Theophrastus' reference to the plowing under of beans in Thessaly and Macedonia. But it was the Romans who were finally convinced of the great value of green manuring, particularly with leguminous green manures.

[2]Probably refers to *Vicia Faba.*

Middle Ages. It is probable that the more primitive naked fallow treatment of land came back into general use in Europe. In the later Middle Ages, however, with the rise of towns there came more intensive agriculture and a return to the use of leguminous crops. Of course, there was never a time when such crops were unknown; vetches, alfalfa, and cowpeas are mentioned as food for cattle by several writers, and economic history shows that peas and beans entered into foreign and domestic commerce more and more as time went on. There is little literary record of medieval agriculture from which to judge of agricultural developments. The classical treatises were not entirely forgotten, as shown by quotation of them by Petrus Crescentius of Bologna (1230-1307) and by numerous translations during the succeeding centuries (Gras, 1925).

With this we may leave the agriculture of the Old World, for it changed very little until the rise of scientific agriculture in the 19th century. Its development at that time will be treated later in this chapter.

Indian and colonial agriculture in the New World. We are only beginning to appreciate the pre-Columbian agriculture of the New World and to realize that ancient America offers an unusually fertile field for the archeologist, ethnologist, and philologist. We are too apt to think that the Indians whom Columbus found were only savages. In reality Columbus was much impressed by the extensive fields of corn, sweet potatoes, mandioca, and peanuts which he saw in the West Indies. The Spanish and Portuguese explorers who followed him were also enthusiastic about the agriculture of the Indians, and it is largely through their reports that we know of the native American plants which the Indians had developed by cultivation and selection. The other, and perhaps more authentic, source of our information is that to be found in the actual remains of Indian civilizations (Spinden, 1917). These are now being worked over by anthropologists and archeologists in an effort to establish the origin of "native" Americans. To the best of our present knowledge, the ancestors of the American Indians came to the Americas at some very remote time and lived here in complete isolation until the coming of Columbus. Perhaps they came from Asia across an ancient land bridge between Siberia and Alaska. The best evidence for this great antiquity of human history in the Western hemisphere is the fact of wide distribution and variation among the peoples and among the native plants which they had developed for their use. Such a process of adaptation must have taken many centuries; indeed some ethnologists venture the opinion that the early civilizations in America were contemporary with the very earliest of the Egyptians and the Sumerians and Akkadians of Mesopotamia, and perhaps of the Chinese and Aryans of northern India. However that may be, we know that the great Maya civilization of Mexico, Yucatan, and Guatemala was well developed and making records and dates of its dynasties, written in stone, as early as 100 B.C. And the Megalithic Empire of the Pre-Inca people of the South American Andes began not later than 200 B.C. It was, of course, preceded by a half legendary period of immigration and was followed, after a period of decadence, by the great Inca Empire (Means, 1917; Morley, 1917).

All of this intensely interesting historical setting is but preliminary to the appreciation of Indian agriculture. Indians from the St. Lawrence and Great Lakes regions down to Chile and the Argentine practiced agriculture. Their methods were appropriate to the climatic variation of this territory, being very crude in

some regions and very much advanced in others. The most elaborate systems of cultivation were those of the Mayas and Pre-Incas, who cultivated miles of terraced hillsides and watered their crops by irrigation. The crops naturally varied from place to place. In the tropics, particularly, certain plants indigenous to the region were brought under cultivation. Certain leguminous plants are native to America. For example, the peanut (*Arachis hypogoea*) is now believed to have originated in Brazil, although it was formerly considered of African origin. Columbus found peanuts in the West Indies. They are supposed to have been cultivated since very early times on the mainland of South America, for they are common among the food offerings in ancient Peruvian graves (Safford, 1917). They are also represented in painted or molded decorations on the terra cotta funeral vases, some of which evidently date from Pre-Inca times. The many varieties of the bean, *Phaseolus vulgaris,* distinct from the *Faba* bean of the Old World, are also American. They, too, are found in the food offerings of the Peruvian graves. These beans include varieties both of *Ph. vulgaris* and of the lima bean, *Ph. lunatus*. The fact that several varieties of beans were in use when Columbus arrived and also that beans had become adapted to the temperate climate of the north and to the deserts and tropical conditions of the south are some of the strongest arguments for the great antiquity of Indian culture (Safford, 1926).

The North American Indians whom the European colonists found on the Atlantic coast were more or less skilled in agriculture. They, too, raised beans among their corn and, according to several early writings of the colonists, also peas. Whitbourne, speaking of Newfoundland in 1618, said, "There is great plentie of greene Pease and Fitches, faire, round, full and wholesome as our Fitches are in England." His "Pease" evidently referred to *Lathyrus* (probably *Lathyrus maritimus*), which grew wild along the coast of Labrador and Nova Scotia. Farther south "Pease" may have meant either small seeded beans or cowpeas. Hariot, writing of the first English Planting in Virginia, said in 1585, "Wickonzour, called by us Pease, in respect of the beans for distinction sake, because they are much less although in form they little differ: but in goodness of taste much like and far better than our English pease." Later botanists identified Wickonzour with the plant we now call the cowpea. It is not an American plant, apparently having been introduced into the West Indies from Africa by the slave traders and from the West Indies to the mainland of North America early in the colonial period (Carrier, 1923). Other leguminous crops, like the various clovers and alfalfa, were introduced from Europe in later colonial times (Erith, 1924; Nessler, 1931).

In New England, where the farmers were forced to consider the maintenance and improvement of the land, there is record that they very early took advantage of the soil-improving qualities of the Leguminosae. In 1801, Bordley, in his *Essays and Notes on Husbandry and Rural Affairs,* made this statement, "Clover plowed in, together with the remains of grain stubble, year after year will gradually meliorate the soil," and also, "Clover is the best preparative for a crop of wheat. . . . Wheat on clover has the best grain and the fullest crop." These statements are typical of several in the books on early American agriculture and may be taken to indicate the continued practical application of a principle for which agricultural science had as yet no explanation.

European agriculture in the nineteenth century. The agriculture of Europe by the time of our colonial period had progressed greatly. After the Middle Ages when the feudal system had been a drag on progress, more rational and economic management of the land came into practice. It would take too long to deal with each country separately. Suffice it to say that the foundations of present day methods had long since been laid, and that better methods of farming were constantly being introduced. There were two great agriculturists of the 19th century whose influence upon European agriculture was profound and whose work in connection with the culture of Leguminosae demands consideration here. We refer to Albrecht Thaer and Schultz-Lupitz.

Albrecht Thaer emphasized the raising of leguminous crops between cereal crops in order to obtain better yields of the latter. His advice must have had some weight, if one may judge from the following sentences in one of his books (Grundsätze der rationellen Landwirthschaft, English translation by Shaw and Johnson 1856): "Latterly the practice of sowing white clover with the last crop has become very general; only a few apathetic and indolent agriculturists, or men who are firmly wedded to old opinions and customs, neglect this practice," and also "Wheat sown on wheat stubble never succeeds . . . But if a crop of some of those plants belonging to the class *diadelphia,* as peas, vetches, beans, or clover, be interposed, then the second crop of cereal plants will turn out well." He further recommended rotation of crops such as the following, in which the Leguminosae actually predominate:

Beans sown in rows
Autumnal corn
Clover for mowing
Spring grains
Peas
Autumnal grains
Pasturage with white clover and grasses

Schultz-Lupitz also was a great advocate of leguminous crops. It was he who persuaded the German farmers to raise lupines extensively on the light, sandy soils of Germany and especially to treat the land by green manuring with lupines. He has been called the "father of modern green manuring in Germany." In his paper of 1881 he clearly stated that such plants as clovers, lupines, and peas are able to utilize nitrogen in some form other than that required by non-leguminous plants. He called the non-leguminous plants "Stickstofffresser" or nitrogen consumers, and the leguminous plants "Stickstoffsammler" or nitrogen accumulators.

SCIENTIFIC AGRICULTURE

For centuries after the farmers of the world were convinced of the value of leguminous crops, science could offer no explanation of their soil-improving qualities. It was not until great advances had been made and methods developed in pure science that the answer could be given. General botany and plant physiology, chemistry, and bacteriology were called upon to solve isolated problems. Only

then was it possible for a single series of experiments, such as Hellriegel performed, to settle the question for all time.

In the early years of the 19th century chemists and physiological botanists were still inquiring into the source or sources of nitrogen available to green plants. Such great scientists as Priestley, de Saussure, Ingen-Housz, Boussingault, Schloesing, Liebig, and Berthelot attacked this problem and arrived at various conclusions. Priestley, 1774-77; Ingen-Housz, 1779; and later Ville, 1885, maintained that plants did absorb free nitrogen from the air; de Saussure, 1804; Liebig, 1852; Mène, 1851; and Boussingault, 1886, emphatically denied it. Boussingault's work in this regard is classical. In 1838 he had found in pot experiments that peas and clover could apparently take nitrogen from the air, while wheat could not. His findings are summarized in the following table:

Plant cultivated	Duration of culture	Weight		Nitrogen		Gain or loss in N
		of seed	of crop	in seed	in crop	
	months	gm.	gm.	gm.	gm.	gm.
Clover	2	1.576	3.220	0.110	0.120	+0.010
	3	1.632	6.288	0.114	0.156	+0.042
Wheat	2	1.526	2.300	0.043	0.040	−0.003
	3	2.018	4.260	0.057	0.060	+0.003
Pea	3	1.211	4.990	0.047	0.100	+0.053

Boussingault himself did not fully appreciate the significance of the different behavior of leguminous and non-leguminous plants. He discounted his results of 1838 because the plants had been grown in open air, and, as he said, there was no way of knowing in what form the nitrogen had been acquired. "Provient-il du gaz azote ou de l'ammoniaque?" Therefore, in 1851 he performed experiments with plants grown in sterilized powdered pumice stone and in a known volume of air trapped in a glass chamber. Later in 1854 he arranged an apparatus for supplying his plants with a continuous flow of purified ammonia-free air. Under these rigorous conditions of culture his plants grew poorly, and he found no absorption of free nitrogen by either leguminous or non-leguminous plants. However, in a repetition of his pot experiments, he again found that leguminous plants in some way acquired more nitrogen than that in seed, soil, and water. Naturally, then, he concluded that the source of this nitrogen must be the ammonia of the air. Such a theory had support from Liebig, 1852; Sachs, 1860-61; Schloesing, 1874; and Mayer, 1874.

Meanwhile, evidence was accumulating that the Leguminosae differed from other plants in some fundamental respect. Lawes, Gilbert, and Pugh in 1861 had published an extensive series of experiments upon plants grown under glass in sterilized soil, supplied with pure air, and watered with pure water. Their non-leguminous plants developed very poorly and the leguminous plants little better. Neither was able to assimilate free nitrogen. But in their conclusion, Lawes and Gilbert cautiously observed that the Leguminosae under the artificial conditions of the experiment were abnormal, for under field conditions it was well known

that they obtained more nitrogen than could be accounted for in the combined nitrogen of soil, rain water, and air. Schultz-Lupitz in 1881 reached practically the same conclusions but had no explanation for the phenomenon. At about the same time Atwater in America was also pursuing a series of experiments on the nitrogen-gathering power of plants. By 1884 he was convinced that pea plants, at least, acquired more nitrogen than could be accounted for from the seed proteins and the nutrient solution of his experiment. The following year he repeated his experiments and reached the same conclusions. He had no explanation for his finding, admitting that such an action was "against the best evidence and opinion at the present time." Berthelot had recently, 1877, reported fixation of nitrogen by organic matter under the influence of static electricity. To Atwater this suggested that perhaps electricity had been the agent of fixation in his experiments. In 1886 he was still at a loss to explain his data, saying, "I am aware of no observed facts to imply that these, separately or together, are able to fix free nitrogen by aid of electricity, microörganisms, or any other means. In the present state of our knowledge, therefore, the balance of probability seems decidedly to favor the assumption that the plants themselves must be factors in the acquisition of atmospheric nitrogen."

His reference to microörganisms as the possible agents of nitrogen fixation was a direct outcome of Berthelot's report of the year before on the direct fixation of atmospheric nitrogen by clay soils. This finding of Berthelot's in 1885 was evidently the first suggestion that bacteria might be concerned in this phase of the nitrogen cycle, even as they had been implicated in the nitrification process by the researches of Schloesing and Müntz,[3] 1877a and b, 1878 and by Warington,[4] 1878, 1879, 1884.

It had been known for some years that the nodules on the roots of leguminous plants contained bodies whose nature was in dispute. Lachmann in 1858 first recognized them and called them "vibro-like," and Woronin, 1866 and 1867, spoke of them as "bacteria-like." But Eriksson, 1873; Frank in a series of studies beginning in 1879; Ward, 1887; and others were of the opinion that the organisms were some sort of fungi.

Such was the status of the problem when Hellriegel reported his great discovery to the Versammlung Deutscher Naturforscher und Aerzte in Berlin on September 20, 1886. The original paper of Hellriegel is entitled "Welche Stickstoffquellen stehen der Pflanze zu Gebote?" and is published in the Tageblatt of the meeting (Hellriegel, 1886a) and in the Landwirthschaftlichen Versuchs-Stationen (Hellriegel, 1887b). Because of its great historical significance, the text of Hellriegel's preliminary report is reprinted herewith:

> Die Gramineen sind mit Bezug auf ihre Stickstoffnährung auf den Boden allein angewiesen. Die einzige Form, in der sie den Stickstoff aufnehmen, ist die der salpetersauren Salze. In dieser Form ist der Stickstoff für die

[3]Schloesing, T., et Müntz, A. Sur la nitrification par les ferments organisés, Compt. Rend. Acad. Sci. (Paris), **84**: 301-303, 1877a.

Schloesing, T., et Müntz, A. Sur la nitrification par les ferments organisés. Compt. Rend. Acad. Sci. (Paris), **85**: 1018-1020, 1877b.

Schloesing, T., et Müntz, A. Recherches sur la nitrification par les ferments organisés. Compt. Rend. Acad. Sci. (Paris), **86**: 892-895, 1878.

[4]Warington, R. On nitrification, Pt. I, II, and III Jour. Chem. Soc. (London), **33**: 44-51, 1878; **35**: 429-456, 1879; **45**: 637-672, 1884.

Gramineen direkt assimilirbar und seine Wirkung quantitativ, d.h. die Produktion steht immer in geradem Verhältnisse zur gegebenen Menge Saltpeterstickstoff. Die Cruciferen, Chenopodiaceen und Polygoneen verhalten sich den Gramineen gleich (näher geprüft der weisse Senf, Rübsam, Zuckerrüben und gemeiner Buchweizen). Die Papilionaceen sind mit dem Bezug der Stickstoffnährung nicht auf den Boden angewiesen. Die Stickstoffquellen, welche die Atmosphäre bietet, können allein schon genügen, dieselben zu einer normalen, ja üppigen Entwicklung zu bringen. Es sind nicht die in der Luft vorhandenen geringen Mengen gebundenen Stickstoffs, welche die Ernährung der Papilionaceen bewirken, sondern der elementare Stickstoff der Atmosphäre tritt hierbei in Mitwirkung; und zwar stehen mit der Assimilation desselben die sogenannten Leguminosenknöllchen in direkter Beziehung. Leguminosenknöllchen und Wachsthum der Papilionaceen in stickstofffreiem Boden lassen sich willkürlich hervorrufen durch Zusatz von geringen Mengen Kulturboden und verhindern durch Ausschluss von Mikroorganismen. Bei verschiedenen Papilionaceenarten wirkt nur der Zusatz von gewissen Bodenarten Knöllchen bildend und Wachsthum fördernd. Salpetersauer Salze werden zwar auch von den Papilionaceen assimilirt, ob aber eine ganz normale Entwickelung der Pflanzen allein mit Hilfe derselben möglich ist, erscheint noch fraglich. (Diese Sätze werden durch Vorlage von Zahlen und Beweispflanzen erläutert, welche Missverständnisse, die aus den kurzen Sätzen entstehen könnten, vermeiden, leider aber des geringen gebotenen Raumes wegen nicht hier Platz finden können.)

A more extensive paper appeared in 1886 in the Zeitschrift des Vereins für Rübenzucker Industrie des deutschen Reichs (Hellriegel, 1886b). It is the text of a paper presented to the Sitzung des Anhaltischen Zweigvereins on October 13th. It bears the same title as the preliminary paper of September 20th, yet the abstract of it in Centralblatt für Bakteriologie und Parasitenkunde, 1887a, has an erroneous title which has been quoted repeatedly. Apparently the abstractor, E. Wollny, added "Ueber die Beziehungen der Bacterien zu der Stickstoffernährung der Leguminosen," to the original title of Hellriegel, "Welche Stickstoffquellen stehen der Pflanze zu Gebote?"

Hellriegel continued his researches in the years following 1886 and with Wilfarth published an extensive report in 1888. This is the classical paper, proving beyond doubt that the root nodules are responsible for the peculiar ability of leguminous plants to use atmospheric nitrogen, and that leguminous plants differ in this respect from gramineous plants. An example of the Hellriegel and Wilfarth data is set out in the accompanying results:

Average results

No.	Nitrogen in calcium nitrate per pot	Oats Average weight of grain and straw	Peas Average weight of seed and vines
	gm.	gm.	gm.
1	none	0.390	4.380
2	0.056	5.876	4.128
3	0.112	10.961	9.132
4	0.168	15.997	-----
5	0.224	21.357	9.725
6	0.336	30.175	11.352

This particular instance clearly shows, on the one hand, the dependence of the oat upon soil nitrate and, on the other, the independence of the pea. The conclusions from this paper are quoted:

1. Die Leguminosen verhalten sich bezüglich der Aufnahme ihrer Stickstoffnahrung von den Gramineen principiell verschieden.

2. Die Gramineen sind mit ihrem Stickstoffbedarf einzig und allein auf die im Boden vorhandenen assimilirbaren Stickstoffverbindungen angewiesen und ihre Entwicklung steht immer zu dem disponiblen Stickstoffvorrathe des Bodens in directem Verhältnisse.

3. Den Leguminosen steht ausser dem Boden-Stickstoff noch eine zweite Quelle zur Verfügung, aus welcher sie ihren Stickstoffbedarf in ausgiebigster Weise zu decken resp., soweit ihnen die erste Quelle nicht genügt, zu ergänzen vermögen.

4. Diese zweite Quelle bietet der freie, elementare Stickstoff der Atmosphäre.

5. Die Leguminosen haben nicht an sich die Fähigkeit, den freien Stickstoff der Luft zu assimiliren, sondern es ist hierzu die Betheiligung von lebensthätigen Mikroorganismen im Boden unbedingt erforderlich.

6. Um den Leguminosen den freien Stickstoff für Ernährungszwecke dienstbar zu machen, genügt nicht die blosse Gegenwart beliebiger niederer Organismen im Boden, sondern ist es nöthig, dass gewisse Arten der letzteren mit den ersteren in ein symbiotisches Verhältniss treten.

7. Die Wurzelknöllchen der Leguminosen sind nicht als blosse Reservespeicher für Eiweissstoffe zu betrachten, sondern stehen mit der Assimilation des freien Stickstoffs in einem ursächlichen Zusammenhange.[5]

Gilbert of Rothamsted was chairman of the meeting at which Hellriegel gave his preliminary report in 1886. He returned to England and there confirmed Hellriegel; Lawes and Gilbert then published their results in 1889 and 1891. Atwater and Woods, 1891 a and b, 1892; and Woods, 1891, in America; Schloesing and Laurent, 1890, and 1892 a and b, in France; and Alpe and Menozzi, 1892, in Italy also contributed confirmatory evidence.

[5]The behavior of the Leguminosae is fundamentally different from the Gramineae in regard to the absorption of their nitrogenous nutrition.

The Gramineae are solely dependent on the assimilable nitrogenous compounds present in the soil, and their development always stands in direct relation to the nitrogenous supply available in the soil.

To the Leguminosae a second source is available besides the nitrogen of the soil, from which they are able in a highly efficient manner to cover their needs entirely or in part, in case the first source is insufficient.

The free elemental nitrogen of the air is this second source.

The Leguminosae do not themselves possess the ability to assimilate the free nitrogen of the air, but the active participation of living microörganisms in the soil is absolutely necessary.

In order to make the free nitrogen serviceable to the Leguminosae for the purpose of nourishment, the mere presence of lower organisms in the soil is not sufficient, but it is necessary that certain kinds of the latter enter into a symbiotic relation with the former.

The root nodules of the Leguminosae are not to be considered merely as reserve storehouses for albuminous material, but stand in a causal relation to the assimilation of free nitrogen.

CHAPTER 2

GENERAL DESCRIPTION OF THE LEGUMINOSAE, THEIR DISTRIBUTION AND IMPORTANCE

"The tree is known by his fruit."
—MATTHEW 12:33

CHARACTERISTICS OF THE LEGUMINOSAE

Before discussing the characteristics of this great group of plants, it seems necessary to explain the term "leguminous plant" as it will be used in this book. In the classical Latin dictionaries of Harper, White and Riddle, Skeats, and Lewis, "legumen" is given the unqualified meaning of "any leguminous plant." This is the sense in which Varro, Pliny, Columella, Cicero, Caesar, and Virgil used the word in their writings. It is derived from the verb "legere," to gather, and is construed to mean plants gathered with the hands and not cut in harvesting. In medieval Latin the original meaning was preserved according to *Dictionarium seu Latinae linguae Thesaurus* (Cum Gallica ferè interpretatione) Paris, 1531, "Legumen" is that which is gathered. The popular term "legume" for the entire plant or loosely for the crop is thus sanctioned by custom and also by original meaning. Botanists, however, have used "legume" in a more restricted sense as meaning the characteristic fruit of the Leguminosae. This meaning is now established, and although it is a departure from the Latin derivation, we prefer to respect its application to the pods and to use "leguminous plants" or "leguminous crops" when speaking of the plants themselves.

Botanical description. The Leguminosae constitute one of the major families of the plant kingdom. They are, of course, members of the Spermatophyta or Seed Plants and more specifically of *Subdivision* II, the Angiospermae, and *Class* II, the Dicotyledoneae. The primary family characteristics are briefly as follows:

The flower of the Leguminosae is typically papilionaceous, that is, resembling a butterfly. It consists of an upper large petal called the banner, vexillum, or standard, which in the bud is folded over the other petals; two lateral petals, called the wings; and two ventral petals, called the keel. Usually the keel-forming petals are united and so enclose the stamens and pistil. The stamens, usually 10 in number, may be monadelphous or diadelphous. They are typically united by their filaments into a tubular sheath surrounding the pistil. The diadelphous forms have usually 9 stamens so united and one free—usually the upper. Because of this sheath around the pistil, and because the keel encloses both stamens and pistil, the pollination of the flowers is largely dependent upon insects. The papilion-

aceous flower is produced by the great majority of the Leguminosae, that is, by Subfamily III, the Papilionoideae. Subfamilies I and II, the Mimosoideae and Caesalpinioideae, bear respectively small, regular, five-petaled flowers and irregular papilionaceous flowers, differing from the true papilionaceous flower in arrangement of petals in the bud and in other minor details.

The leaves of the Mimosoideae and Caesalpinioideae are simply or doubly pinnate and of the Papilionoideae usually simple or simply compound, and are usually arranged alternately upon the stem.

The seed pod is as characteristic of the family as any single feature. It is essentially a single free pistil, which in the fruit develops into a pod or legume, dehiscent by one or two sutures into two valves. Rarely the pod is indehiscent, constricted between seeds, or jointed.

Chemical composition. The secret of the preëminence of leguminous plants in respect to food value lies in their chemical composition. The seeds of the Leguminosae, as compared with those of cereal grains, for example, are rich in reserve proteins. It is significant that they are able to store up nearly the same protein reserve in spite of varying conditions of culture. Iwanoff, 1927, has reported a stable protein level in the seeds of the pea, lentil, vetch, and horse bean produced under widely different conditions of soil, moisture, and climate. The proteins of the leguminous seeds are principally globulins, insoluble in pure water, but readily soluble in neutral salt solution. They are variously named legumin (as first reported in peas), phaseolin, vignin, arachin, glycin, etc., according to the generic names of the plants in which they occur. Like most vegetable proteins, the legumins are not of first grade in biological or nutritive value, because they are deficient or lacking in certain of the essential amino acids. The proteins of the lima, navy, and adsuki beans, and of the cowpea, field pea, and lentil, for example, are deficient in cystine (Jones, Gersdorff, Johns, and Finks, 1922; Johns and Finks, 1920; Jones, Finks, and Gersdorff, 1922; Finks, Jones, and Johns, 1922; and Jones and Murphy, 1924). Like other vegetable proteins, the legumins tend to be high in glutamic acid, arginine, and ammonia nitrogen.

The fact that the legumins are not complete in all amino acids is no disgrace to them. Few proteins are complete in themselves; but they are valuable in the diet for all that, as any ordinary mixed diet necessarily contains many proteins which are mutually supplementary. This being so, it is economically advisable to use leguminous food products generously in the human diet, because they are considerably less expensive than are the animal protein foods. For animal feeding, of course, leguminous hays are highly prized as sources of nitrogen, as pointed out by Henry and Morrison, 1927, and Leppan, 1924. The paper of Davies, 1926, should be consulted for detailed analyses of the proteins of some leguminous forage crops.

The Leguminosae furnish valuable mineral constituents also. Newton in 1923 compared the mineral matter of leguminous and non-leguminous plant parts and found some remarkable differences even between plants grown side by side. His results with pea and barley tops from plants grown in adobe soil in California are illustrative.

	Ca	K	Mg	P
	per cent	per cent	per cent	per cent
Barley tops	0.67	5.37	0.66	0.29
Pea tops	1.57	5.93	1.03	0.37

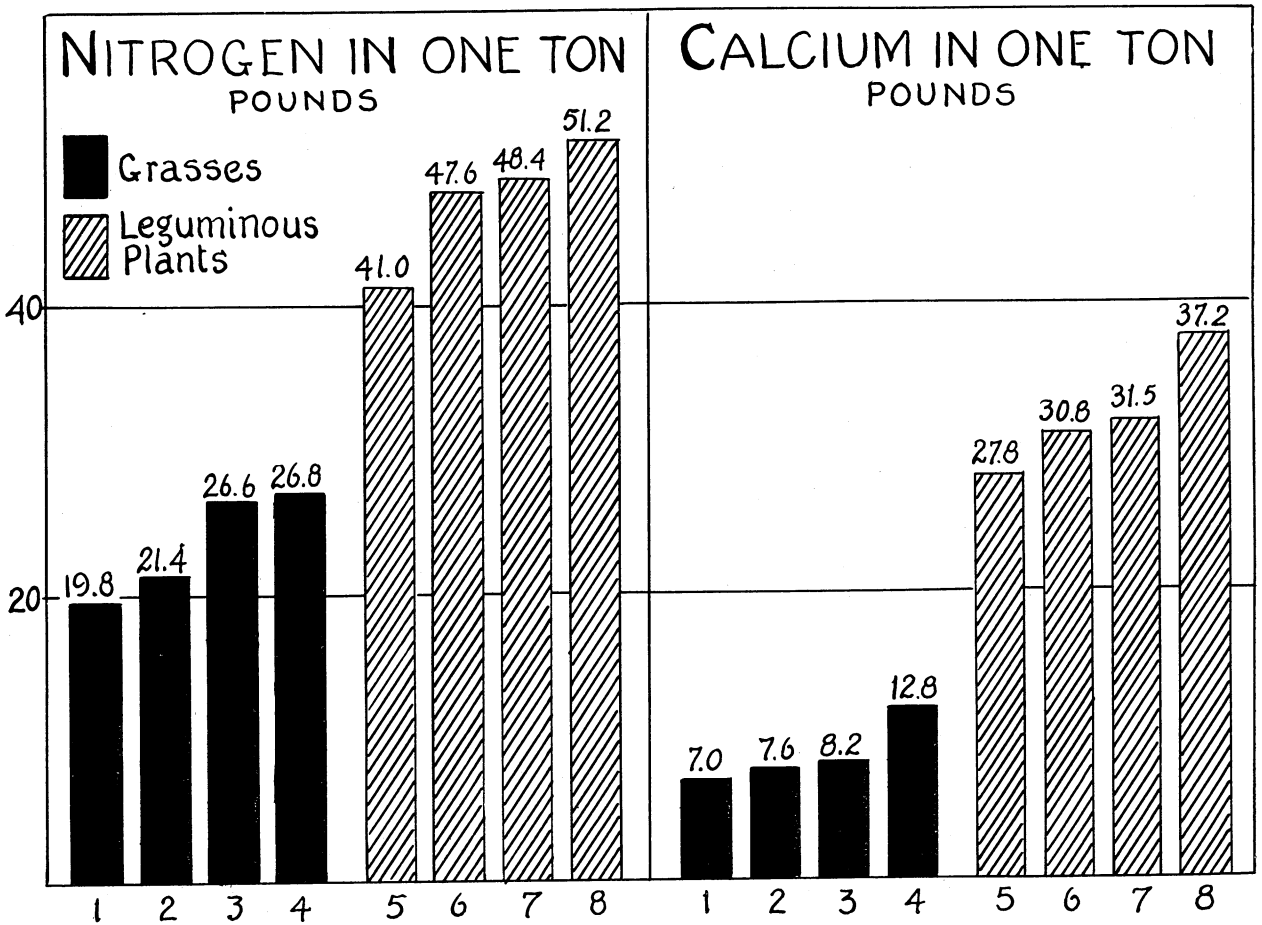
It is clear that the pea tops are distinctly superior in calcium and magnesium content and slightly higher also in potassium and phosphorus. The difference in potassium is only slightly in favor of the leguminous plant, and yet a recent paper by Weigert and Hiltner, 1930, gives evidence of marked difference in the potassium requirements of leguminous plants and grasses. According to their data, potassium may be the limiting factor in the growth of a leguminous crop. An example of their data will prove this point.

Air-dry crop yield

	No fertilizer	Fertilizer			
		without P	without N	without K	with NKP
Grass	100	146.3	115.2	185.8	214.0
Leguminous crop	100	310.5	324.8	**127.9**	353.1

The high calcium value of the leguminous plant as reported by Newton is apparent in many analyses. Calcium is especially significant in nutrition and in part accounts for the good results of feeding leguminous products to stock. In Chart 1 this high lime value of several leguminous hays is graphically contrasted with the low lime value of some common grass hays and corn fodder. The same graph compares the nitrogen content of these representative leguminous and non-leguminous cattle feeds.

Iodine is another mineral element recently reported as occurring in significant amounts in the Leguminosae. The work is by Mitchell, 1929, of the South Carolina Experiment Station. The location of the station on the Atlantic seaboard is a factor to be remembered, for it is well known that plants raised in regions of iodine-bearing soil and water are distinctly higher in iodine content than similar plants grown in iodine-deficient soils. The figures given by Mitchell are none the less of comparative value and indicate a remarkable iodine absorption by the Leguminosae. A few of his data are given, with the iodine values expressed as parts per billion. It is evident that certain of the Leguminosae are extremely active in iodine absorption; others, like the cowpea, lespedeza, and alfalfa, show no special aptitude in this respect.


NITROGEN IN ONE TON
POUNDS
Grasses
Leguminous Plants
40
20
19.8
21.4
26.6
26.8
41.0
47.6
48.4
51.2
1 2 3 4 5 6 7 8
CALCIUM IN ONE TON
POUNDS
7.0
7.6
8.2
12.8
27.8
30.8
31.5
37.2
1 2 3 4 5 6 7 8

Iodine in plants

Leguminous		Non-leguminous	
Plant	Iodine P.P.B.	Plant	Iodine P.P.B.
Cowpea	162	Oats	219
Soybean	224	Grass hay	150
Vetch	418	Bermuda grass	100
Alfalfa	170	Paspalum	150
Lespedeza	150	Johnson grass	350
Winter field pea	880		
Clover	380		

The carbohydrates of leguminous plants are not particularly noteworthy. In kind they are lignin, celluloses, hemicelluloses, pentosans, starches, and various free sugars as commonly found in plants. The actual amounts of these carbohydrates in the seed, for instance, vary according to the kind of plant and the stage of its development. In the seed, the carbohydrate of the tissue is necessarily somewhat reduced on account of the enhanced protein storage. The fat content of the leguminous seed is not particularly high except in the case of the soybean and peanut. The oil content in the former varies from 13.21 to 19.91 per cent, according to analyses by Leith, 1924. It usually ranges between 16 and 18 per cent.

Important physiological characteristics. There are one or two important features in the physiological processes of the leguminous plants which set them apart from all other plants. Newton in 1923 reported that pea plants in sand culture give off much more CO_2 than do barley plants of comparable age. For example, in 90 hours the CO_2 excreted by the roots of the barley was sufficient to neutralize an average of 46.9 cc. of 0.02N $Ba(OH)_2$, whereas the value for the pea plant was 88.8 cc. Reinau in 1927 carried the investigation of CO_2 output one step further. He discovered that the presence of nodules on the roots of the Leguminosae greatly increases the amount of CO_2 which they contribute to the soil atmosphere. The actual increase in CO_2 according to Reinau's experiments amounted to from one-third to two-fifths of the total carbon dioxide evolution of the soil.

According to Kostytschew, 1922, the Leguminosae are extremely active in CO_2 assimilation, the rate being from two to three times that of the other plants with which he compared them. This difference is of fundamental importance, inasmuch as photosynthetic activity must be governed by the energy requirement of the plant, plus that of the bacteria in the nodules. Further work should be done upon this point as a means of studying the energy relationships in the process of nitrogen fixation. If the product of this excessive carbon-dioxide assimilation be starch, it might be supposed that large amounts of diastase would be present to accomplish its digestion. The results of Brown and Morris, 1893, some of which are cited below, are in support of this supposition. They record the amount of

maltose which 10 gm. of air-dried leaf powder produce from the hydrolysis of soluble starch in 48 hours at 30° C.

Pisum sativum ---------- 240.30 gm. of maltose
Phaseolus multiflorus ---------- 110.49 gm. of maltose
Trifolium pratense ---------- 89.66 gm. of maltose
Vicia hirsuta ---------- 53.23 gm. of maltose
Lotus corniculatus ---------- 19.48 gm. of maltose

The value for the leaves of the non-leguminous species studied, chosen at random, ranged from 2.01 gm. for *Humulus lupulus* to 9.64 gm. for one of the determinations on *Tropaeolum majus.*

A particularly interesting line of work on the nitrogen nutrition of leguminous plants is being developed by Ziegenspeck, 1922; Rippel and Ludwig, 1926; and Storck, 1930. Assuming that the nitrogen of a growing plant adequately supplied with nitrate-nitrogen is taken up entirely as nitrate, the above investigators consider that the nitrate is absorbed in combination with bases. From the amount of nitrogen present and the amount of base present compared with the amount expected according to the combining power of the total nitrogen as nitrate, a nitrogen-base ratio is derived. This value has been determined for a number of leguminous and non-leguminous plants, and it has in general been found that leguminous plants tend to a base-excess, whereas non-leguminous plants as they approach maturity show a diminishing surplus of base. Nodulated leguminous plants, on the contrary, show a large nitrogen-excess.

In 1907 Lemmermann published an elaborate comparison of leguminous and non-leguminous plants, in which he maintained that the roots of the Leguminosae are significantly more acid in reaction. For example,

Acidity of 100 gm. of root tissue (cc. 0.1 N acid)

Graminae		Leguminosae	
Rye	16.3	Bean	18.9
Oats	12.2	Red clover	20.0
Timothy	8.6	Vetch	31.4
Buckwheat	9.9	White clover	24.9

This root acidity together with the habit of deep rooting renders the Leguminosae peculiarly able to absorb mineral nutrients from the soil. Lemmermann further suggested that mycorrhiza often found with the Leguminosae also aid in absorption.

DISTRIBUTION AND IMPORTANCE OF THE LEGUMINOSAE

Fossil Leguminosae. The Leguminosae, now so numerous upon the earth, have an ancient lineage. There are fossil records of them back through the Quaternary Era (Recent and Pleistocene), through all four periods of the Tertiary Era (Pliocene, Miocene, Oligocene, and Eocene), and even into the Cretaceous or last division of the Secondary or Mesozoic Era. These periods are estimated to have

covered 95-120 millions of years. In terms of animal history, they represent the entire Age of Mammals and a part of the Age of Reptiles. In terms of plant history, they cover the time that Angiospermae (the Leguminosae are a family of the Angiospermae) have lived upon the earth. In fact, Spermatophyta, flowering plants in general, are said to have appeared only in the Lower Cretaceous period. Evidently, then, the Leguminosae are among the oldest of the so-called higher plant families.

Two remarkable facts stand out in the fossil record of the Leguminosae. We refer first to the wide distribution of fossils even in the early periods. For instance, fossils of *Cassia* (senna) reputed to date from Cretaceous and Tertiary periods have been found in Bohemia, Greenland, France, Germany, Switzerland, valleys of the Andes of South America, and North America (MacMillan, 1892). Such wide distribution in early times foreshadows the world-wide distribution of today. In the second place, a great many types are represented among the fossils. Sargent, 1892, and MacMillan, 1892, alone list fossils of 11 certain and 3 doubtful genera. There are fossils of leguminous trees like *Robinia, Acacia, Gymnocladus,* and *Gleditsia,* and there are those of herbaceous Leguminosae like *Phaseolus, Lathyrus, Vicia,* and *Amorpha.*

The authors have not found any reference to the presence of nodules on these ancient Leguminosae. It would seem highly desirable, therefore, that an examination of the fossils be made from this point of view, in order to establish the antiquity of the plant-bacterial association.

Leguminosae of to-day. The leguminous plants of our day show a great range of adaptation to varying conditions of temperature, moisture, and soil. They are most numerous in the torrid and temperate zones, although members of the family may be found wherever other flowering plants exist, from the coldest arctic region to the hottest region of the tropics. Their great abundance in nature may perhaps be judged from some quantitative figures on the size of the family. The Spermatophyta, or seed plants as a whole, comprise approximately 130,000 known species. The largest single family, the Compositae, contains about 800 genera and 12,000 species. The Leguminosae are second with no less than 10,782 species, described as in 487 genera. Third come the Gramineae with about 400 genera and 4500 species. On virgin soils the Leguminosae constitute a considerable portion of the vegetation, and it may fairly be asked what part they have played in building up the natural fertility of the soil. In 1905 Norton and Walls made a survey of the wild Leguminosae of Maryland and found that in many parts of the state the Leguminosae make up one-fourth to three-fourths of the wild flora. They suggested that the wild Leguminosae, nearly all of which are nodulated in Maryland, are important agents for adding nitrogen and humus to the soils. Four years later Warren, 1909 and 1910, independently arrived at similar conclusions. He particularly emphasized that the Leguminosae are abundant both in number of individuals and in number of species on poor soils and slopes where grass is thin. On richer soils and on regular grasslands, the Leguminosae are largely displaced. Campbell, 1927, studied the re-vegetation of gravel wastes and of "worn out" and abandoned farm land. In the first period of return to nature the Leguminosae may constitute 75-100 per cent of the plant population. But as such soils increase in fertility in consequence of the growth of the Leguminosae themselves, the flora

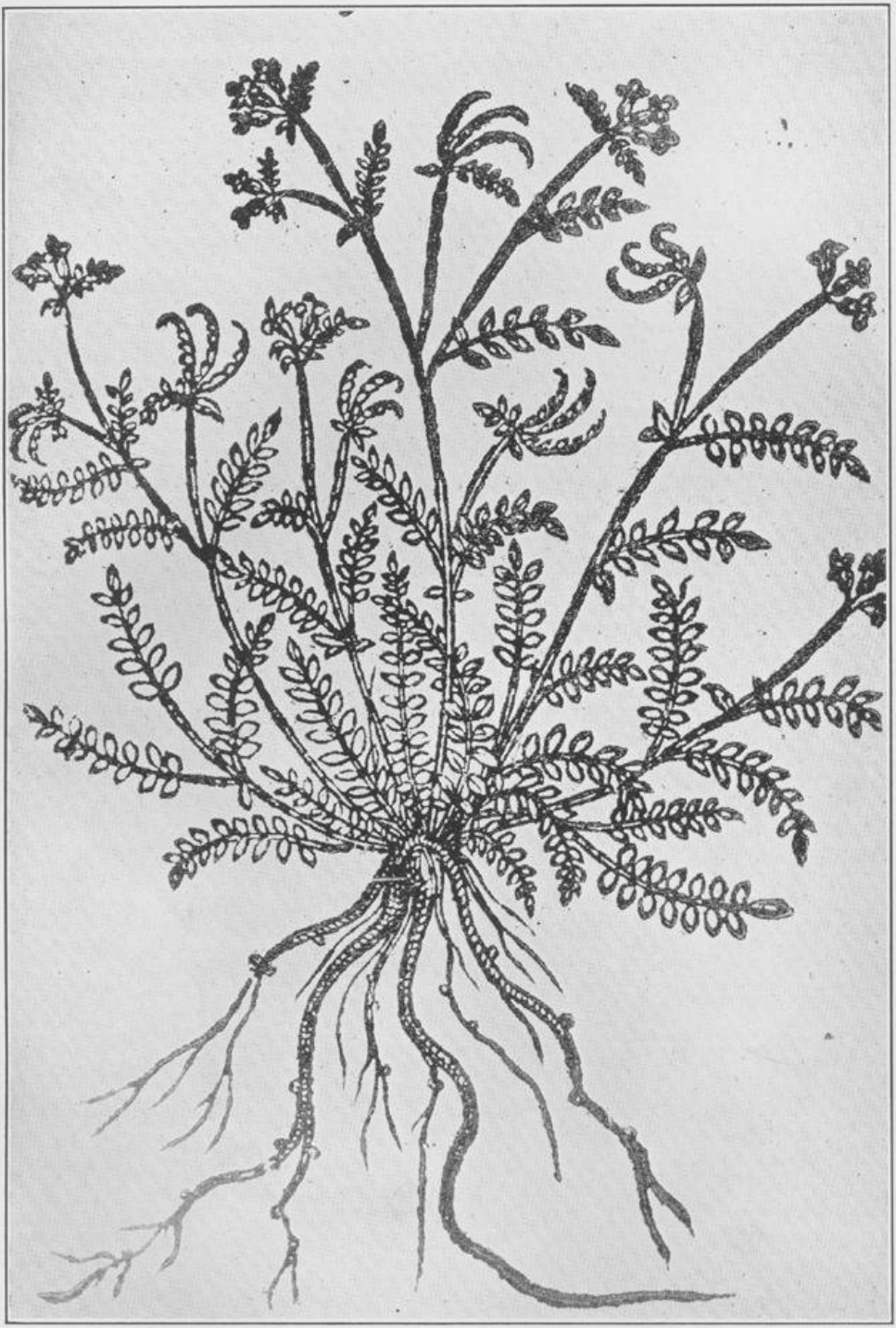
changes. The Leguminosae recede, and other plants creep in. On such grounds Campbell suggested that the relative abundance of Leguminosae in any wild association of plants constitutes an ecological index to the fertility of the soil.

While it is thus extremely probable that the wild Leguminosae are instrumental in building up and maintaining the fertility of the soil in which they grow, there is very little available data on the amount of nitrogen which they actually assimilate. Alway and Pinckney, 1909, have determined the nitrogen content of the aerial portions of wild Leguminosae gathered from a unit area of upland prairie soil in Nebraska, and have estimated the annual return of nitrogen by wild Leguminosae to be about 8.5 to 10 lbs. per acre.

Granted that the Leguminosae are of unparalleled importance in the natural flora, what can be said of them in the cultivated areas where man has to his own profit disturbed the balance of nature? Of the vast number of species of the Leguminosae, comparatively few, in fact less than 200, are cultivated, and of these less than 30 are grown in the United States. Perhaps 10 others are cultivated in Europe and the Mediterranean region and in the Orient certain others which are indigenous to that region. (See Akemine, 1931, for a list of Leguminosae cultivated in Japan, probably representative of the Orient in general.) It is almost impossible to make an estimate of the extent to which these crops are raised. In the United States the crop data of the Bureau of Crops and Markets, United States Department of Agriculture, are available to those interested, but they are admittedly incomplete and are of comparative value only.

It is equally difficult to estimate the *economic value* of leguminous crops, for their market value, great as it is, is a small part of their intrinsic value in the system of agriculture. Their value as food for man and animals is well known and is a natural consequence of their composition. (See the discussion of the chemical analyses of these plants in the early part of this chapter.) Certain of the Leguminosae also yield valuable woods, oils, dyes, and medicinal products. Recently there has appeared a curious new use for leguminous plants, proposed by Krysto, 1930, who offers to rid the world of malaria by the extensive introduction of leguminous plants into malaria-infested regions. It is claimed that *Anopheles* mosquitoes feeding partly upon juices of leguminous plants are unable to spread malaria though carrying the parasite.

The value of the Leguminosae to the farmer, however, has at least two other aspects which will be emphasized again and again in this book. Firstly, there is the fertilizing effect upon the soil. This results from direct addition of nitrogenous and organic matter to the soil, the effect, of course, varying with the amount of such matter added. When the whole leguminous crop is plowed under as a green manure, this benefit is at its maximum. When part of the crop is removed as hay or seed, the effect is much less. In Chapter 12 there is further discussion on this economic aspect under the caption of Green Manuring. Secondly, the Leguminosae have long been used as cover crops. As such they are not without effect upon the associated crop. This phase of the problem also is treated more extensively in Chapter 12.



CHAPTER 3

THE OCCURRENCE OF ROOT NODULES

"We see only what we know."
—GOETHE

PART I. NODULES OF LEGUMINOUS PLANTS

Early observations of nodules. The question of who first observed nodules on the roots of leguminous plants provokes more curiosity than can be easily satisfied. If we are to judge from the first published picture of nodules which we have been able to find, we must give the credit to Fuchs. The first edition of his *De Historia Stirpium Commentarii Insignes* in 1542 pictured nodules on the roots of four Leguminosae: *Aphacia,* p. 110; *Vicia,* p. 172; *Faba,* p. 389; and *Foenograecum,* p. 798. The reproduction which we give of *Faba* (Plate 1) is photographed from the original colored plate in the 1542 edition of the Fuchs' herbal. Neither this text nor that of Bock, 1556, from whose book Plate 2 is taken, mentions nodules; but they are specifically named by Dalechamps in his celebrated work, *Historia Generalis Plantarum,* published in two volumes in 1587 and 1586. He described a species of the genus *Ornithopodium* as distinguished by its nodule-bearing habit. He named it *Ornithopodium tuberosum* Dalech., and evidently considered the nodules to be normal plant structures. Plate 3 is a reproduction of his original wood cut. His text description of the nodules is interesting and indicative of keen observation.

> *Ornithopodium* aliud ex Dalechampio pictum damus, quod in aridis et sabulosis, nascitur, radice multiplici huc illus fusa, tenuibus fibris capillata, crebris tuberculis nodosa duris, rotundis.[1]

Nearly a hundred years later Malpighi, 1679, published his elaborate *Anatome Plantarum,* in which he referred to the swellings on the roots of leguminous plants as galls caused by insect larvae.

> In *Pisi,* et similium siliquis, persaepe exterius strumosi eminent tumores cum cicatricum vestigiis, crassefacto ibidem pericarpio: intus verò copiosi vermiculi conduntur, qui semen etiam vorantes, tandem foràs viam sibi faciunt.[2]

[1]We give another *Ornithopodium,* described by Dalechamps, which grows in dry and sandy soil, its roots extended here and there with many branches, hairy with slender fibers, and knotty with abundant tubercles hard and round.

[2]In *Pisum* and similar leguminous plants there protrude tumors, often strumous on the outside, with marks of wounds, the pericarp at that point having been thickened; on the inside, in truth, are many worms which devour the shoot and finally make a way out for themselves.

Plate 4 is taken from Malpighi's book and shows clearly his conception of the root nodules of *Vicia Faba.* After the time of Malpighi there is another gap in the records of observations on nodules. Not until the early part of the 19th century did botanists take an active interest in the occurrence and significance of root nodules. Since that time the root nodule problem has been very much in the spot light of science.

The nature of nodules. It is interesting to trace out the changing conceptions of the nature and function of nodules from Dalechamps to the present time. Most of the suggestions and theories which appeared from time to time are not worthy of detailed description and are therefore simply listed in outline below. The grouping is based on that of Mattirolo, 1899.

Theories of the nature and function of nodules

1. Simple outgrowths of the tissue of the root or diseased outgrowths of non-specific nature
   - Dalechamps, 1587, (normal outgrowths)
   - De Candolle, 1825, (morbid tumors)
   - Tuslane, 1851, (morbid tumors or fleshy protuberances)

2. Galls caused by insect larvae or annelid worms
   - Malpighi, 1679
   - Cornu, 1879

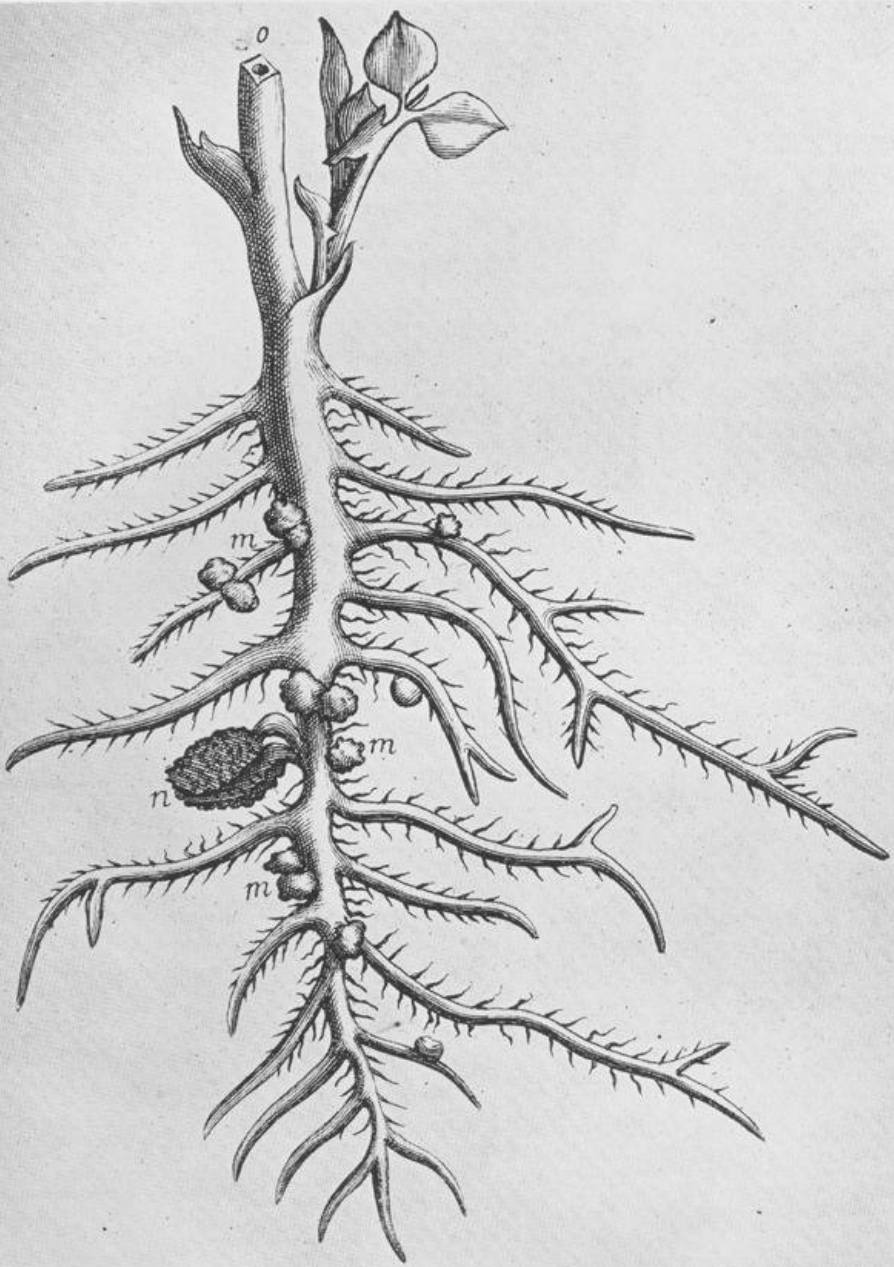
3. Sclerotia
   - Bivona, 1816 (due to distinct species, *i.e., Sclerotium lotorum, Sclerotium medicaginum,* etc.)
   - Persoon, 1818 (due to *Sclerotium rhizogonum*)
   - Fries, 1821

4. Lenticels
   - Clos, 1848, 1849, 1852

5. Abortive gemmae of latent fruits
   - Doody (cited by Treviranus p. 396)
   - Trinchinetti, 1837
   - Treviranus, 1853 (may develop if normal fruiting is prevented)

6. Abortive roots or rootlets
   - Gasparini, 1851 (absorbing tubercles)
   - Kolaszeck, 1856 (absorbing organs)
   - Cornu, 1879
   - DeVries, 1877
   - Van Tieghem and Douliot, 1888

7. Normal organs for storage
   - Lachmann, 1858
   - Mattirolo and Buscalioni, 1887
   - Frank, 1887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Brunchorst, 1885a
Benecke, 1887
Tschirch, 1887
Van Tieghem and Douliot, 1888

8. Products of endotrophic mycorrhiza or endomycorrhiza of Chytridiaceae
Vuillemin, 1888

9. Defective formation of cortical parenchyma of the root
Eriksson, 1873
Prillieux, 1879

10. Products of association between nitrogen-fixing bacteria and the plant.
All workers since Beijerinck's isolation of the bacteria, 1888, and Ward's, 1887, proof of nodule formation resulting from infection.

"Association between bacteria and plant" is a purposely noncommittal phrase. It is not exact. In a broad sense, it is equivalent to the term *symbiosis* as originally used by de Bary in 1878.[3] *Symbiosis* is derived from the Greek, *συμβίωσις*, meaning "living together." It was used by de Bary to cover all cases involving the living together of dissimilar organisms, regardless of how loose the association. It would thus include all cases of parasitism as well as all cases of mutual benefit or of no benefit. But today symbiosis has come to mean particularly the association of mutually beneficial organisms and is sometimes called mutualism. The case of the Leguminosae and their nodule bacteria is often cited as a classical example of symbiosis. In a later Chapter, 10, there will be further discussion of this relation between plant and bacteria. It will there be pointed out that in some cases, at least, there is little or no benefit to the host plant, because of inherent qualities in the strains of bacteria. In Chapter 9 on cytology of the root nodules also, there is the statement that in early stages, at least, the plant is actually contributing most to the partnership. In other words, the bacteria are temporarily parasitic. Surely the relation between plant and bacteria is infinitely complex. Was not Beijerinck right when he wrote:

> Wenn die lebende Pflanzenzelle Nutzen von einem anderen Organismus ziehen soll, welcher, wie im vorliegenden Falle, als Theil des Protoplasmas auftritt, so muss ein subtiles Gleichgewicht zwischen Wachsthum von beiden möglich sein.

Non-occurrence of nodules. So general is the phenomenon of nodulation of leguminous plants that one might suppose all members of the family to bear nodules if exposed to the proper bacteria. This is generally true of the subfamilies Papilionoideae and Mimosoideae (one recently reported exception will be mentioned below), but among the Caesalpinioideae there are several well-known exceptions. It was Lachmann in 1858 who first reported that nodules do not form on *Cercis,*

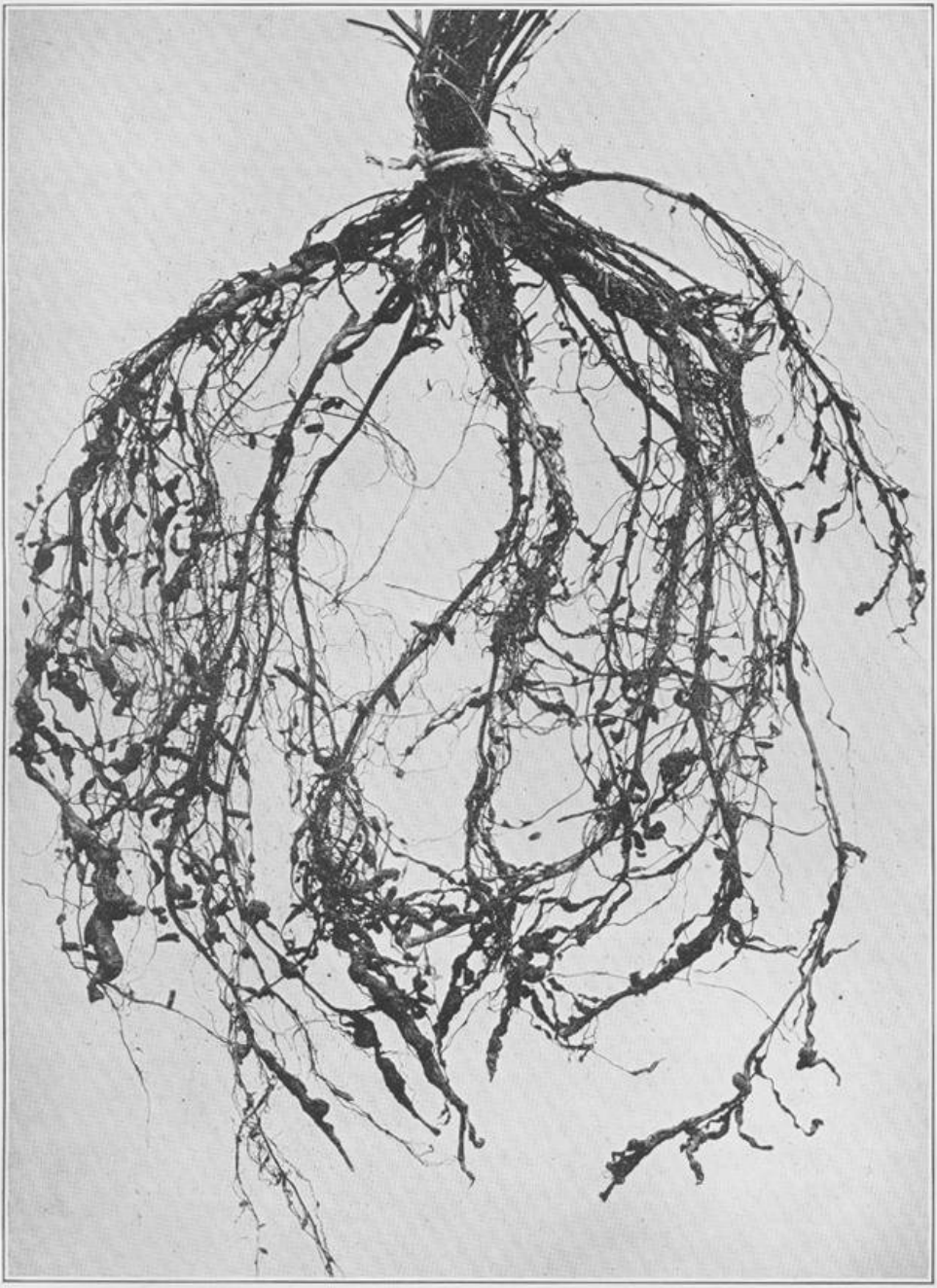
[3]Bary, H. A. de. Ueber Symbiosis. Tageblatt der 51 Versammlung Deutscher Naturforscher und Aertze, Cassel, Nr. 5, Sept. 16, 121-126, 1878.

the redbud or Judas tree. Buckout, 1889, and Harrison and Barlow, 1906, confirmed him in respect to *Cercis canadensis* L. and Clos, 1893, also in the case of *Cercis siliquastrum.* A recent report by Leonard, 1925b, also indicated that *Cercis canadensis* L. is devoid of nodules. The only indication to the contrary is that of Buchanan, 1909a, who reported studies of the physiology of a culture of *Cercis* bacteria. Another plant which is without nodules is the Kentucky coffee tree, *Gymnocladus dioica* (L.) Koch. As early as 1890 Buckout observed the lack of nodules on the *Gymnocladus* and recently also Leonard, 1925b, confirmed the report. One of the sennas or *Cassia barclayana* Sweet was reported devoid of nodules by Clos, 1893, and recently also *C. marylandica* L., *C. medsgeri* Shafer, *C. tora* L., and *C. occidentalis* L. in the field and *C. corymbosa* Lam., *C. bicapsularis* L., *C. emarginata* L., *C. laevigata* Willd., *C. tomentosa* Wall., *C. artemisoides* Gaudlich., and 5 unidentified species in the greenhouse by Leonard, 1925b. Still another plant, *Gleditsia triacanthos* L., or the honey locust, has been reported to be non-nodulated by Nobbe, Schmid, Hiltner, and Hotter, 1891; Harrison and Barlow, 1906; and Leonard, 1925b.

Members of these four genera, *Gymnocladus, Gleditsia, Cassia,* and *Cercis,* then, are the recognized exceptions to the habit of nodule formation. Leonard, 1925b, added to the list *Acacia baileyana* F. Muell. as the single exception among the 24 species of *Acacia* examined. The interesting thing about the *Acacia* case is that the genus *Acacia* belongs to the Mimosoideae, most of which are conceded to be nodule-forming. One previously reported exception is *Mimosa pudica,* the sensitive plant (Buckhout, 1889). The authors, however, have observed that *Mimosa pudica* sometimes bears nodules under greenhouse conditions.

There is no well-established explanation for the lack of nodulation in the cases just discussed. McDougall, 1921, suggested that the unusually thick-walled and lignified root hairs produced by *Gleditsia* are immune to infection. Recently Friesner, 1926, and Fehér and Bokor, 1926, working independently, have reported that *Gleditsia* produces cylindrical swellings on the main axis of the smaller roots. These swellings contain bacteria which apparently function as do the bacteria of true root nodules. Whether this is the true explanation remains to be shown by further investigation. Moreover, there is as yet no explanation for the absence of nodules on senna, redbud, and the Kentucky coffee tree.

Shape, number, and size of nodules. Just as there are differences in the shape and size of the flowers of different plants, so are there differences among nodules. Nodules may be simple or compound, round, elongated or club-shaped, single or in clusters. Generally the cultivated annual Leguminosae have large spherical or irregular nodules, often grouped about the tap root or first-formed lateral roots. Perennial or biennial Leguminosae tend to develop smaller nodules, elongated and in clusters. These may be more widely distributed over the roots, as the plant continues to form new nodules on young parts of the root throughout its growing period. The total number of nodules to be found on any single plant ranges from a few to several thousands. Perhaps the largest number ever reported is 5000 on a garden-bean plant examined by Löhnis and Leonard, 1926. Other high counts are given by Nobbe, Schmid, Hiltner, and Hotter, 1891 who found 4572 nodules on a pea plant; and by Whiting, 1915. For a time it was



thought that a large number of nodules formed by a plant was an outward sign of satisfactory association between the plant and the bacteria. Recent experiments on strain variation on the part of the bacteria (see the discussion of this question in Chapter 10) indicate that this is not necessarily true. Some thrifty plants are furnished with few nodules, while others apparently receiving less benefit have many nodules.

False nodules. Under certain conditions there may appear on the roots of leguminous plants nodule-like growths distinctly different from true nodules. These so-called false nodules are produced by nematodes or eelworms, and also by the crown-gall bacterium. The nematode gall, as shown in Plate 5, is an enlargement of the root cylinder, an effect which has suggested the term *root-knot* for the nematode disease. The disease is not common in many parts of the world, but in nematode-infested soil it may become very serious (Godfrey, 1923). It is particularly widespread in the South in light sandy soils which are continually warm and moist. Also in commercial-greenhouse soils the disease may become a serious menace. The causal organism is *Heterodera radicicola,* a small nematode or eelworm. The crown-gall type of lesion is a swelling at or near the crown of the root; it is caused by *Phytomonas tumefaciens.* Crown gall occurs most commonly on woody nursery stock but may occur on a variety of other plants, including the Leguminosae (Smith, Brown, and Townsend, 1911). Kellerman, 1911b, warned against the confusion of crown-gall lesions and true root nodules. There is a superficial resemblance, but close examination will show that the true nodule is an outgrowth of tissue *within* the root cortex, whereas crown gall is an irregular deformation of the root itself. Moreover, a laboratory examination will show the cells of the nodule to be packed with living bacteria, whereas very few or none can be seen in the crown gall. The organism is present, however, and may be isolated at some stages of the disease.

PART II. NODULES OF NON-LEGUMINOUS PLANTS

Certain non-leguminous plants are found in nature to bear root nodules which superficially, at least, resemble the root nodules of the Leguminosae. Probably there are several, perhaps many, types of these non-leguminous nodules, for they have not yet been adequately studied. Root swellings as produced by the mycorrhiza, crown-gall organisms, and nematodes are beyond the scope of this monograph. For information on the mycorrhiza the monographs of Melin, 1925, and Rayner, 1927, should be consulted. Concerning crown gall there is a large literature, from which may be cited the early paper of Smith, Brown, and Townsend, 1911, and the recent papers of Riker *et al.,* 1930; and Wright *et al.,* 1930. The present discussion will be limited to an account of the nodules of supposed bacterial origin. Generally speaking, these are distinguished from the true leguminous nodules by their lack of masses of bacteria in the bacteroidal form, by point of origin and arrangement of tissues, and so on. It must be remembered, however, that there are many errors and confusion in the reports on non-leguminous nodules. For lack of authentic information therefore it is in most cases impossible to give an accurate picture either of the causal agents or the structure and function of

such nodules. Brief consideration will also be given to the bacteria which are regularly found in the leaf tissues of some tropical genera of the Rubiaceae and of *Ardisia* and *Dioscorea* species.

ROOT FORMS

Alnus nodules. The curious root nodules of the alder attracted the attention of Meyen as early as 1829 and of Woronin in 1866. These nodules are warty, brownish masses, sometimes as large as a man's fist, and are especially abundant on trees growing in shady or damp places. The internal structure of the nodules was briefly described by Woronin as follows: The nodules show essentially the same structure and possess the same arrangement of tissue elements as the roots of normal trees. The cortical parenchyma, however, is thicker and contains the organisms which are supposed to initiate the nodules. According to Meyen, the vascular system consists of only a few meagerly pitted vessels extending little more than half way through the center of the nodule. Gravis, 1879 and 1880; Woodhead, 1900; Shibata, 1902; Bottomley, 1907; and Spratt, 1912a, agreed in the main with the foregoing. Miss Spratt particularly emphasized that these nodules are perennial. Schacht, 1853; Harshbarger, 1903, and Atkinson, 1892, have also reported general observations on the occurrence of alder nodules.

The nature of the organism causing nodules on *Alnus* has been a point of great contention in the literature. Meyen, 1829, considered it probably of the order *Lathraea* or *Balanophora,* and von Jäger, 1860, suggested that the nodules of the alder are insect galls. Woronin, 1866, introduced the mycelial fungus theory, which has prevailed to the present. According to the observations of Woronin, most cells of the cortical parenchyma are filled with very small, colorless, spherical closely packed vesicles (*Bläschen*). These spherical bodies are borne upon branches of delicate colorless hyphae which extend between and into the plant cells. Woronin supposed the vesicles to be the spores of the fungus, although he did not observe germination of them. He called the fungus *Schinzia alni,* by virtue of its supposed, though admittedly remote, relation to the genus *Schinzia,* other members of which occur in plant roots. Gravis, who studied the organism again in 1880, agreed on the whole with Woronin's description. Moeller, 1885, declared that the fungus, previously supposed to be composed of hyphae, is in reality a plasmodium, finely granular in the young stages, but aggregated into small lumps in the older stages and eventually into sporanges in which spores are regularly arranged. The name *Plasmodiophora alni* was therefore proposed and was accepted by Woronin, 1885, with the reservation that there might also be another organism in the nodules. Brunchorst, 1885b, and 1886, showed by lucid description and figures that if sections are treated with hydrochloric acid, the younger portions of the fungus in the tip of the nodule, which look like plasmodia in material fixed in alcohol, are resolved into a network of very fine hyphae. Like Moeller, Brunchorst considered that the vesicles in the older parts of the nodule function as sporanges. Moeller, 1890, and Wolpert, 1910, claimed to have observed germination of the spores, but Shibata, 1902, thought it more likely that the vesicles and their contents are not sporanges containing spores, but degeneration forms of the fungus. Brunchorst considered the fungus different from any previously

described and therefore gave it the name *Frankia subtilis*. Unfortunately from our modern codal viewpoint, he ignored the specific name, *alni*, which Woronin had given.

The question whether or not cross walls are present in this fungus continually vexed those who were now agreed concerning the gross morphological characteristics. Brunchorst, 1886, found septation; Moeller, 1890, found none; Björkenheim, 1904, reported that a few walls can be found by careful observation, and Wolpert, 1910, stated that they are discernible in some material.

All investigators were not yet convinced of the mycelial nature of the inhabitant of the alder nodule. Frank particularly seems to have vacillated in his conception of the organism. In the first edition of his book, *Die Krankheiten der Pflanzen,* 1880, he agreed with Moeller's designation, *Plasmodiophora*. In 1887 he thought the contents of the nodule not a foreign organism at all, but a mass of material organized by the tree itself for protein storage. In 1891 he still did not believe the fungal hypha theory, for the fineness of the filaments reminded him of a Schizomycete; but because of the similarity which he thought to exist between the alder organism and the orchid mycorrhiza he was forced to accept the views of Brunchorst. In the second edition of *Die Krankheiten der Pflanzen,* 1895-96, therefore, he classed the organism as *Frankia subtilis*. Zach, as late as 1908, accepted this classification. Other workers were still not convinced. Hiltner, 1898, regarded the causal agent as one of the Actinomycetales or higher bacteria. He secured a pure culture of the organism, which he described as a "bacillus" of the Streptothrix type—a type which by its possession of sporanges forms a bridge between the bacteria and the higher fungi. By inoculation experiments with an infusion of crushed alder nodules, he was able to induce infection. Shibata, 1902, also saw resemblance to bacteria and claimed the organism to be related to *Mycobacterium tuberculosis,* as did also Peklo, 1909. Pelko isolated the organisms and was the first to obtain nodulation by pure-culture inoculation. On the basis of morphology and cultural reactions, Peklo placed the organism with the Actinomycetales, calling it *Actinomyces alni*. For this name he had the support of Hiltner, 1898; Shibata, 1902; and Ross and Hedicke, 1927. Maire and Tison, 1909, proposed *Frankiella alni*.

Bottomley is almost alone in considering the alder organism identical with the rhizobia of leguminous root nodules. In 1906 he reported the formation of nodules on sweet peas and tares, following application of mixed cultures from the nodules of *Acacia, Alnus,* and *Elaeagnus*. In 1907 in a short article on the morphology of root tubercles, he referred to "bacteroid tissue" in the cortex of alder nodules. He was confirmed by Spratt, 1912a, who found no conflict in the obvious morphological differences between the alder organism and the rhizobia. The spherical "sporanges" she described as coccoid forms of the organism, more resistant to adverse conditions than the usual rod form. Burrill and Hansen, 1917, criticized Miss Spratt's observations, but McLuckie, 1923a and b, was inclined to accept the work of both Bottomley and Spratt. Kellerman, 1911a, also considered that bacteria similar to the rhizobia are the cause of alder nodules, but he gave only morphological similarity as evidence for the opinion.

Almost every alder tree possesses nodules, a fact which suggests some vital importance of the association. Hiltner, 1896, tried the experiment of growing

Alnus glutinosa in sterilized nitrogen-free soil and inoculating some pots with an extract of alder nodules. After one year the plants without nodules were unhealthy and appeared to be suffering from want of nitrogen. The nodule-bearing plant, on the other hand, was larger and growing thriftily, as apparent from Plate 6, a photograph of the alder plants of Hiltner's experiment. In 1904 Nobbe and Hiltner published a second article with much the same results.

Frank, 1891, drew comparisons between an endotrophic mycorrhiza and the alder organisms; by analogy he said that the fungus was absorbed by the tree. Thereafter the alder organism was frequently referred to as a form of endotrophic mycorrhiza, notwithstanding the warnings of Shibata, 1902, and of Hiltner, 1903, that the alder nodule is somewhat different from a true mycorrhizal tubercle. The evidence for digestion of the alder-root invader by the tree is almost entirely cytological. It is agreed that the older parts of the nodules show the organism to be disorganized and scarcely identifiable. Shibata, 1902, observed metamorphosis of the nucleus of the host cell to an amoeboid form, accompanied by formation of small drop-like round or oval bodies in the cytoplasm. These droplets he called *Sekretkörperchen,* for he supposed that they secreted an enzyme for digestion of the fungus. In support of this theory he demonstrated the proteolytic properties of nodule extract in digesting fibrin. His work was confirmed by Maire and Tison, 1909; and Arzberger, 1910. Zach, 1908, interpreted the drop-like bodies not as *Sekretkörperchen,* but as waste products or *Exkretkörper.* Ziegenspeck, 1929, gave a confused account of digestion of the endophyte by the host plant, involving amoeboid transformation of the nuclei of the plant cells and appearance of amoeboid phagocytes of endophytic origin. In 1903 Hiltner questioned the complete digestion of the fungus, since it is evident that parts at least must survive for years if the nodule is to remain active.

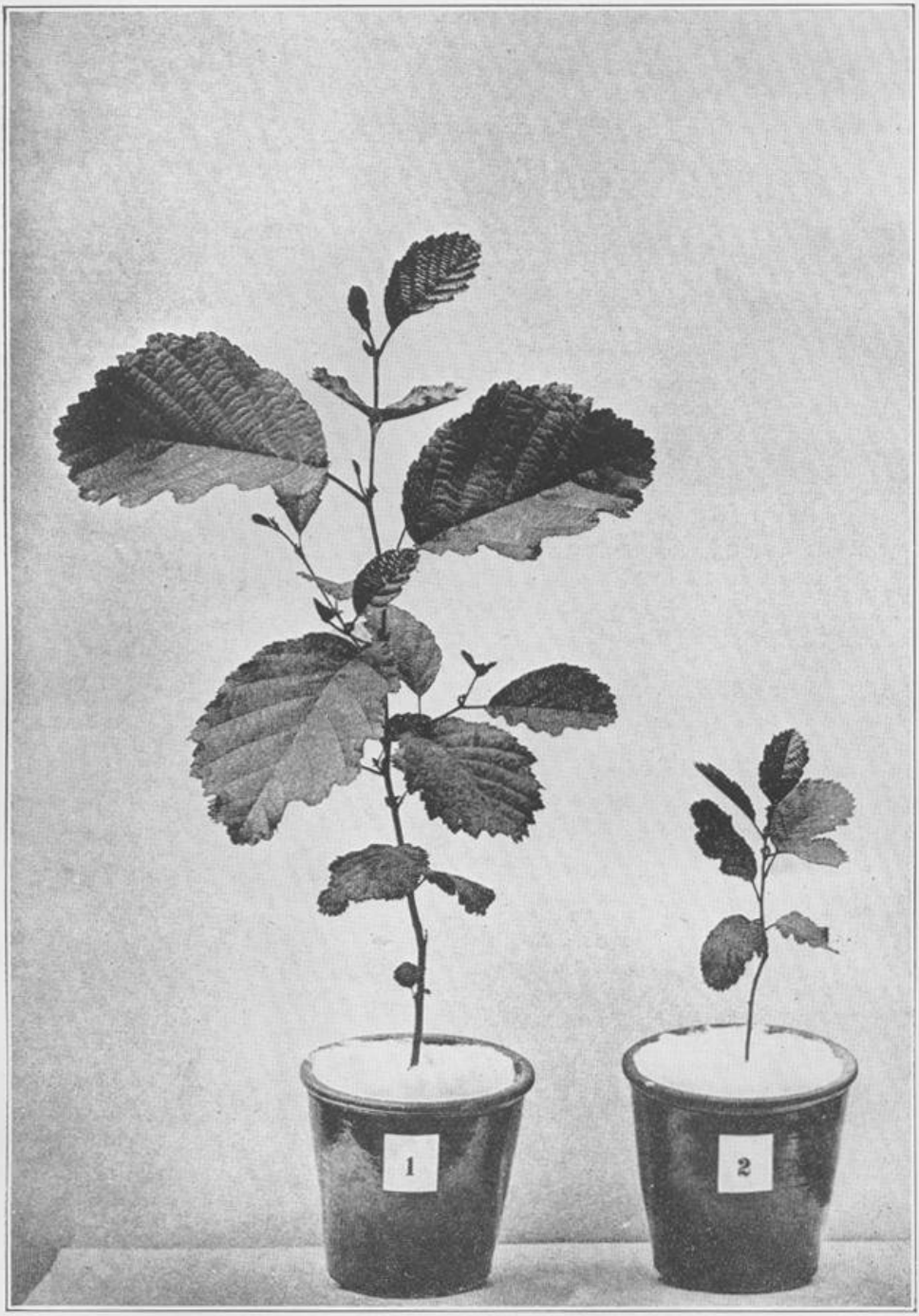
Coriaria nodules. In a recent paper Kataoka, 1930, has reported that the nodules of *Coriaria japonica* A. Gr. are essentially similar to those of *Alnus, Elaeagnus, Ceanothus,* and *Myrica.* He stated on the basis of previous work by Shibata, 1902, and Shibata and Tahara, 1917, that the causal organism is an actinomycete. His chief thesis, however, is that nodulated *Coriaria* plants do actually assimilate atmospheric nitrogen. An example of his data follows:

Comparison of Coriaria plants with and without nodules, grown in a nitrogen-free sand for one year.

Plant	Total sum of the lengths of off-shoots	Weight of plants air-dry	Total nitrogen content
	cm.	gm.	gm.
With nodules	225.5	9.51	0.1012
Without nodules	68.5	0.42	0.0034

He also furnished some very good pictures of the vigorous nodule-bearing plants and of the weak sickly nodule-free plants.

Nodules on the Elaeagnaceae. Warming, 1876, was first to describe the root swellings on the various members of the genera *Hippophaê, Elaeagnus,* and *Shepherdia* in the family Elaeagnaceae. Plate 7 illustrates a typical nodule cluster on


1
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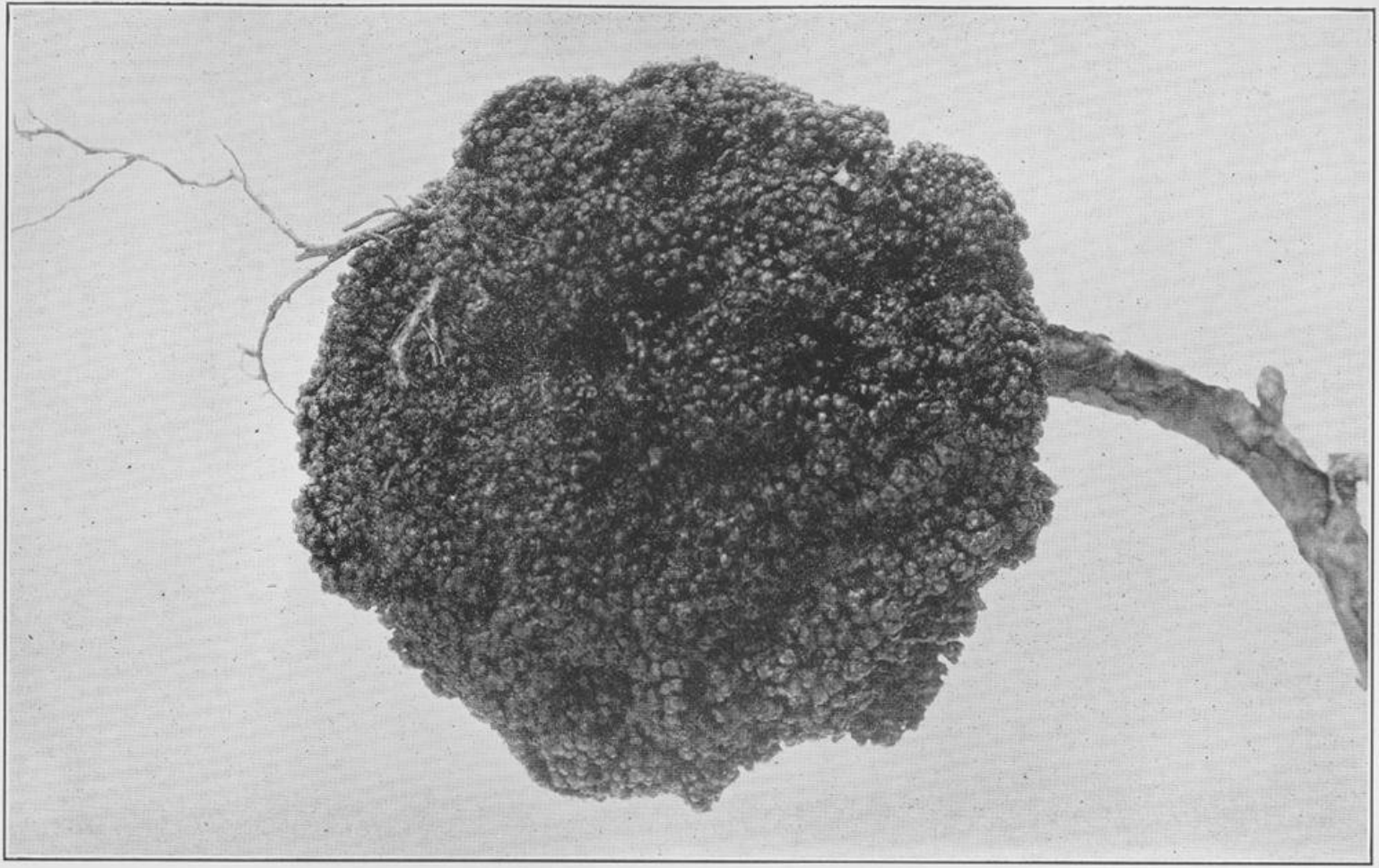


Elaeagnus multiflora. According to Brunchorst, 1885b and 1886; Frank 1887; Moeller, 1890; Bottomley, 1907; Zach, 1908; Maire and Tison, 1909; and Spratt, 1912a, the causal agent of these nodules is very similar to that of alder nodules. Arzberger, 1910, pointed out also the structural similarity of the nodules. Nobbe, Schmid, Hiltner, and Hotter in 1892b, working with *Elaeagnus angustifolius* and Nobbe and Hiltner, 1904, with *Shepherdia canadensis,* demonstrated that nodule-bearing plants make distinctly better growth than nodule-free plants. The function of nitrogen assimilation on the part of the nodules is therefore suggested. Snyder, 1925b, considered it probable in a case of the Russian olive tree, *E. argentea.*

Ceanothus nodules. Very little work has been done on the nodules of *Ceanothus.* They were not discovered until 1890 by Beal, and work on their etiology was not reported until the following year, when there appeared the following statement (Atkinson, 1891): "The tubercles discovered by Prof. Beal were found upon further study to be caused by a parasitic fungus allied to *Schinzia Alni* found upon the root of *Alnus* and *Elaeagnus,* and now transferred to the genus *Frankia.*" The same worker in 1892 found that the organism differed in a few minor points from *Frankia subtilis* of Brunchorst, and accordingly placed it as a new species *Frankia Ceanothi.* In 1910 Arzberger reported the regular occurrence of nodules on *Ceanothus americanus,* whereas Bottomley in 1915 failed to find them on the *Ceanothus* species in the botanical gardens at Kew and in the Chelsea Physic Gardens. The function of *Ceanothus* nodules is even less certainly known than that of *Alnus* or *Elaeagnus.* Arzberger, 1910, demonstrated a proteolytic enzyme as in the alder nodule, but did not claim to have thereby established that nitrogen fixation necessarily occurs. Bottomley, however, in 1915 did claim so; incidentally he regarded the causal agent of *Ceanothus* nodules as identical with that of leguminous root nodules. Snyder, 1925b, reported that Petry at Michigan also found a bacterium-like organism in *Ceanothus* and evidence of higher nitrogen content in nodule-bearing plants. Burrill and Hansen, 1917, failed to isolate the *Ceanothus* organism at all or even to induce nodule formation by the use of an infusion of crushed *Ceanothus* nodules.

Myrica nodules. The nodules on the roots of *Myrica* are unique in having at their tips elongated, slender, root-like processes which are in reality extensions of the central cylinder of the modified root. They were reported by Brunchorst in 1886. Moeller, 1890, considered the causal organism an individual species, *Frankia Brunchorstii.* Harshbarger, 1903, classed it with the Oomycetes and hence termed it a mycorrhizal form. He used the term "mycodomatia" to describe the infections. In this classification he has not been substantiated by other workers. Shibata, 1902, claimed an *Actinomyces* relationship for the fungus, a conclusion in which he was backed by Peklo, 1909 and 1910; and Arzberger, 1910. The most recent work has been done by Dangeard and Trnka, 1929. They agreed that the etiologic agent is a filamentous form, but they would class it with the true bacteria rather than with the Actinomycetales. *Rhizobacterium Myricae* is their name for the organism. Bottomley, 1911a and b, and 1912a, reported that a hyphal fungus occurs in the base of the nodule, and in the tip bacteria giving the cultural reactions of true rhizobia. He tested cultures of the organism for nitro-

gen fixation and reported fixation of 2.05 mg. of N per 100 cc. of culture, incubated for seven days at 25° C. No one has confirmed this work, and since it is the only published report on the nitrogen-fixing ability of the *Myrica* organism, the hypothesis cannot be considered established. Cytological evidence of Arzberger, 1910, would indicate a pathogenic rather than a symbiotic relationship with the infected plant. The cytoplasm of infected cells is destroyed, and there is no digestion of the fungus elements as has been observed in instances of *Alnus* and *Elaeagnus* invasion. Yet there is nothing to disprove that adjacent cells do not benefit from the invader; relatively few cells are infected in the *Myrica* root, the parasite being confined to one or two layers beneath the cork.

Podocarpus nodules. The work which has been done on the root nodules of *Podocarpus* and allied genera is insufficient to warrant conclusions as to the etiologic agent. Hiltner, 1903; and Nobbe and Hiltner, 1899a, classed these nodules definitely with the mycorrhizal infections. Bottomley, 1912b; Spratt, 1912b; and McLuckie, 1923a, on the contrary, found bacteria very like the rhizobia of leguminous nodules. Miss Spratt explained the hyphae of Hiltner and Shibata as belonging to a secondary invader. The function of nitrogen fixation has been attributed to the *Podocarpus* nodules by Nobbe and Hiltner, 1899a; Bottomley, 1912b; and Spratt, 1912b. Shibata, 1902, reported the occurrence of a proteolytic enzyme in the nodules.

Nodules on the Cycadaceae. Schacht, 1853, is credited with the discovery of root nodules on the Cycadaceae. They occur on members of all the genera with the possible exception of *Microcycas,* according to Spratt, 1915, and Kellerman, 1911a. The first organism found in nodules of the cycads is an alga of the genus *Anabaena* (Reinke, 1879). It occurs in a special layer in the cortex of the nodules and is supposed to enter through lenticels of the root. Miss Spratt, 1911, confirmed the classification of the alga and made notes upon its life history. From the fact that the alga is not always present Brunchorst, 1886, concluded that it is not the etiologic agent. He found in addition a fungus, which, being present in even the youngest stages of nodule formation, appeared to him to be the causal agent. Schneider, 1894a, presented a still more complicated picture—a nodule made up of six tissue layers which in cross-section appear as follows: an outer dermal layer of irregular corky cells containing rhizobia in small numbers; a dermatogenic layer of thin-walled angular cells; a subdermal layer with intercellular spaces and air passages; a fourth layer of palisade cells containing algae (*Nostoc*); a fifth layer of starch-bearing parenchyma cells; and a sixth and inner tissue which is the vascular cylinder as in an ordinary root. The algae may be lacking in some nodules; the bacteria, however, are constantly present, largely in the dermal layer but also in nearly all of the cells of the nodule. In cultures of the bacteria, Schneider found a coccus, a rhizobium which he believed to be *Rh. Frankii,* and a large Indian-club-shaped organism resembling *Rh. mutabile.* Life, 1901, found several types of bacteria—the rhizobia of Schneider, a very large rod-form, and a coccus. Pampaloni, 1901, found bacteria in the older portions but not in the younger. Miss Spratt, 1915, on the contrary considered the bacteria as the primary infecting agents and the fungi, if present at all, as later invaders. She regarded *B. radicicola* as the causative agent but stated that *Azotobacter* is

also present in some cases. From nodules of *Cycas revoluta* containing algae, Burrill and Hansen, 1917, isolated three forms of bacteria, none of which resembled the rhizobia of the Leguminosae culturally or in ability to induce nodules on leguminous plants.

With so many organisms in the nodules, experiments to prove the function of any one species become difficult, for the opportunities for symbiotic relationship are manifold, and the separation of organisms may lead to results very different from those of the nodule. In truth, no such experiments have been reported. All attempts to solve the question of interaction in the nodule have been based upon *a priori* reasoning. Thus Life, 1901, thought that the algae present might help in aeration of the root and in nitrogen fixation (nitrogen fixation by a *Nostoc* species having been previously reported by Prantl in 1889). Digestion of the endophytic fungus by the host cells in the manner discussed for the alder nodules was described by Zach, 1910, who compared the process to phagocytosis in animals.

Casuarina nodules. The literature relating to *Casuarina* is comparatively meager and contradictory. Janse, 1897, who first called attention to the nodules in this genus, emphasized their similarity to the nodules of *Podocarpus* and *Elaeagnus*. Miehe, 1918, reported that *Casuarina* nodules lack definite hyphae and are filled with bacteria-like elements in rod or branched form. Kamerling, 1915, stated that the *Casuarina* nodules are in all respects like those of the Leguminosae, but gave few details. Hutchinson, 1922; McLuckie, 1923b; and Rao, 1923a, used the term bacteroid in describing the morphology of the *Casuarina* organism and suggested that it is more closely related to the rhizobia of the Leguminosae than are any other non-leguminous types. Rao's contribution, 1923a, lies particularly in the field of nitrogen fixation. With pure cultures of the organism he was able to obtain 7.12 to 17.74 mg. N per 100 cc. of a two per cent mannitol solution.

LEAF FORMS

Among biologists the idea is prevalent that the interior of normal, undiseased plant tissues is free from bacteria, yet the members of the genera *Pavetta, Psychotria, Ardisia,* and *Dioscorea* are recognized exceptions to the rule. Not only do bacteria regularly occur in the leaf tissues of these plants, but they pass on from generation to generation in the seeds. (This may not be true for *Dioscorea macroura.*) How these plants first became infected and adapted to harboring these bacteria are questions as fascinating as, and perhaps more futile than, the question of how any root-nodule-bearing plant first came to harbor a root symbiont.

Rubiaceae. In 1902 Zimmerman described the peculiar knots—we may call them nodules—scattered over the blade of the leaves of *Pavetta* or along the midrib of *Grumilea mikrantha*[4] leaves. These leaf nodules are abundant, occurring even in the white parts of variegated leaves of *Pavetta indica.* Zimmermann had no difficulty in demonstrating that bacteria occur in the nodules and are presumably the cause of them. In 1911 Boas discovered leaf nodules on *Psychotria*

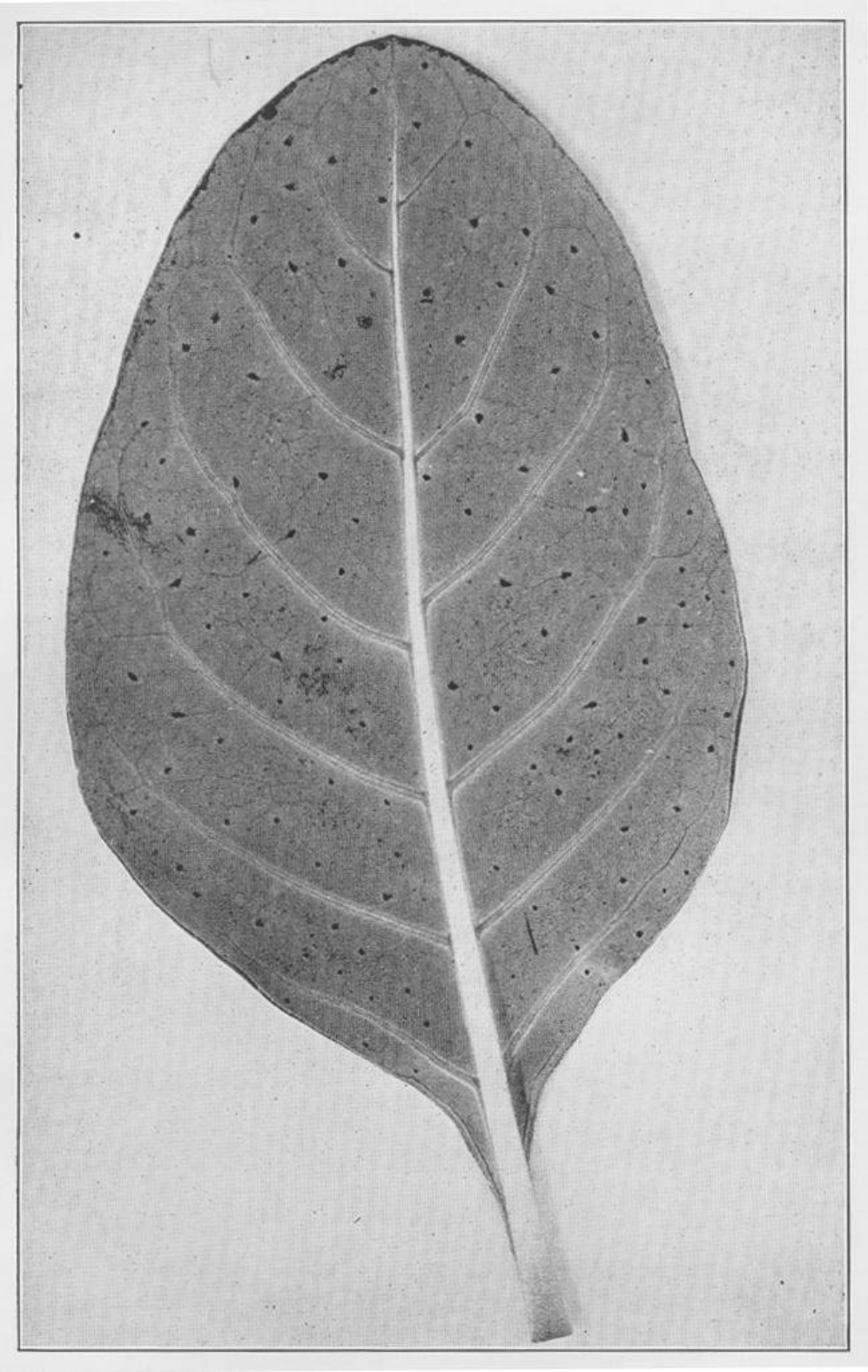
[4]Probably *Pavetta Zimmermanniana* (von Faber, 1912).

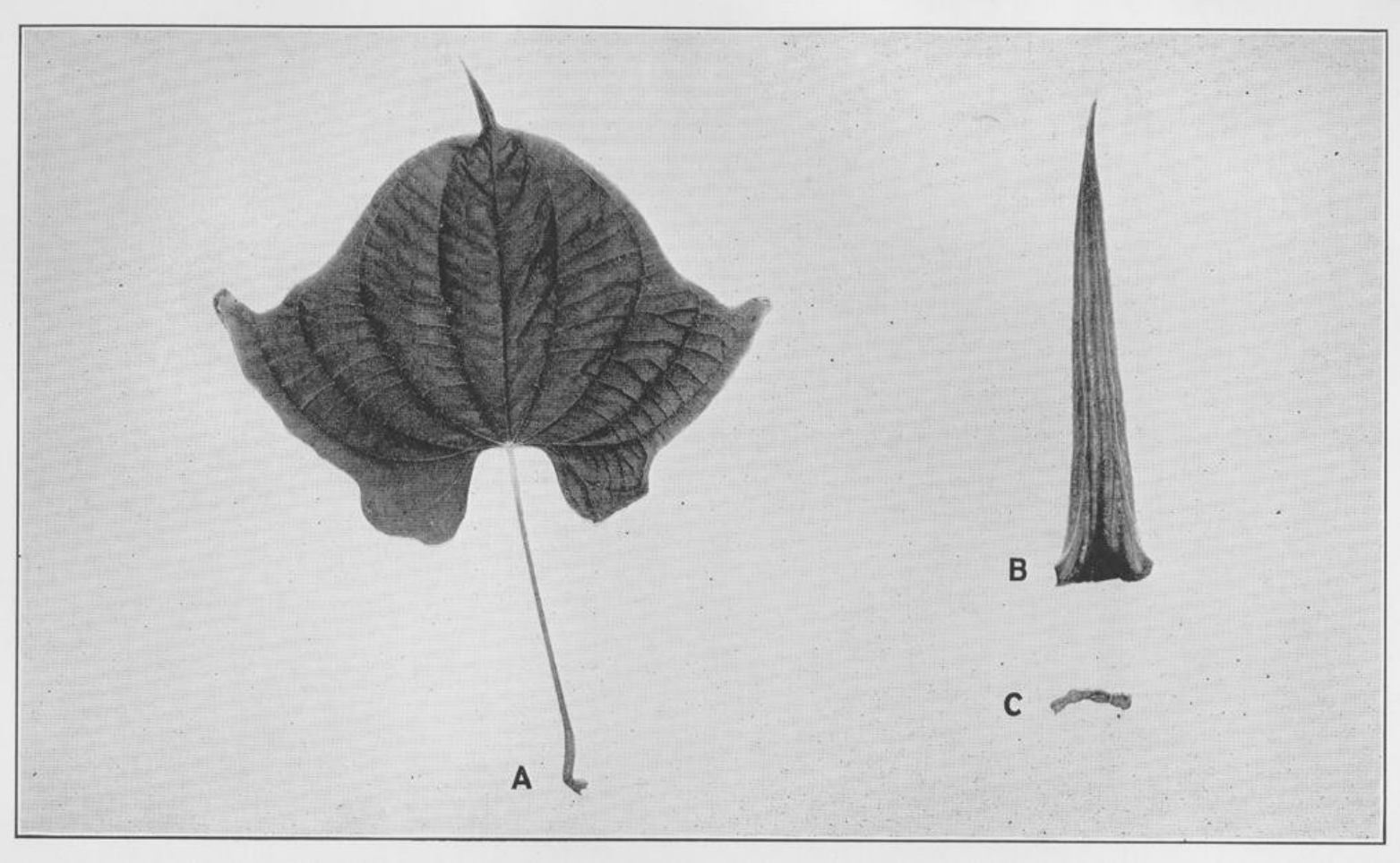
alsophila and *Psychotria umbellata*. The paper of von Faber, 1912, is the most extensive yet published on the development and function of leaf nodules and the morphological and physiological nature of the bacteria both in the plant and in artificial culture. From his account it appears that the bacteria are present in a foamy, gum-resinous mass found in the leaf buds. They gain entrance to the leaf tissue through special openings (not true stomata) and are then surrounded by a proliferation of cells, which eventually close the portals of entry. Since the bacteria are consistently present in the growing tips of the shoots, it is possible for them to enter also into the developing blossoms, thence through the pistil into the carpel, and so into the ovule, where they remain near the egg cell outside the embryo sac during the development and rest period of the seed. At germination they are ready to infect the plumule. In an effort to understand the balance of symbiosis between *Psychotria bacteriophila* and its leaf nodule bacteria, Kořínek, 1928, investigated the fate of the bacteria at the death of their host. He found that the bacteria die with the plant. It is thus not possible to regard the *Psychotria* symbiosis as a case of balanced parasitism, where either symbiont may take advantage of weakness in its confederate.

The bacteria of *Pavetta* and *Psychotria* are small, often curved, non-motile, Gram-negative rods. In the leaf nodules and in liquid culture, branched forms are common. Colonies are at first milk-white, or semi-translucent and shiny. Later they become distinctly gummy. According to von Faber the organisms from both plants constitute a single species called *Mycobacterium Rubiacearum*. Miehe, 1912, contested this classification on the grounds that von Faber's cultures were not pure and that inoculation experiments had not been carried out. By 1914 von Faber replied that inoculation of plants grown from sterilized seeds resulted in the development of nodules. The sterilization of seeds was ingeniously accomplished by immersing the seeds in water at 50° C. for 25 minutes. Some of the seeds so treated are able to germinate and to produce plants free of leaf nodules. Such plants grow poorly in nitrogen-poor sand and show marked chlorosis. The difference between them and normally infected plants growing vigorously in the same sand, von Faber cited as evidence of nitrogen fixation on the part of the leaf nodules. The conclusion is valid only if the hot water treatment of the seeds does not permanently injure the sterile plants. Unfortunately there is no proof that it does not. The contention that the bacteria are able to assimilate nitrogen of the air is not entirely without other support, however. Rao, 1923b, obtained a distinct increase in nitrogen by the culture of bacteria from *Pavetta indica* and *Chomelia asiatica* in liquid media free of combined nitrogen. In the light of his results, he recommended the use of leaves of rubiaceous plants of the types associated with bacteria as green manures for soil.

Leonard, in a personal communication, reported failure to isolate bacteria from the leaves of *Psychotria bacteriophila,* although he tried all of the common laboratory media and that recommended by von Faber. Plate 8 illustrates the leaf nodules on the under surface of a young leaf of *Psychotria bacteriophila.*

Ardisia. In old taxonomic works *Ardisia crispa* is described as possessing protein glands along the margins of its leaves. In 1911 Miehe discovered that these glands and also those of the sub-genus *Crispardisia* and the genera *Amblyanthus* and *Amblyanthopsis* contain bacteria. As in the members of the Rubiaceae,




A
B
C

the bacteria are transmitted from generation to generation through the seeds. The organisms in this case are motile in young stages and in the nodule produce branched forms abundantly. In culture the organism called *B. foliicola* by Miehe, 1913, does not utilize ammonia or nitrate but requires organic nitrogen such as asparagin, peptone, or other products of protein decomposition. It does not grow in a nitrogen-free medium and shows no ability to utilize the free nitrogen of the air when grown in a culture medium for which pea extract forms the base. An additional organism, *B. repens,* is reported by Miehe, 1913, as occurring in *Ardisia* leaf nodules. It is not regularly found and is considered a secondary invader.

In 1917 Miehe performed experiments with seeds sterilized in hot water by a modification of the von Faber method. The plants developing from such seed appear normal at first but later become stunted and sickly. Whether the unhealthy condition results from injury to the seedling or from lack of nitrogenous food is debatable here, as in the case of von Faber's *Psychotria.* Miehe's attempts at inoculation gave poor results, partly because of the technical difficulties involved. Because of the peculiar germination of *Ardisia* seeds, it is impossible to inoculate the seedlings until they are well advanced. Only in the case of a heat-treated cutting was any satisfactory result obtained. In this instance the branch which was inoculated with a pure culture of *B. foliicola* resumed growth at its apex, while another branch, uninoculated, on the same plant remained in its stunted, crippled condition. This result would indicate, not so much a functional relation of the bacteria to the nitrogen nutrition of the plant, as importance in the growth processes of the plant. A recent summary of the work on *Ardisia* and the Rubiaceae is given by Wilson, 1924.

Dioscorea. The leaf nodules of *Dioscorea* have received comparatively little attention. In 1923 Orr described the long accuminate leaf tips of *Dioscorea macroura,* a vine native to West Africa. These tips contain glands in the lumen of which is a mucilaginous secretion harboring bacteria in great numbers (see Plate 9). They are present also in the fluid which bathes the growing apex of the stem and leaf primordia. There is no explanation of how the bacteria get to the leaf glands, unless they are in the tubers and grow up with the plant. The organism is a small Gram-positive rod and is motile by a single polar flagellum. Large irregular bodies resembling the bacteroids of leguminous nodules are found in the glands and sometimes in liquid culture. According to Leonard (personal communication), the organism of *Dioscorea* is easily isolated on beef agar.

The results of leaf analyses show a striking difference in the nitrogen content of the lamina (bacteria-free blade tissue) and acumen (bacteria-bearing tip tissue).

Nitrogen content of 100 gm. of leaf tissue (Orr 1923).

Time of sampling	Lamina	Acumen	Excess of N in acumen
	mg.	mg.	mg.
10:50 A.M.	246	560	314
4:30 P.M.	173	486	313

Orr also reported a remarkably high nitrogen fixation by the organisms in culture; i.e., 25.43 mg. N per 100 gm. of medium. Some analyses of *Dioscorea* tissues by E. W. Hopkins (unpublished Wisconsin data) indicate somewhat less difference in the leaf-tip and blade tissues. In terms of percentage nitrogen (dry basis) his results are: leaf lamina 4.65 per cent; leaf acumen 5.87 or only 26+ per cent excess in favor of the bacterial tissue.

Milovidov, 1928a and b, reported cytological studies of *Dioscorea bulbifera.* Leaf tips of this species also contain short, almost coccoid rods, lying in the intercellular spaces of the gland ridges.

There are one or two miscellaneous reports of bacterial association in non-leguminous plants about which very little is known. Beijerinck, 1888, mentioned nodules on the roots of *Melampyrum pratense* and *Rhinanthus major,* members of the Scrophulariaceae. This report appears only in a footnote; no further reference to these forms has been discovered. According to work done by Mahdi Hassan and reported in a footnote by Přibram, 1925, rhizobia-like organisms are found in the juices of the rubber tree. It is suggested that these bacteria make nitrogen available for the plant and also by their proteolytic activity make plant proteins available for certain insects which harbor yeasts as intestinal parasites. These observations are interesting but of uncertain importance.

THE POSSIBLE RELATION OF LEGUMINOUS NODULE BACTERIA TO NON-LEGUMINOUS PLANTS

It is evident from the foregoing that in no case have the rhizobia of the Leguminosae been conclusively proved to inhabit nodules on plants of other families. Whether they may in any way benefit such plants without nodule formation is not known; the present weight of evidence is negative. In the few published experiments on this subject, the usual procedure has been to attempt to adapt the rhizobia to foreign environment by growing them in media containing progressively greater proportions of an extract of the plant to be inoculated. This was first attempted by Schneider, 1893a, who reported that he was able to induce invasion of corn root hairs by bacteria originally isolated from *Melilotus alba.* No nodules were formed, however, and there was little, if any, beneficial effect on the plant. Oat plants showed no effect of inoculation.

Stutzer, Burri, and Maul, 1896, accustomed alfalfa nodule bacteria to growth on a medium containing extract of seedlings of white mustard. Such bacteria used to inoculate mustard plants did not provide nitrogenous food for the plants. Grosbüsch, 1907, working with Gramineae, also had no success.

Bottomley, 1909, reported increased yield in oat, barley, and hyacinth crops as a result of inoculation with a mixed culture of *Azotobacter* and *Pseudomonas radicicola,* but no nodules were produced. Blunck, 1920 and 1924, modified the adaptation procedure somewhat by introducing active enzymes into the media in the later stages of the process. With nodule bacteria thus adapted he claims to have obtained increased yields of sugar beets. Knudson in 1922 obtained a beneficial effect upon germination of orchid seed, following inoculation with the nodule organism of alfalfa. Burrill and Hansen, 1917, made extensive attempts to inoculate non-leguminous plants with rhizobia from leguminous species. Tomato

seedlings, morning glory, and strawberry plants were used, but no infection and no benefit resulted.

In summary it may be said that there is no substantial evidence of infection of non-leguminous plants by bacteria isolated from leguminous nodules nor of any benefit occurring to the non-leguminous plants without nodule formation. Kordes, 1925, in a review of the subject, pointed out that if such infection were possible, instances of it might be expected in nature. Whether there is any benefit to plant growth from inoculation with general soil bacteria without infection, is hardly within the province of this chapter. The reviews by Vogel, 1917, 1920; Barthel, 1920; Makrinoff, 1924b; Kordes, 1925; and Behn, 1928, give references to such work.

CHAPTER 4

THE ISOLATION AND STUDY OF THE ROOT NODULE BACTERIA

"Living things are found by a simple experiment to have powers undreamt of, and who knows what may be behind?"

—W. BATESON

METHODS AND MEDIA SUITABLE FOR THE ISOLATION AND STUDY OF RHIZOBIA

The widespread occurrence of nodules on the roots of the leguminous plants and the connection between these swellings and the nitrogen-nutrition of the higher plant were well established by the last decade of the nineteenth century. Interest was thus awakened regarding the nature of the organisms found within the root nodules, and investigations were undertaken in various parts of the world to isolate and study the characteristics of this interesting and highly important group of bacteria. Because of the peculiarities in their morphology and cultural reactions, it is not surprising that the literature of that period is a mass of uncorrelated and conflicting observations. Controversies resulted among the early botanists and bacteriologists, and much was published that is not supported by later carefully controlled experiments. These discussions centered chiefly around the nature of the microörganisms and their mode of entrance into the plant.

Naturally the study of the nodule organism[1] began with its isolation and involved some determination of favorable conditions for its growth. As early as 1888 Beijerinck found that meat-peptone gelatin is not well adapted to the root-nodule bacteria. He succeeded in the isolation of these organisms on a substrate prepared from a water extract of the leaves of leguminous plants such as peas, beans, or alfalfa, plus 7.0 per cent of gelatin, 0.25 per cent of asparagin and 0.5 per cent of sucrose. In the same year Bréal, 1888b, cultivated nodule bacteria in a root-extract ammonium phosphate medium. Two years later Beijerinck, 1890, recommended 2.0 per cent instead of 0.5 per cent of cane sugar and the aqueous

[1]We use the expression, "the root-nodule organism," rather loosely. It was at first supposed that a single species was responsible for nodule formation upon all of the Leguminosae. Later it was recognized that there are permanent and significant differences between the organisms causing nodulation of certain groups of leguminous plants. The bases of differentiation of the bacteria will be discussed in detail in Chapter 8. Meanwhile the following specific names will be used, without explanation, wherever an exact designation of the organism is desirable.

Rhizobium leguminosarum Frank	Pea group
Rh. trifolii Dangeard	Clover group
Rh. phaseoli Dangeard	Bean group
Rh. meliloti Dangeard	Alfalfa group
Rh. japonicum (Kirchner) comb. nov	Soybean group
Rh. lupini (Schroeter) comb. nov	Lupine group

Wherever the specific type is unknown or immaterial, the more general terms, rhizobia, root-nodule bacteria, nodule organisms, etc., will be used.

extract of various young leguminous plants. A dilute extract of malt or a small amount of peptone was found greatly to increase growth. These organisms he found to develop best in a medium slightly acid, containing about 0.6 cc. of normal malic acid in 100 cc.

In 1918 Beijerinck described a favorable medium for the isolation of nodule bacteria, prepared from:

Agar	20.0 gm.
Sugar, cane	10.0 gm.
Starch	10.0 gm.
Dipotassium phosphate	0.5 gm.
Tap water	1000.0 cc.

On this substrate he found that the colonies of the nodule bacteria remain small, while those of *Azotobacter* continue to grow. The addition of small amounts of nitrogen as saltpeter or ammonium sulphate to the medium brings about a decided increase in the size of the colonies of nodule bacteria.

A modification of his plant extract medium as used at the present time is given below:

Pea-extract Sucrose (Modified Beijerinck)

Pea seedlings about 3-5 in. tall (green)	300.0 gms.
Water (tap)	1000.0 cc.

Heat the plant tissue in a steamer for 4 to 5 hours and then boil for 1 hour over a free flame. Filter and make up to 1000.0 cc. Add 1 per cent of sucrose or other carbohydrate, and 0.5 per cent of $CaCO_3$. This medium contains about 50 mg. of nitrogen in 100 cc. To prepare a solid substrate, add about 12.5 per cent of gelatin or 1.5 per cent of agar.

During the closing years of the nineteenth and the first ten years of the twentieth centuries, the findings of Beijerinck were confirmed in their broad aspects, and our knowledge of various phases of this subject was extended by the researches of Prazmowski, Laurent, Nobbe and Hiltner and their associates, Mazé, and others. Prazmowski, 1890, also noted that meat-extract peptone gelatin is unsatisfactory for the isolation of the nodule bacteria. Much better results were secured from the use of an extract of pea leaves plus 0.5 per cent of asparagin, 1.0 per cent of glucose, and 7.0 per cent of gelatin.

It is significant that in 1891 Prazmowski particularly emphasized the ability of nodule bacteria to grow, though somewhat more slowly, in a nitrogen-free medium. A similar statement appears in the 1891 paper of Laurent, who recommended the use of a nitrogen-free medium with sucrose, maltose, glucose, or other sources of carbon.

Laurent's Nitrogen-Free Phosphate Solution

Potassium phosphate	1.0 gm.
Magnesium sulphate	0.1 gm.
Sucrose	5.0 gm.
Water (distilled nitrogen-free)	1000.0 cc.

Laurent, 1890 and 1891, also cultivated the nodule bacteria on a medium prepared from the extracts of peas or lupines, with and without gelatin. He noted the occurrence of the bacteroid forms, Y and T shapes, in the slimy deposit at the bottom of his liquid cultures.

Mazé in 1897 devised a bean-infusion medium prepared from haricot beans heated ½ hour at 100° C. (weight of beans and volume of water to be used not specified). Sucrose 2 per cent, NaCl 1 per cent, and a trace of $NaHCO_3$ were added. A modification of this medium as used by Miss Löhnis, 1930a, is prepared as follows:

Navy bean seed (dry)	250 gm.
Tap water (cold)	500 cc.

Soak the beans in cold water two to three hours; then pour off the water and steam in fresh water (2000 cc.) for two to three hours. Filter and make up to volume. Add 1 per cent sucrose and, if desired, 0.5 per cent of $CaCO_3$. This medium contains about 40 mg. of nitrogen in 100 cc.

Zinsser, 1897, found the culture media commonly used unsuitable for the isolation of nodule bacteria. He tried silica gel and found this substrate entirely satisfactory. Three years later, Dawson, 1900b, reported the isolation of nodule bacteria from *Desmodium* and *Pisum* on a silica gel medium containing sugar, ammonium sulphate, potassium phosphate, magnesium sulphate, and calcium chloride. Moore, 1905, and de' Rossi, 1907, also made use of silica gel for the culture of nodule bacteria.

After trying various kinds of culture media, Greig-Smith, 1899, adopted a glucose-peptone solution and a solid medium of glucose-glycerol agar or gelatin.

Glucose-Peptone

Peptone	10.0 gm.
Calcium chloride (crystals)	5.0 gm.
Monopotassium phosphate	2.5 gm.
Glucose	50.0 gm.
Water, tap	1000.0 cc.

Neutralized with KOH until 10 cc. requires 0.7 cc. of 0.1 N. acid.

Glucose-Glycerol Agar or Gelatin

Gelatin	100.0 gm.
or Agar	10.0 gm.
Glucose	20.0 gm.
Glycerol	10.0 gm.
Lupine extract[2]	1000.0 cc.

Add calcium chloride and potassium phosphate and neutralize as described above.

In a later paper, Greig-Smith, 1912, gave the formula of a synthetic medium which he deemed selective for rhizobia.

[2]Lupine extract is prepared by chopping 1000 grams of leaves and stems and boiling with 1000 cc. of water. Allow to boil for several hours, and press out the juice by means of a meat press.

Fructose-Asparagin-Citrate

Fructose	20.0 gm.
Asparagin	0.6 gm.
Sodium citrate	1.0 gm.
Potassium citrate	1.0 gm.
Agar	20.0 gm.
Water, tap	1000.0 cc.

The reaction should be faintly acid. Add 0.06 to 0.1 cc. of 1 N Na_2CO_3 to 10 cc. of the medium when used.

The value of a truly "selective" medium would be great. By its use it would be possible to determine directly the approximate number of the nodule bacteria in any soil, without resorting to the long and laborious procedure of selecting probable cultures and testing them for ability to form nodules on appropriate host plants. Careful tests carried out by Kellerman and Leonard, 1913, unfortunately failed to support the claims of Greig-Smith. They found that the nodule bacteria grew sparingly on the medium made according to the formula of Greig-Smith and that the medium failed to show any differential characteristics. Kellerman, 1911b, found the following Congo red synthetic medium more selective for rhizobia than the rich nitrogenous medium described by Greig-Smith.

Congo-Red Agar

Agar	15.0 gm.
Sugar	10.0 gm.
Monopotassium phosphate	1.0 gm.
Magnesium sulphate	0.2 gm.
Congo red	0.1 gm.
Water	1000.0 cc.

Neumann, 1902a, made a study of the influence of the composition of culture medium on the production of branched forms by the organisms from *Vicia Faba*. In all, he used 70 different kinds of culture media prepared from urine, extracts of roots, leaves, seeds, and soil, with and without nitrates and various carbon sources. Unfortunately, he failed to find a medium which would regularly give branched forms and concluded that in culture solutions the bacteroids are transition forms found only at certain periods of growth.

Hiltner and Störmer, 1903a, and Hiltner, 1904a, recommended the use of two kinds of culture media for the two groups of nodule bacteria. For Group 1, to which belong the bacteria from alfalfa, clover, common bean, locust, sweet pea, pea, and vetch, they suggested:

Group 1
Rhizobium radicicola

Agar	15.0 gm.
Root extract	2.0 gm.
Glucose	10.0 gm.
Asparagin	1 to 2 gm.
Water	1000.0 cc.

Gelatin may be used in place of the agar.

Group 2 includes the organisms from soybean, lupine, serradella, and Genista.

Group 2
Rhizobium Beijerinckii

Agar	15.0 gm.
Root extract[3]	2.0 gm.
Glucose	10.0 gm.
Calcium carbonate	in excess
Water	1000.0 cc.

Gelatin can not replace the agar.

In a United States Letters Patent No. 755,519 taken out in 1904 and in later publications from the United States Department of Agriculture, 1905, Moore emphasized the great value of culturing the nodule bacteria in a medium free from combined nitrogen. Instead of selecting the medium giving the most abundant growth, he believed it best to use a medium which would give a fair growth and at the same time retain or increase the ability of the nodule organisms to fix nitrogen. An agar medium without added nitrogen as suggested by Moore follows:

Phosphate-Maltose Agar

Agar	10.0 gm.
Maltose	10.0 gm.
Monopotassium phosphate	1.0 gm.
Magnesium sulphate	0.2 gm.
Water, distilled	1000.0 cc.

He maintained that cultures grown on this medium are more active in the assimilation of nitrogen than similar cultures grown on a rich nitrogenous medium. A liquid medium of very similar nutrient content was also recommended by Moore, 1905.

Phosphate-Sucrose Solution

Potassium phosphate	1.0 gm.
Magnesium sulphate	0.2 gm.
Sucrose	10.0 gm.
Water	1000.0 cc.

It will be noted that this formula is rather like one suggested by Laurent in 1891.

Löhnis, 1905, used a soil-extract medium in which he obtained a good growth of the nodule organisms from red clover and vetch.

Soil-extract, Glucose or Mannitol

Glucose or Mannitol	10.0 gm.
Dipotassium phosphate	0.5 gm.
Stock soil extract[4]	1000.0 cc.

[3]This extract is prepared from the roots of the desired leguminous plant and contains 2 per cent of dry substance. The extract should be made from the species of plant from which the bacteria are to be isolated.

[4]Stock Soil Extract—Heat 1000 grams of a rich garden or field soil with 1000 cc. of water for 30 minutes in an autoclave at 15 to 20 pounds steam pressure. Filter (if turbid add a small amount of talc) and make up to 1000 cc.

The advantages of nitrogen-free media were also stressed by Ferguson, 1906. This investigator prepared liquid cultures for seed inoculation, for which he secured the inocula without plating by direct transfer of material from the center of superficially sterilized nodules.

The Canadian investigators, Harrison and Barlow, 1907, described in detail methods for the isolation of the root nodule bacteria. They found a substrate prepared from maltose and wood ashes very suitable for these organisms.

Ash Maltose Agar

Ash (maple, beech, elm, etc.)	50.0 gm.
Maltose	10.0 gm.
Agar	10.0 gm.
Water, tap	1000.0 cc.

The reaction should be slightly alkaline. Add the ashes to cold water, stir well and boil for at least one minute, then filter through absorbent cotton.

Because of variations in the composition of ashes from different plants, this medium is not uniform.

Zipfel, 1911, recommended a medium prepared from the seed of leguminous plants.

Leguminous Seed-extract Agar

Agar	30.0 gm.
Glucose	20.0 gm.
Extract of leguminous seed[5]	1000.0 cc.

The reaction should be slightly acid. Zipfel recommended the addition of 10 cc. of 1 N malic acid to 1 liter of the medium.

The following root-extract medium, one of several which have been used, is provisionally recommended by the authors.

Root-extract Agar

Dipotassium phosphate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.2 gm.
Manganese chloride	0.01 gm.
Ferric chloride	0.01 gm.
Calcium carbonate	3.0 gm.
Mannitol or sucrose	10.0 gm.

Prepare the above salt mixture and carbohydrate, weighing quantities for one liter; dissolve in 500 cc. of distilled water; add 500 cc. of root extract, and if a solid medium is desired, 12.5 to 15 gm. of agar. The root extract is prepared from ground, dry root tissue such as that of alfalfa (roots from the field may be

[5]Weigh out 20.0 gm. of seed; powder in a mortar and add 20 cc. of 1 N KOH. Rub well, make up to 1000 cc. and allow to stand for 24 hours. Now siphon off the clear liquid, neutralize with phosphoric acid and make up to 1000 cc.

dried in the fall and stored for year-round use). Boil in excess of distilled water for one hour and allow to stand over night; filter through cotton; and dry an aliquot portion to determine the approximate content of dry matter. About 2-4 gm. of dry matter per liter is best; dilute if necessary. To approximate the desired concentration in the extract, 10-15 gm. of root powder in 1000 cc. of water for the original infusion are suggested.

Instead of the extract of leguminous plants, pods, or roots, Müller and Stapp, 1925, obtained excellent growth of the various cultures of the root-nodule bacteria on carrot-extract agar. Their method of preparing this medium follows:

Wash the carrots in running water until all soil particles are removed, then chop (meat chopper) into small pieces and prepare as follows:

Carrot-extract Agar

Carrots	250 gms.
Water, tap	500 cc.

Cook in a steamer for 30 minutes and filter. Neutralize to pH 7.0 — 7.2 with a strong solution of sodium carbonate (avoid NaOH) and make up to 500 cc. Now add 500 cc. of a 3.6 per cent neutral water solution of agar. Tube and sterilize. Avoid excessive heating, because the carrot extract becomes more acid after long heating.

Allison, 1927, carried out a study of the growth-promoting properties of various plant extracts for the root-nodule bacteria. He found that the extracts of 26 different leguminous and non-leguminous materials including vegetables, fruits, and field crops all increase bacterial growth providing they are used in the proper concentration. This beneficial effect varied widely for different plants and different parts of the same plant; the extracts of leguminous crops particularly favored the growth of the nodule bacteria. Of the non-leguminous extracts, those from lettuce, cabbage, carrots, spinach, tomato, green corn tops, blue grass, and oranges gave the best results.

From a review of the various studies carried out with the nodule bacteria, it is well established that plant or soil extract is not essential for the growth of these organisms. Many kinds of synthetic culture media may be used. Perhaps one of the best-known examples is the nitrogen-free mannitol medium of Ashby, 1907.

Mannitol-Phosphate Solution (Ashby modified)

Mannitol	10.0 gm.
Magnesium sulphate	0.2 gm.
Monopotassium phosphate	0.2 gm.
Sodium chloride	0.2 gm.
Calcium sulphate	0.1 gm.
Calcium carbonate	5.0 gm.
Water, distilled	1000.0 cc.

Dissolve the phosphate separately in a little water, and make the solution neutral to phenolphthalein with 1 N NaOH, then add to the other ingredients. For a solid medium add 12.5 to 15 gm. of agar to each liter.

In order to secure a more abundant growth, various modifications of this nitrogen-free synthetic agar medium have been proposed. The addition of a small amount of an inorganic nitrogenous salt, the water extract of germinated seedlings or the extract of pressed yeast have all been found of decided value. Two examples are given.

Nitrate-Mannitol Agar

Mannitol	10.0 gm.
Potassium nitrate	0.5 gm.
Dipotassium phosphate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.2 gm.
Manganese sulphate	trace
Ferric chloride	trace
Water, distilled	1000.0 cc.

For a solid medium add 12.5 to 15 gm. of washed agar to each liter.

Yeast-extract-Mannitol Agar

Agar	15.0 gm.
Mannitol	10.0 gm.
Dipotassium phosphate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.1 gm.
Calcium carbonate	3.0 gm.
Yeast water (Reaction pH 6.8)	100.0 cc.
Water, distilled	900.0 cc.

Prepare a water extract of starch-free bakers' yeast, 10 per cent (wet basis). Mix the yeast with cold water and let stand at room temperature for 1 to 2 hours. Then autoclave for 40 to 60 minutes and allow to settle for several days.

Repeatedly it has been the experience of the authors that the addition of neutral yeast extract greatly increases the rate of growth of all groups of the nodule bacteria.

Of the almost innumerable formulae for culture media recommended for the rhizobia, the authors have found several which appear to be superior. They are of three general types and are to be recommended for different purposes such as maintenance of stock cultures, study of fermentation characteristics, gum production, and so on. The yeast-extract mannitol medium, above, is used perhaps most extensively in the Wisconsin laboratories and that for a four-fold reason; first, it supports very good growth of all species of the rhizobia; second, it is easy to prepare; third, it is easy to sterilize; and fourth, it is uniform in composition and may be depended upon for reproducible results. This medium or a slight modification of it (with sucrose replacing the mannitol and with the yeast extract reduced to 10 cc. per liter) is commonly used in our laboratories for carrying stock cultures. For fermentation tests the nitrate-salt medium, above, has been found advantageous. The soil-extract medium of Löhnis, p. 38, is also very useful. For special work, such as study of gum production, the bean-extract formula, p.

36, is to be recommended, as are also other plant-extract media, p. 39, for their growth-stimulating properties.

The use of dyes in media for the rhizobia. It is sometimes desirable in isolating rhizobia or in testing the purity of laboratory cultures to use media containing certain dyes. Generally speaking, the best basis for a dye medium is a synthetic substrate such as the mannitol-nitrate agar described above, since growth upon it is not too abundant and the color-absorption reactions are more delicate. One of the most commonly used dye media is a Congo red agar, prepared by adding just before tubing 10 cc. of a 1:400 aqueous solution of the dye to the synthetic mannitol-nitrate medium above referred to. According to Kellerman, 1911b, Congo red agar offers a means of detecting a frequent contaminant, *Bact. tumefaciens,* since it produces deep red colonies in contrast to the whitish colonies of the true rhizobia. More recent tests of the absorption of Congo red by the crown gall, root nodule, and *B. radiobacter* cultures show that there is only slight difference between the members of these three groups of organisms. Absorption of Congo red by many of the common soil bacteria is, however, much more marked, and hence Congo red in the plating medium for rhizobia is by no mean worthless.

A number of other dye media have been proposed for the differentiation of *B. radiobacter,* another contaminant often met in work with rhizobia (Idaho Bulletin, 1928; Ruehle, 1928; Anderson, 1929; and others). Perhaps the most recent suggestion of the kind is that of Leonard (1931 and personal communication) in which use is made of a glycerol agar containing crystal violet. The medium is:

Soil extract[6]	1000.0 cc.
Dipotassium phosphate	1.0 gm.
Sodium nitrate	1.0 gm.
Glycerol	10.0 gm.
Agar	10.0—12.5 gm.

The reaction is usually alkaline; 1 N HCl should be added to pH of 6.8. The dye, crystal violet, should be used in 1:80,000 concentration and for best results should be added aseptically after sterilization. On plates of this medium *B. radiobacter* produces large colonies with deep blue or violet centers and clear rims. Colonies of rhizobia are small and evenly colored throughout.

Brom thymol blue agar is of value in quite a different way. It offers an easy and accurate means of detecting change in reaction of the medium and is thus an index to the fermentation characters of the organism growing therein. A detailed description of this use will be found in the paper by Baldwin and Fred, 1927. It is recommended that the indicator be added as 5 cc. of a 0.5 per cent alcoholic solution per liter of medium.

More details concerning special media to be used for studies of the physiology of rhizobia will be found in Chapter 6. In closing this section relating to culture media, attention is called to the effect of long cultivation on the efficiency of the nodule bacteria. This is an important practical question, as it may conceivably be one of the factors affecting the value of commercial cultures for

[6]Soil extract. This is prepared by heating 1000 gm. of garden soil with 1000 cc. of tap water in the autoclave for 30 minutes. A small amount of calcium carbonate is added, and the whole is filtered through a double filter paper. The turbid filtrate should be poured back on the filter until it comes through clear.

the inoculation of leguminous plants. Unfortunately bacteriologists are not yet agreed as to what *is* the effect on the rhizobia of cultivation in artificial media. Several investigators, Gonnermann, 1894; Nobbe and Hiltner, 1893 and 1899c; Frank, 1898; Hiltner, 1900a, for example, have claimed that gelatin in media has a marked effect on the bacteria and in some cases causes a change in the "virulence." Simon, 1907, also reported a distinctly toxic effect of gelatin medium and in 1908-09 recommended sterilized soil as the ideal substrate for long time culture of rhizobia. A similar statement was made by Barthel, 1919, to the effect that "virulence" of rhizobial cultures is unchanged by long culture on sterilized soil. Moore, 1902, 1904, and 1905, spoke of the bad effects of any medium rich in combined nitrogen and advised a practically N-free medium for the maintenance of virulent cultures. Prazmowski, 1890; Laurent, 1891; Zinsser, 1897; and Dawson, 1900b, all recommended media poor in nitrogen, while Greig-Smith, 1912; Kellerman, 1911a; Ferguson, 1906; and Harrison and Barlow, 1907, actually used synthetic media with no added nitrogen. On the other hand, Hiltner and Störmer, 1903a, asserted that extracts of leguminous plants may counteract to some extent the toxic effects of gelatin and exert a favorable effect on the cultures. Since then numerous investigators have successfully used plant-extract media. One of the best of these in the opinion of the authors is the yeast-extract mannitol agar cited above. It has been their personal experience that stock cultures carried on this medium, in some cases for more than 17 years, are unchanged as far as virulence or effectiveness in nodulation is concerned.

THE TECHNIQUE OF ISOLATION OF RHIZOBIA FROM NODULES

The bacteria of the root nodules of the common leguminous plants are not difficult to isolate, providing certain conditions are observed. In the selection of the nodule, one should consider the general vigor of the plant as compared with near-by plants and also the condition of the nodule. The plant should be young, of a dark green color, and apparently well supplied with nitrogen. The nodule should be plump, healthy, and either rosy or flesh colored. A grayish or brownish nodule is usually old and should be avoided.

Hiltner, 1904b, claimed that especially active cultures are to be obtained from nodules of plants grown several years in the same location. He thought that in this way an increase of efficiency of the organism resulted from repeated plant passage. For later reports on this subject, see the papers of Wunschik, 1925; Allen and Baldwin, 1931a.

Assuming that some healthy young nodules have been found, the next step, and one of great importance, is to remove outside contamination by thorough washing. This may be accomplished by washing with a brush or with a strong stream of water, the nodules being held in a Gooch crucible.

After the nodules are washed, remove a nodule with forceps and immerse in a solution of mercuric chloride.

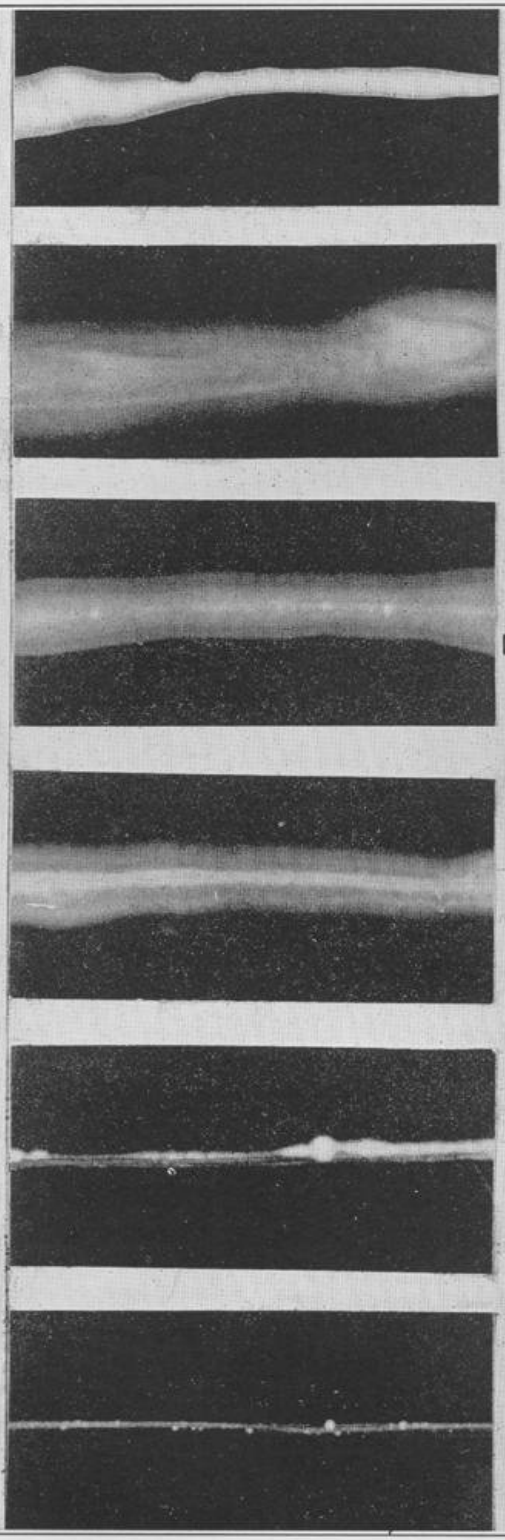
Hydrochloric acid	2.5 cc.
Mercuric chloride	1.0 gm.
Water, distilled or tap	500.0 cc.

If the nodule floats, it must be held beneath the surface of the liquid. About 2 to 5 minutes, depending upon the nature of the surface of the nodule, is usually sufficient to destroy surface organisms. Naturally, large nodules may be held in the solution much longer without injury to the nodule contents; e.g., soybean or cowpea nodules may safely remain in the chloride solution for more than 30 minutes. If the nodules have been washed in a Gooch crucible, it will be found convenient to dip the whole vessel with the nodules into a large dish containing the $HgCl_2$ solution, then into successive dishes of sterilized water. A sterilized dilute solution of ammonium sulphide may be used to remove the remaining $HgCl_2$ in order that there may be no danger of its adverse effect on the bacteria when the nodule is opened. With sterile forceps pick up the nodule. Cut away one side with a red hot chisel-shaped needle. A platinum-iridium needle, which has been flattened by hammering and filing the end, will be found most satisfactory. If this chisel is then thrust into the nodule tissue near the center of the broken surface and rotated slightly, it is comparatively easy to secure masses of the bacteria free of any outside contamination. The material is transferred to a drop of sterilized water in a petri dish or directly to agar melted and cooled to 40° C. Sometimes it is preferable to spread the culture on the surface of the agar medium by means of a bent glass rod, Drigalski spatula. If the solid agar method is followed, the same glass rod is then rubbed on the surface of several petri dishes. De'Rossi, 1907, strongly recommended this method of spreading on the surface of the hardened agar. Greig-Smith, 1899, advised that the emulsion from the nodule be distributed with a sterilized camel's hair brush over the surface of the solid culture medium.

If the dilution is carried out in liquid, either melted agar or sterilized water, it is well to make two or more loop transfers from the first petri dish to two or three others in series. Now add the desired agar and mix thoroughly before allowing to cool. After the agar has solidified, label, invert the plates, and incubate under a bell jar at room temperature. The relatively low temperature of incubation, approximately 20° C., is especially desirable because the rhizobia grow well at this range, while many contaminating organisms are somewhat retarded.

A rather different method of isolation was described by Zinsser in 1897. After the removal of surface contamination by washing in water, then in alcohol, and then in mercuric chloride solution (1:1000) for 10 minutes, he again washed the nodules in water and then ground them in a sterilized mortar. From a suspension of the bacteria prepared in this way, Zinsser inoculated plates of silica gel. The plates were incubated for 8 days and the colonies fished off. Richmond, 1926a, described an ingenious way of getting bacteria from the interior of a nodule, from which it is desired to make isolation. The nodule is imbedded in paraffin which is trimmed down to convenient size for handling; the block is then broken or cut in two across the nodule and material from the interior of the nodule removed by sterile needle and used for dilution plates in the usual manner.

Colony formation. The shape, size, and appearance of the colonies depend upon the source of the culture, their position in the solid medium, and the kind of medium, as well as the age of the colonies. In general, colonies of the alfalfa, clover, pea, and bean groups grow rapidly on agar plates, producing well-defined surface colonies in 5 to 7 days, while the soybean, cowpea, and lupine groups grow


RHIZOBIUM
MELILOTI
RHIZOBIUM
TRIFOLII
RHIZOBIUM
LEGUMINOSARUM
RHIZOBIUM
PHASEOLI
RHIZOBIUM
JAPONICUM
RHIZOBIUM
LUPINI

slowly, often requiring 10 to 20 days before the colonies are large enough to pick. Plate 10 shows the relative growth of alfalfa, clover, pea, garden bean, soybean, and lupine bacteria.

The well-isolated colonies on the surface of agar are raised, wet, shining, and somewhat translucent, with smooth edges, while the imbedded colonies are lens-shaped or elliptical, gradually becoming more turbid and opaque with age. The colonies grow steadily for a long period of time; those of the slowly growing group, however, never attain the size of the others. The consistency of these colonies varies according to the plant species. Those from red clover nodules are decidedly mucilaginous, especially when old; often they become so viscid that they may be drawn out in long threads when touched with the needle. The colonies ordinarily exhibit no chromogenesis, although they may appear somewhat chalky or opaque, depending upon the group and the age of the cultures. The brownish color which develops in old cultures of the slow-growing group may be due to the production of tyrosinase. Wunschik, 1925, found that the colonies of bacteria from clover nodules on clover-extract medium are at first an opaque, grayish color and later a light brown. The yellowish and pinkish colors noted by certain investigators are probably due to contamination. Variations in growth are to some extent recognizable among organisms of the several cross-inoculation groups, but it is unwise to place too great emphasis on any such methods of differentiation.

A recent paper by Israilsky and Starygin, 1930, pointed out that the rhizobia may exhibit the dissociation phenomenon, and that "rough" as well as "smooth" cultures exist. The usual form of the organism, which has been described above, is the "smooth" culture. The "rough" variants of the cultures studied by Israilsky and Starygin differ in physiological as well as cultural characters from the "smooth" cultures. After 4 to 5 days' growth on bean-extract media, smooth cultures show a large proportion of bacteroids, whereas rough cultures show few if any. The relationship of this variation to the efficiency of the organisms in aiding plant growth has not been studied.

THE TECHNIQUE OF ISOLATION FROM SOIL

The isolation of free-living rhizobia directly from soil is much more difficult than from the nodule. Because of the resemblance of the nodule bacteria to other bacteria commonly present in soil, and because of the absence of any well-defined colony characteristic, only a few investigators have secured cultures in this way. Unfortunately no one has as yet devised a medium highly specific for rhizobia; Greig-Smith, 1912, claimed to have found such a medium, but as noted above, later tests did not agree with his findings.

Beijerinck, 1888, reported the isolation of nodule bacteria from soil and water. He called attention to the fact that the small size of the organism and the lack of definite cultural characters made direct isolations from soil difficult. No further details of his studies are given.

A report from Mazé, 1898, is of interest in this connection. He was unable to isolate nodule-forming bacteria directly from soil, but he did obtain a spore-

forming organism which he called Bacillus *a*. By artificial culture he claims to have transformed Bacillus *a* to forms *b* and *c*, which he thought to be actually two different forms of the nodule bacteria, although their cultural and physiological characteristics differ in several respects. Forms *a*, *b*, and *c* alone did not form nodules in Mazé's experiments, but a mixture of *b* + *c* is said to have produced nodules on vetch plants.

In 1891 the German investigators, Nobbe, Schmid, Hiltner, and Hotter, prepared a gelatin medium containing pea extract, glucose, and asparagin according to the formula of Beijerinck and succeeded in the isolation of cultures of the root-nodule bacteria of *Pisum sativum, Robinia Pseudacacia, Cytisus laburnum,* and *Gleditsia triacanthos*[7] directly from soil. Budinov, 1907, described an ingenious way of enriching rhizobia from soil by means of capillary tubes. His method has been used with excellent success by Allen and Baldwin, 1931b. Capillary tubes approximately 1 mm. in diameter and 50 to 60 mm. in length are sealed at one end and autoclaved in a dish of the medium (*i.e.* yeast-water mannitol). These tubes, filled with the sterile medium, are then suspended through corks with the open ends down in a water suspension (1-100) of the soil to be examined. The contents of one of these tubes are plated after 1 hour, of others after 12 and 24 hours on a brom-thymol-blue yeast-water mannitol agar. The plates poured from the capillary tubes after a period of 12 hours showed the largest percentage of colonies resembling the rhizobia, frequently as high as 90 per cent. Such colonies were picked and tested culturally and for nodulation on suitable host plants. In this way a number of cultures of *Rhizobium trifolii* and *Rh. meliloti* were secured. The Budinov method was not widely known after its appearance in 1907, and reports of failure or great difficulty in the isolation of rhizobia from soil continued to appear.

In this connection the report of Kellerman and Leonard, 1913, is of interest. By means of Greig-Smith's technique, Kellerman and Leonard attempted to isolate the nodule bacteria directly from three types of soil: (1) soil from greenhouse, (2) soil from around the roots of *Astragalus falcatus,* and (3) soil which had been sterilized and inoculated with the bacteria from alfalfa nodules. From colonies which resembled pure cultures of rhizobia, selections were made and tested for their ability to infect the proper host plant. They were unable to secure nodules except in the case of number 3, the sterilized soil to which alfalfa bacteria had been added. They concluded that the Greig-Smith medium is no more selective than the synthetic agar commonly employed for rhizobia, and that improvements in technique and culture media must be made before it will be possible to secure reliable data concerning the distribution of the nodule bacteria in soil.

The California investigators, Lipman and Fowler, 1915, poured dilution plates (soil-extract maltose agar) of soil which had previously grown *Vicia sicula.* From the colonies which appeared to be characteristic of rhizobia, they made 44 transfers to slants of soil-extract agar. Of these 44 cultures tested for their power to form nodules, 21 gave positive results, that is, induced nodule formation.

Joshi, 1920, isolated the nodule bacteria of *Crotalaria juncea, Cajanus indicus, Vigna catjang,* and *Phaseolus aconitifolius* directly from soil. He used four dif-

[7]This culture never produced nodules on the honey locust, however.

ferent kinds of media, of which soil-extract mannitol agar seemed most suitable, although all of the media used gave colonies other than rhizobia.

In a paper dealing chiefly with serological investigations of the root-nodule bacteria, Vogel and Zipfel, 1921, claimed to have isolated directly from the soil three cultures of bean bacteria and four cultures of pea bacteria. The soil samples for their isolations were drawn near the roots of the respective plants and after dilution plated out on glucose-leguminous-seed-extract agar. Since they relied only on serological tests to prove the identity of their cultures, it is not certain that they obtained bacteria capable of producing nodules on the host plants claimed.

Müller and Stapp, 1925, made use of both liquid and solid media in their attempts to isolate root-nodule bacteria directly from soil. After several unsuccessful efforts, they were forced to the conclusion that no suitable method for the direct isolation of rhizobia had been developed. Pohlman, 1931b, arrived at a similar conclusion following his failure to isolate and count rhizobia from soil by means of nitrogen-free or yeast-extract media, with or without the addition of dyes. Nevertheless, the possibility of developing some culture medium or some condition of incubation so highly specific as to favor the growth of rhizobia seems to the writers to be worthy of careful consideration. Several old problems, such as the distribution of rhizobia in nature, longevity under field conditions, the flora of clover-sick soils, and the flora in the immediate vicinity of leguminous plants, call for attention as soon as a dependable technique shall be found. The Budinov method seems to offer possibilities and should be more thoroughly tested.

THE OCCURRENCE OF RHIZOBIA IN THE SEEDS AND STEMS OF LEGUMINOUS PLANTS

Although the evidence is by no means convincing, there are a number of reports in which it is claimed that nodule bacteria may exist in the leaves, stems, and seeds of the host plant. Hiltner, 1887, observed the bacteria in the seeds of clover and peas, and Frank, 1890b, reported the presence of bacteroids in bean seeds. Laurent, 1889, however, denied that microörganisms are present in normal healthy tissues of plants, especially seeds. Beijerinck, 1888, found the bacteria in the stems of nodulated leguminous plants, and more recently Wallin, 1922b, has reported them in leaves, stems, and root tissue. At least the last of these reports is vitiated by confusion of mitochondria with the bacteria. No one who has used fixing and staining methods for differentiating between mitochondria and bacteria is prepared to say that infection extends beyond the limits of the nodule. Yet in another sense, it is not easy to dispute the reports of bacteria within the seed, at least within the seed coat, for it is entirely possible for infection to occur through the micropyle. Such infection, however, cannot be general, for it is comparatively easy to raise nodule-free plants from seeds superficially sterilized with mercuric chloride, alcohol, hypochlorite solutions, or hydrogen peroxide. The ease with which such sterilization is accomplished suggests that the bacteria are present on the surface of the seed coat rather than within it. Wilson, 1929a, has obtained evidence of this in another way. By a process of washing leguminous and other seeds in water and after suitable enrichment of the bacteria thus obtained by culture in sterile soil, he was able to prove the presence of nodule bacteria in the washings by production of nodules on leguminous plants.

CONTAMINANTS IN THE NODULE

Generally speaking, one considers that normal young nodules are free from organisms other than rhizobia, but that old nodules may contain various foreign organisms. Perhaps the evidence in the case is not conclusive in either direction. On the one hand, *B. radiobacter* is very frequently encountered in isolation of nodule bacteria from apparently normal nodules, and Löhnis and Leonard, 1926, have reported that it is "even to be found fairly regularly in nodules of soybeans, cowpeas, and related plants." On the other hand, the finding of a foreign organism in culture does not necessarily prove its origin from the inside of the nodule. Particularly in the case of old nodules, a rough cortex may make it very difficult to remove surface contaminants. It is highly probable that faulty technique may account for some of the contaminants reported. Some of these reports are shown below.

Kind of organism other than Rhizobium

1. Beijerinck, 1888

   Yellow pigment-forming organism, probably *Bacillus luteo-albus,* and a brownish-colored form, *Bacillus agglomerans;* also *Bacillus fluorescens putidus* and *Bacillus Trimethylamin.*

   In 1918, Beijerinck reported the presence of *B. herbicola* "within the living cells" of nodules.

2. Klein, 1894

   Cultures from lupine nodules show two kinds of bacteria.

   1. A motile organism which liquefies gelatin and produces a green color, *Bacillus fluorescens liquefaciens.*
   2. A non-motile rod form which liquefies gelatin very slowly. Perhaps *B. radicicola.*

3. Gonnermann, 1894

   Found ten species of bacteria in the nodule of lupine. He classified these as follows:

   1. *Bacillus fluorescens non-liquefaciens.*
   2. " *tuberigenus* (1)
   3. " " (2)
   4. " " (3)
   5. " " (4)
   6. " " (5)
   7. " " (6)
   8. " " (7)
   9. *Micrococcus tuberigenus* (1)
   10. " " (2)

   Gonnermann believed that the nodule organisms exist as various forms rather than as a single definite species described by Beijerinck.

4. Mazé, 1898 — Found a spore-forming organism, *Bacillus a.*

5. Greig-Smith, 1899 — Reported that other organisms may be found within the nodule, but these may be looked upon as accidental. In the nodules of peas, lupines, and vetches he frequently found an organism similar to *Bacillus megatherium.* From cultural tests Greig-Smith suggested that this *B. megatherium* helps to dissolve the starch in the nodule and thus aids the nutrition of *Rhizobium.* The accidental occurrence of *Bacillus fluorescens liquefaciens* in pea nodules was also reported.

1905 — From the nodules of *Lupinus luteus* Greig-Smith isolated a slime-producing organism which he called *Bacillus alatus* n. sp.

6. Hartleb, 1900 — *Pseudorhizobium ramosum,* an organism very similar to the nodule bacteria but not capable of forming nodules.

7. Burrage, 1901 — Five species of bacteria isolated from the nodules of alsike, buffalo, and red clover, vetch, and beans.

8. Schneider, 1902 — Motile bacillus resembling *B. anthracis* or *B. subtilis.*

9. Hiltner and Störmer, 1903a — Found contaminating organisms within the nodule. One of these contaminants is quite similar to rhizobia. This foreign species grows slowly on gelatin medium and does not cause liquefaction. It produces a very tenacious slime which holds the cells in the form of the colony.

10. Lewis and Nicholson, 1905 — Found on plates from a cowpea nodule an organism with the same general characteristics as rhizobia, except that it produces a scum and does not form nodules.

11. Heinze, 1907 — Found microörganisms belonging to the genera *Clostridium* and *Plectridium* regularly in serradella and more rarely in lupine nodules.

12. Rodella, 1907 — *Cl. Pastorianum* or an organism closely related to this species regularly present.

13. De'Rossi, 1907	Found four species of bacteria in the nodules of *Vicia Faba.* 1. Small motile non-spore-forming rods which do not liquefy gelatin (found 3 times). 2. Small thick capsulated non-motile rods, other characteristics as for No. 1 (found once). 3. Short, thin rods, motile, forming yellow colonies (found twice). 4. Small, very thin non-motile rods, other characters as for No. 1 (found once).
14. Simon, 1907	Reported that certain bacteria commonly present in the soil in comparatively small numbers may enter the nodules and, because of the favorable conditions, multiply rapidly. These normally saprophytic forms now become parasitic, feeding upon the contents of the nodule, perhaps at the expense of the rhizobia. The suggestion is offered that in certain cases soil sickness may be due to the enormous development of these contaminants of the nodule.
15. Löhnis and Westermann, 1909	Plates poured from the nodules of vetch frequently show colonies of *Bacillus danicus.*
16. Georgevitch, 1910	Described two kinds of bacteria from *Vicia sativa* nodules: a short motile spore-former and a long, immotile non-spore-former.
17. Simon, 1913	Contaminating bacteria present in nodules.
18. Manns and Goheen, 1916	In plating out old, but apparently sound, nodules, found *Aztobacter.*
19. Löhnis and Hansen, 1921	*B. radiobacter* regularly present.
20. Beijerinck and den Dooren de Jong, 1922	*B. polymyxa* commonly found in the bark of the nodules of Leguminosae.
21. Fehér and Bokor, 1926	Found in the root nodules of *Amorpha fruticosa* two organisms living symbiotically, *Bacterium radicicola* and *Bacillus mycoides* (Flügge).

22. Löhnis and Leonard, 1926	*B. radiobacter.*
23. Skinner, 1928	*B. radiobacter* often found.

B. radiobacter is by far the most frequent organism found as contaminant in isolations from the nodule. Furthermore, it is difficult to distinguish from the nodule organism, for its general appearance and cultural characteristics are very similar; but there are certain tests which may be used for differentiating. In plain milk *B. radiobacter* usually produces a brown color; litmus, if present in the milk, is slowly reduced, and later the brown coloration appears. On potato there is a distinctly brown color. *B. radiobacter* is in general marked by more vigorous growth, and by ability to produce much acidity from various carbon compounds. The recently proposed medium of Leonard (p. 42) is also useful in detecting *B. radiobacter* in mixture with rhizobia. Upon his crystal violet agar *B. radiobacter* produces large colonies with deep blue or violet centers and clear rims, whereas colonies of rhizobia are small and evenly colored.

CHAPTER 5

THE MORPHOLOGY AND LIFE CYCLE OF THE ROOT NODULE BACTERIA

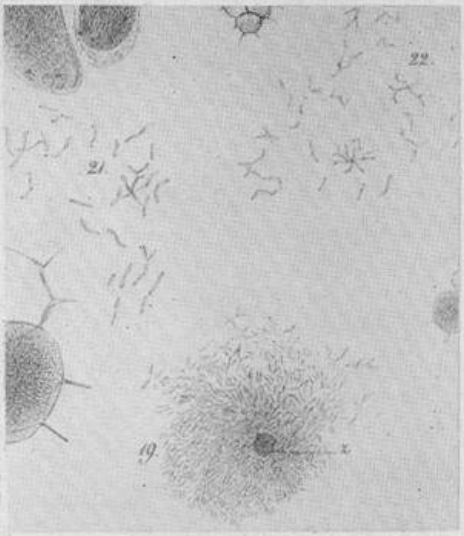
"Occasionally, and frequently, the exercise of the judgment ought to end in absolute reservation. We are not infallible, and so ought to be cautious."
—FARADAY

MORPHOLOGY

Probably the first observations regarding the morphology of the organisms responsible for nodule formation were those of Lachmann, 1858. He noted that the inner cells of the nodules are filled with numerous small, elongated bodies resembling the so-called vibrios. Other cells showed a mass of very fine granular bodies. A few years later, 1866, Woronin described these bodies more fully. He reported that the nodules of *Lupinus mutabilis* are filled with "vibrio-like bodies," 0.0016 — 0.0028 mm. in size. While Woronin stated that he was never successful in cultivating these bodies in either water or sugar solution, he did observe reproduction of the individual bodies suspended in water. When first placed in water they are independently motile, but after 3-20 hours lose their motility and come to rest. "Soon after coming to rest, they show changes of a characteristic type. At first they lengthen and then break up into separate small particles, which also have the appearance of rods; or they produce buds, which either separate from each other at once, or frequently remain hanging together for a long time, forming short, thin, rosary-like strings or small clusters, as is to be seen from figures 21 and 22" (in Plate 11). Although Woronin did not definitely state that these bodies were bacteria and the agents responsible for nodule formation, his observations of their motility and methods of reproduction are very suggestive.

The next recorded observations on the morphology of the organisms are those of Eriksson, 1873, who observed "bacteria-like" bodies in the older cells of the nodule tissue. He also noted the infection threads, which he considered to be mold hyphae and the cause of nodule formation. He did not attempt to answer the question of the relationship existing between the mold hyphae and the "bacteria-like" bodies. He did note, however, that the hyphae are not present in the older cells of the nodule which are filled with the bacteria-like bodies. His figures, No. 40 and 41 (in Plate 11), of these bodies from the nodules of *Vicia Faba* are very characteristic of the forms occurring in the nodules.

Beijerinck, 1888, isolated and cultivated the organisms from the nodules of a number of species of leguminous plants. In his studies Beijerinck noted the presence of several different morphological forms of the organism. He recognized the usual rod forms, 1μ by 4μ, and small, ovoid, or irregular forms, 0.18μ by 0.9μ, which he called swarmers, and lastly the irregular vacuolated forms or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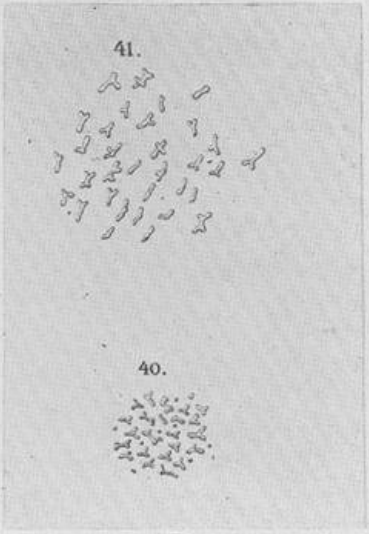
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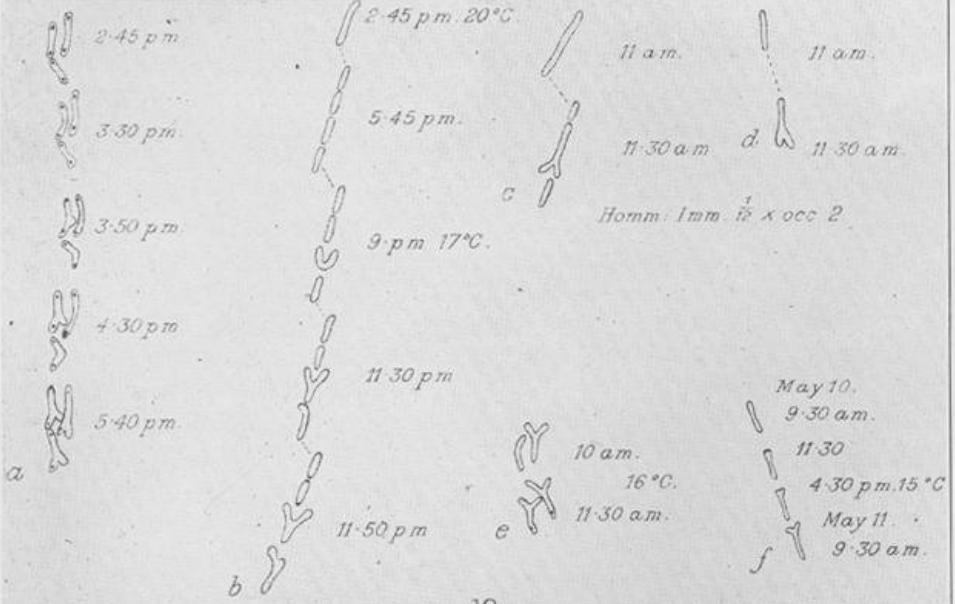
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11·50 p.m.
b
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c
11 a.m.
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d
Homm. 1mm. 1/12 x occ. 2.
10 a.m.
16°C.
11·30 a.m.
e
May 10.
9·30 a.m.
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May 11.
9·30 a.m.
f

12

C

bacteroids. These forms were all observed in the nodules and also in cultures grown on a *Vicia Faba* stem-extract gelatin medium. Later, 1890, he reported the presence of star-shaped forms on a gelatin medium containing peptone, asparagin, cane sugar, and extract of *Vicia Faba* stems. He stated that these stars arose in a manner similar to that in the *Actinomyces,* to which he thought the nodule bacteria closely related.

The pleomorphism of the organism both in the nodule and in culture media has been established by many workers. The form varies from small, round bodies, 0.4μ in diameter, through simple rod forms, 0.8μ by 2-4μ, to larger irregular organisms up to 6μ. The younger cells are motile by means of one or more flagella. The cells are Gram negative, and when stained with the common aniline dyes, they often present a vacuolated appearance. The occurrence and significance of the various forms and the flagellation and staining characters will be discussed in more detail under separate headings.

Infection threads and bacteroids. From the date of Woronin's paper, 1866, until after the appearance of Beijerinck's, 1888, and Prazmowski's, 1890, there was little agreement as to the nature of the nodules or of the agents responsible for their formation. Of those who held that the nodules were formed as a result of outside infection, the majority believed that the so-called infection threads running through the nodules were the hyphae of a fungus. Nodule studies by Beijerinck, 1888, with his study of the organism in pure culture, and by Prazmowski, 1890, with the production of nodules by pure-culture inoculations, demonstrated that the threads are not the hyphae of a true fungus, but rather a product of the bacteria or of an interaction between the bacteria and the host plant. Under suitable conditions the bacteria can be seen within the thread. Little definite information is available, however, as to the way in which the infection threads are formed or as to their chemical nature. A more detailed account of these structures is presented in Chapter 9.

The irregular bacterial bodies tightly packed in the inner cells of the nodules were referred to by a variety of names in the early literature. Woronin, 1866, spoke of them as "vibrio-ähnlichen Organism" and "vibrio-ähnlichen Körper." Frank, 1879, called them "Sprösszellchen." Kny, 1879a, and Prillieux, 1879, thought that the strands running through the cells were plasmodial strands similar to those of *Plasmodiophora Brassicae,* and that the independent bodies in the cells were the spores. In 1885a, Brunchorst applied the term "bacteroid," which has been almost universally used since that time. There has, however, not been complete agreement as to its definition. Brunchorst applied the name to "the organized protein bodies of the full grown cells of the nodules, the 'bacteria-like bodies', 'Sprösszellchen' etc.," which according to his views were natural protoplasmic formations of the plant. He definitely recognized differences in the size and shape of the bacteroids as follows:

> Die aus Knöllchen verschiedener Leguminosen gewonnenen Bacteroiden sind nicht von derselben Form. Bei den meisten sind sie wohl einfach, langgestreckt; bei anderen aber verzweigt durch eine Art Sprossung (daher der Name Frank's "Sprössellchen"), wodurch sie Q-Form annehmen oder gar das Bild zweier in irgend einer Weise mit einander verbundenen Y-s zeigen. Neben diesen zusammengesetzten Formen kommt aber in denselben Zellen

auch die ganz einfache Stabform vor, was auf Theilung der Körperchen hindeutet. Auch aus dem Grunde ist eine Vermehrung der Körperchen durch Theilung anzunehmen, weil die Zellen, wo sie zuerst auftreten, klein, sind sich Später aber stark ausdehnen, und auch nach der Vergrösserung gleich dicht, mit den Bacteroiden erfüllt sind, wie vorhin. Bei weider anderen Spezies sind die Körperchen rundlich, auch die Semmelform kommt daneben vor, was ebenfalls wieder auf Theilung deutet. Die Grösse der Körperchen ist ziemlich verschieden bei verschiedenen Species.

Dangeard, 1926, used the term to apply only to branched forms. In lupine, soybean, and garden bean nodules, branched forms do not occur, and Dangeard would not consider the enlarged vacuolated forms of these bacteria as bacteroids.

Certain investigators, Zipfel, 1911, and Barthel, 1921, felt that there is a physiological difference between the irregular forms in the nodule and those produced on the ordinary culture media. Those found in the nodules are called the true bacteroids and are thought to possess the power of reproduction. The irregular forms found in ordinary culture media are considered to be either carried over directly from the nodule, or true involution forms (dead or dying) which are produced as a result of unfavorable conditions. By the addition of certain alkaloids, caffein, etc., to the media, Zipfel and Barthel were able to produce forms which they felt to be identical with the true bacteroids of the nodule.

Löhnis and Smith, 1916, objected to the use of the term "bacteroid" for the irregular forms of the organism, since it is now generally recognized that they are forms of the bacteria and not plant products resembling bacteria. In spite of the confusion regarding the use and meaning of the term, it seems desirable to retain it because of its established place in the literature and because of the lack of a preferable term to replace it. The term is therefore used by the authors to designate the enlarged, frequently club-shaped or branched, vacuolated or banded forms of the root nodule bacteria, both as they occur in the nodule and in culture media.

Viability of the bacteroids. For a number of years after the true bacterial nature of the organism causing nodule formation was recognized, discussion continued as to the viability of the bacteroids.

Beijerinck, 1888, believed the bacteroids to be metamorphosed bacteria which have lost their power of development but function as organized protein bodies. Prazmowski, 1888, published the first of a series of papers in which he stated that the bacteroids may produce simple rods which in turn develop into bacteroids. Later, 1890, he agreed with Beijerinck that the bacteroids are involution forms incapable of growth, and that the appearance of these abnormal branched forms is usually the first indication of the death of the bacteria.

Earlier than this, Tschirch, 1887, had decided that the bacteroids were not bacterial in nature, since he was unable to cultivate them as such in any of the media he tried. He stated, however, that due to the difficulty in maintaining aseptic conditions, it was not uncommon to find the development of contaminating rod forms. It is probable that he had the true nodule bacteria in some of his cultures, but failed to recognize them because he was searching for branched forms as in nodule tissues.

Frank, 1889, stated that the bacteroids are protein bodies of the Leguminosae, which are produced by the plant and enclose the micrococcoid organisms. He was

unable to produce bacteroids in artificial culture media but was able to follow the development of swarmers, the micrococcus forms, from bacteroids.

Frank later, 1891, thought the bacteroids to be true fungi, although much modified through the assimilation of cell protoplasm.

Atkinson, 1893, believed that the fully formed bacteroids were no longer capable of growth and that "The death of the organism in its passage to the sterile condition of the perfect bacteroid is first indicated by a firmer condition of the organism, probably brought about by the increasing presence of protein matter which in many cases finally becomes centered in different parts of the bacteroids and forms bodies which possess a very high power of refracting light." These protein bodies which Atkinson thought to be lifeless storage bodies are probably identical with the micrococcus organisms of Frank, 1889, and with the spores of Prazmowski's early paper, 1888.

Laurent, 1890, and Mazé, 1898, considered the bacteroids to be normal reproductive forms of the organism. They described the formation of coccoid cells as a type of dichotomous budding. It seems probable, however, that these observations were made on impure cultures.

Schneider, 1892, stated that the modified rhizobia (bacteroids) would not reproduce and develop outside of the host. The next year, 1893b, he stated that "*Rhizobium mutabile* (*Rhizobium radicicola,* bacteroids, etc.) can be cultivated with great difficulty. It multiplies by division and from spores. *Rhizobium Frankii* develops very readily both by division and from spores." Schneider in his 1892 paper established five species of the nodule-forming organism, based on morphological characters of the organisms as they occur in the nodule. *Rhizobium mutabile* and *Rhizobium Frankii,* referred to above, are two of these species. Hartleb, 1900, stated that the bacteroids are normal forms of the organism and reproduce by the liberation of spore-like bodies which later develop into swarmers. Greig-Smith, 1901, and 1906b, held the view that the bacteroids represent a capsule-like structure enclosing the coccus forms of the organism.

Lewis and Nicholson, 1905, stated that the branched and vacuolated forms of the bacteria represent degenerate stages of the organism, and that they do not grow and form colonies. Their evidence for this is indirect and based on the fact that few colonies appear on plates made from nodules or cultures containing a large percentage of bacteroids. Moore, 1905, on the other hand, believed that the bacteroids are not degenerate forms.

Most of the more recent workers agree that the bacteroids represent viable forms of the organism and in many cases have associated them with a definite life cycle; Buchanan, 1909b; Peklo, 1910; Fred, 1913; Löhnis and Smith, 1916; Bewley and Hutchinson, 1920; Thornton and Gangulee, 1926; Káš, 1927; Gibson, 1928; Śnieszko, 1928; and Schönberg, 1929, are of this opinion.

Certain others, however, believe that bacteroids represent teratological (malformed) or degenerate forms which are incapable of further growth and development. Bazarewski, 1927, stated that the structure of the bacteroids does not indicate that their organization is in any way superior to that of the rods, and that generally the bacteroids do not reproduce, but that very young bacteroids may transform themselves into simple rods. He concluded that the bacteroids are involution forms incapable of multiplication.

Müller and Stapp, 1925, regarded the bacteroids as teratological forms incapable of growth and reproduction. Microscopic examination of hanging drops and single-cell cultures all failed to show any growth or reproduction. The number of trials is not recorded, and comparatively short incubation periods were used. It is possible that variations in the medium, conditions of growth, prolonged incubation periods, and so forth might result in growth of the bacteroid cells. The need for further work on this subject is indicated.

Cappelletti, 1926a and b, considered the bacteroid form a reaction against the immune mechanism of the plant and stated, "This form has for the most part lost the capacity of reproduction and in some cases it has the tendency to transform itself into forms similar to spore-bearing cells."

Pfeiffer, 1928, differentiated between the normal branched forms and the abnormal irregular forms, the latter distinguished by clavate form, knobs, and irregular swelling. He considered the normal branched forms the true bacteroids, and believed that these bacteroids are neither special reproduction forms nor degenerate forms but are induced by specific chemical and physical influences of certain compounds, particularly those concerned in surface tension effects.

Unpublished studies at this station indicate that the bacteroid form is usually incapable of reproduction. Over 100 single bacteroid forms were picked with a micromanipulator and placed in media under conditions which are known to be favorable to the growth of single cells of the rod forms. Only two of the bacteroids so studied initiated growth. More work is in progress. To summarize these findings and to draw a clear-cut conclusion is difficult. From the evidence given in the literature and from the results of the preliminary studies at this station, the writers believe that the weight of evidence points to the fact that bacteroids are usually incapable of reproduction.

Production of bacteroids in laboratory media. A summary of the data relating to the production of irregular forms in laboratory media is presented in Table 1. Although many observations have been reported, very few careful studies have been made on the factors influencing the production of bacteroids. Of these, the work of Stutzer, 1900a and b, and 1901; Neumann, 1902a; Hiltner and Störmer, 1903a; Süchting, 1904; Buchanan, 1909b; Zipfel, 1911; Barthel, 1920, 1921; Müller and Stapp, 1925; and Śnieszko, 1928, may be mentioned.

The medium used and the nutrition of the organism seem to determine the production of the irregular cell forms. In media which are unfavorable to the life and activities of the organism, it is probable that the irregular forms which are produced are non-viable. On the other hand, certain media in which the organisms grow very well for many generations produce quite regularly the bacteroidal forms.

Bacteroids are produced more readily in liquid than on solid media. Süchting, 1904, suggested that the lowered oxygen tension in liquid medium might be important in determining the formation of bacteroids, whereas Bewley and Hutchinson, 1920, observed a marked rise in proportion of cocci in cultures placed under anaerobic conditions. It will be remembered that in Bewley and Hutchinson's cycle the bacteroids immediately precede cocci. Budinov, 1907, concluded from his experiments, however, that oxygen tension bears no relation to the production of bacteroids. The influence of oxygen tension and oxidation-re-

TABLE 1

Summary of reports concerning bacteroid formation

Author	Organisms from	Grown on	Forms observed	Notes
Beijerinck 1888, 1890		Leguminous leaf-extract gelatin plus sugar and asparagin	Swarmers, rods, and bacteroids	
Laurent 1890, 1891	Pea and lupine	Decoction of pea leaves and synthetic solutions	Y and T shaped forms	Believed that the branches separate to form new individuals
Atkinson 1893	*Vicia sativa*	Vetch-vine infusion agar	Various oval, oblong ,amoeboid, and forked forms	
Mazé 1898	Clover	Bean-infusion agar plus sucrose, tartaric, or oxalic acids, 1 to 1000	Rods, ovals, club-shaped, and branched forms	Incubation at 35 °C. tended to increase the number of abnormal forms
Greig-Smith 1899	Lupine	Lupine extract, glucose or glycerol liquid, agar or gelatin	Typical bacteroids	Believed that the organism reproduces by budding
Hiltner 1900a	*Pisum sativum* *Robinia pseudacacia* *Trifolium repens* *Ornithopus sativus* *Lupinus luteus*	Plant extract and other liquid media	Typical bacteroids	
Hartleb 1900, 1901a		Glucose, mineral salt solution, plus lactic or succinic acid	Swarmers, rods, and branched bacteroids	Observed the formation of bacteroids under hanging drop
Stutzer 1900a, 1901	*Vicia Faba* *Pisum sativum* *Trifolium hybridum* *Trifolium pratense* *Trifolium incarnatum* *Vicia sativa* *Vicia villosa* *Phaseolus vulgaris* *Lupinus luteus* *Lupinus angustifolius* *Ornithopus sp.* *Soja hispida*	Leguminous extracts plus glucose, asparagin, and citric, succinic, lactic, tartaric, or acetic acids. Also several salts, agar	Bacteroid forms produced with all organisms, in certain media	Liquid media were generally more effective in producing bacteroids than agar media

TABLE 1 (Cont.)

Author	Organisms from	Grown on	Forms observed	Notes
Dawson 1900a and b	*Pisum sativum* *Desmodium gyrans*	Pea-leaf decoction and agar	X, Y, and swarmer forms	Observed in hanging drop the formation of bacteroids. See Plate 11
Neumann 1902a	*Vicia Faba*	Diluted urine, soil extract, and leguminous extract	Typical bacteroids	No bacteroids on solid media. Secured bacteroids in hermetically sealed tubes
Hiltner and Störmer 1903a	Pea, vetch, clover, soybean, locust, lupine, serradella, and bean	Media with various sugars, nitrate, asparagin, peptone, phosphoric acid, organic acids, HCl, H_2SO_4 plus $MgSO_4$	Bacteroids	Carbon source seemed to be most important in determining bacteroid formation
Süchting 1904	Horse bean and lupine	Faintly acid plant extracts, liquid and solid	Branched forms with organism from horse bean in liquid, and enlarged forms with that from lupine	Suggested that lowered oxygen content of liquid and old nodules may be responsible for bacteroid formation
Moore 1905		Faintly acid medium containing potassium phosphate	Irregular branched forms	Stated they may be readily produced but failed to give details of media or source of cultures
Lewis and Nicholson 1905	Alfalfa, clover, soybean, and cowpea	Agar and bouillon	Vacuolated branched forms	Called involution forms
Clark 1905		Media poor in nitrogen	Large rod and branched forms	Branched forms were observed only in old cultures
Maassen and Müller 1906	27 different species	Solid media	Typical bacteroids	Stated that the same forms found in nodules can be produced on solid media. No details given
Harrison and Barlow 1907		Wood ash, maltose liquid	Branched forms	

TABLE 1 (Cont.)

Author	Organisms from	Grown on	Forms observed	Notes
de'Rossi 1907	*Vicia Faba*	*Vicia Faba* extract gelatin	Branched and vacuolated forms	Claimed that the forms previously described by others are not the true nodule organisms. Conclusion probably unjustified
Budinov 1907	Clover	Bean extract plus sucrose	Bacteroid-like bodies	Grown at 30 °C. Does not believe bacteroid formation to be influenced by either oxygen pressure or by-products of growth
Buchanan 1909b	*Trifolium pratense* *Medicago sativum* *Petalostemon candidus* *Lathyrus odoratus*	Salts of organic acids, glycerol, asparagin, sodium asparaginate, urea, peptone, glucosides, carbohydrates, leguminous extracts	Typical bacteroids as they occur in plants and many other irregular forms	Specific character of the substance used in the medium seemed to be the determining factor in bacteroid formation, rather than acidity, osmotic pressure, temperature, light, oxygen tension or the accumulation of metabolic products
Gage 1910	Clover	Nitrogen-free liquids and solid media with various carbohydrates	Branched and vacuolated forms	
Zipfel 1911	*Pisum sativum* *Vicia Faba* *Trifolium pratense* *Phaseolus vulgaris*	Agar or gelatin with organic acids, carbohydrates, proteins, or protein split products	Typical bacteroids and other enlarged forms	
Fred 1913	Alfalfa, pea, white clover, red clover, sweet clover, sweet pea, cowpea, soybean	Leguminous extract agar plus caffein or cumarin	Branched, club-shaped, and enlarged forms	Caffein tended to produce typical bacteroid forms
Prucha 1915	Canada field pea	Solid media plus sugars	Typical bacteroids	Bacteroid formation dependent upon nutrition

TABLE 1 (Cont.)

Author	Organisms from	Grown on	Forms observed	Notes
Löhnis and Smith 1916	Vetch	Several different media	Nine different stages in life cycle	
Burrill and Hansen 1917	A large list of species	Ash agar plus sugar	Swarmers and branched forms	Bacteroids occur in old cultures
Wilson 1917	*Soja Max*	Agar medium with sucrose	X and Y forms	
Barthel 1920 1921	Vetch	Gelatin, leguminous extract plus caffein, guanine, guanidine, pyridine, or chinoline	Typical bacteroids	True bacteroids are only produced in artificial culture media which contain certain alkaloids
Bewley and Hutchinson 1920	Red clover, broad bean, alfalfa, and lupine	Many media	Cocci, rods, and vacuolated forms	A large number of factors studied
Löhnis and Hansen 1921	11 different species	6 different media	Several different stages in life cycle	
Müller and Stapp 1925	25 different cultures	Tested the effects of antiseptics, alkaloids, glucosides, neutral salts, acid phosphates, and organic acids	Wide variety of enlarged, branched, and vacuolated forms	Arbutin, caffein, and the chlorides of caesium and magnesium were very effective in producing these changes
Whiting, Fred, and Helz 1926	*Dalea alopecuroides*	Agar media with various sugars	Irregular and branched forms	
Friesner 1926	*Gleditsia triacanthos*	Both liquid and solid media	Rods, cocci, and various irregular forms	
Thornton and Gangulee 1926	*Medicago sativa*	Soil with milk and $CaH_4(PO_4)_2$	Rods, cocci, and vacuolated forms	
Gangulee 1926b and c	*Crotalaria juncea* and alfalfa	Several media	Cocci, rods, and vacuolated forms	

TABLE 1 (Cont.)

Author	Organisms from	Grown on	Forms observed	Notes
Alicante 1926	Pea, red clover, and soybean	Liquid synthetic media plus phosphates, carbonates, aluminum chloride, or mineral acids	Various abnormal forms	Phosphates and carbonates stimulated the formation of bacteroids
Bazarewski 1927	Pea	Liquid media plus organic acids and purine bases	Typical bacteroids	Stated that bacteroids are involution forms incapable of multiplication
Káš 1927	Not stated	Various media	Several stages in life cycle	
Gibson 1928	*Trifolium pratense* *Trifolium hybridum* *Medicago sativa* *Melilotus alba* *Pisum sativum* *Vicia sativa* *Phaseolus vulgaris*	Various media	Several stages in life cycle	
Pfeiffer 1928	Pea	Numerous media with alkaloids, varying pH, and salts	Branched, vacuolated, and coccus forms	Normal branched forms are not involution forms
Śnieszko 1928	Bean, pea, vetch, and red clover	Soil extract, glucose, asparagin, mineral salts, liquid, and agar. Varying pH	Several stages in life cycle	Changes in acidity cause differences in morphology
Schönberg 1929	17 different species	Soil extract, mineral salts, with various carbohydrates	Several stages in life cycle	Age of culture and acidity of media are important
Israilsky 1929	Pea and others	Liquid and solid media with bacteriophage	Swollen, vacuolated, branched rods, and cocci	Bacteriophage produces irregular forms

duction potentials on the growth, physiology, and morphology of the nodule organism has never been adequately studied, but it is probable that this may be one of the factors concerned in the production of bacteroids. With a closely related form, *Ph. tumefaciens,* Stapp and Bortels, 1931, have shown that the addition to media of small amounts of oxygen carriers, as iron and manganese salts, results in a definite increase in the production of the "Bakteriensterne," star or radial-shaped arrangements of the organisms, and that these forms are apparently only formed in the surface films of growth in liquid media.

The use of water extracts of leguminous plants or seeds in media favors the production of bacteroids. It is probable that this effect is due in part to the alkaloid content of such extracts, since a number of the alkaloids, caffein, cumarin, etc., are very effective in inducing bacteroid formation.

Certain of the sugars and glucosides as well as the organic acids tend to give irregular forms. Growth in fairly acid media also produces bacteroid forms. Müller and Stapp, 1925, found that low concentration of either magnesium chloride or caesium chloride in carrot agar produces a wide range of irregular vacuolated forms. Israilsky, 1929, noted that the bacteria tend to produce irregular forms under the influence of bacteriophage. Israilsky and Starygin, 1930, found that the smooth races of their cultures of rhizobia form many more bacteroids than the rough races.

Bacteroids and nitrogen fixation. Many of the early workers felt that bacteroids represent protein storage bodies and that these are absorbed by the plant to furnish its nitrogen. Brunchorst, 1885a, originally applied the term to what he called "the organized protein bodies of the full-grown cells of the nodules," and he felt that these are in due time absorbed by the plant in its growth.

With the assumption that the bacteroids are a stage in the growth of the nodule organism, the idea was carried over that they are bodies high in protein and that they are absorbed by the plant in its growth. Prazmowski, 1890, and Frank, 1891, both stated that the plant profits from the symbiotic relationship by absorbing the bacteroids.

The theory that nitrogen fixation is definitely associated with the change of the rod forms to the enlarged vacuolated forms in the nodule was probably first suggested by Prazmowski, 1891. He stated that the material in the bacterial bodies is not absorbed by the plant until it has undergone a series of deep-seated changes —transformation into bacteroids and characteristic protein substances—under the influence of the cell plasma. Neumann, 1902a, disagreed with Prazmowski and stated that the bacteroids are living transition forms and are not absorbed. Moeller, 1892b, stated that the bacteria change into bacteroids under the influence of the plant and that these dead cells then undergo a fatty degeneration, ultimately being absorbed by the plant. Nobbe and Hiltner, 1893, developed this idea further when they stated that the unchanged bacteria are not effective in nitrogen assimilation, and that assimilation only begins with bacteroid formation. They arrived at this conclusion from a study of the morphological characters of the bacteria in nodules from plants at different stages of development and nutrition. It was noted that when plants are grown in a nitrogen-rich soil, there is little or no nitrogen fixed, and that bacteroids are not present in the nodules of such plants. And conversely, when the plants are grown in nitrogen-poor soils, the nodules

contain many bacteroids, and considerable nitrogen is fixed. Nobbe and Hiltner, 1893, also worked with a strain of the nodule organism which formed nodules on peas, but which did not aid the growth of the host plant by nitrogen fixation. This strain was described as purely parasitic. Morphological studies of this organism in the nodules showed no bacteroids at any stage of plant growth. These factors were also correlated with nodule size in their experiments. Apparently the nodules remained small if the bacteria were quickly changed to bacteroids or grew large if this change did not occur.

The views of Nobbe and Hiltner were rather generally accepted for a number of years. Hartleb, 1900, stated that neither the coccoid nor rod forms are able to fix nitrogen, and that only the bacteroids can do this, and in 1901a he was granted United States Letters Patent No. 674,764 covering a medium in which it was claimed that bacteroids were produced in abundance. Moore, 1905, thought that the importance of the bacteroids lies in the fact that they can be dissolved by the plant, whereas the rod forms can not. The fixation of nitrogen, however, was not considered an attribute peculiar to the bacteroids. He stated, " . . . the branched forms . . . are of the greatest service to the plant in supplying it with nitrogen." And, ". . . the nodule-forming organism in the large rod stage has the property of fixing free nitrogen independent of any host plant . . ." Also, "The nodule organism of most legumes, so long as it retains the rod form, is insoluble and the plant must be supplied with bacteria capable of passing into the branched stage under the conditions existing in the nodule if they are to be of service." Löhnis and Smith, 1916, made the statement, "However, nitrogen fixation takes place only when these branched forms develop . . ."

Recent students of this problem lay less stress on the value of the bacteroid form in the phenomenon of nitrogen fixation. Müller and Stapp, 1925, after a careful investigation of the production of bacteroids in artificial media, could find no evidence that they are the only forms concerned in nitrogen fixation. Barthel, 1926, studied nitrogen fixation by pure cultures in media containing caffein which induced bacteroid formation, but found no nitrogen fixation either with or without caffein. According to Bazarewski, 1927, the bacteroids are involution forms incapable of multiplying and probably unable to fix the nitrogen of the air. In a second paper, Bazarewski, 1929, used caffein and theobromine to produce bacteroids, but did not note nitrogen fixation in such cultures.

Pfeiffer, 1928, studied the progress of nitrogen fixation in the pea plant during its development and at the same time carefully followed the morphological changes of the organisms within the nodules. In some of his experiments there was an apparent correlation between the transformation of the rods into bacteroids and the accumulation of nitrogen by the plant. In many cases, however, no such correlation could be found, and with certain strains of the nodule organism no bacteroids were formed in the nodule, although there was a considerable fixation of nitrogen.

Pfeiffer's results with plant studies and those of Müller and Stapp, Barthel, and Bazarewski with pure cultures of the bacteria indicate that there is little or no connection between bacteroid formation and nitrogen fixation. Cappelletti, 1924a and b; 1926a and b; and 1928, has reported that the formation of the bacteroids in nodules is coincident with the development of specific antibodies.

He found this to be very noticeable in the case of the pea and horse bean. In the kidney bean, the antibody is developed only to a slight extent, and he stated that bacteroid-like forms are never found. Only the branched forms are considered by him to be true bacteroids, forms resulting from the immunity processes in the plant. The physiological importance of the bacteroids, however, has never been satisfactorily explained, and it is probable that a continuation of painstaking studies by microchemical technique on the relationship between morphology and physiology will prove profitable.

Variations in bacterial form. It was recognized early in the study of the root nodule organisms that the form of the organism in the nodule varies with the plant species. Frank in 1879 described and pictured the organisms as they occurred in the nodules of *Lathyrus pratensis, Orobus tuberosus, Lupinus, Ononis repens,* and *Genista germanica.* He described the organisms in the last three plants as being similar, oval or almost round. The organisms in the first two species were pictured as slender, long rods, usually branched.

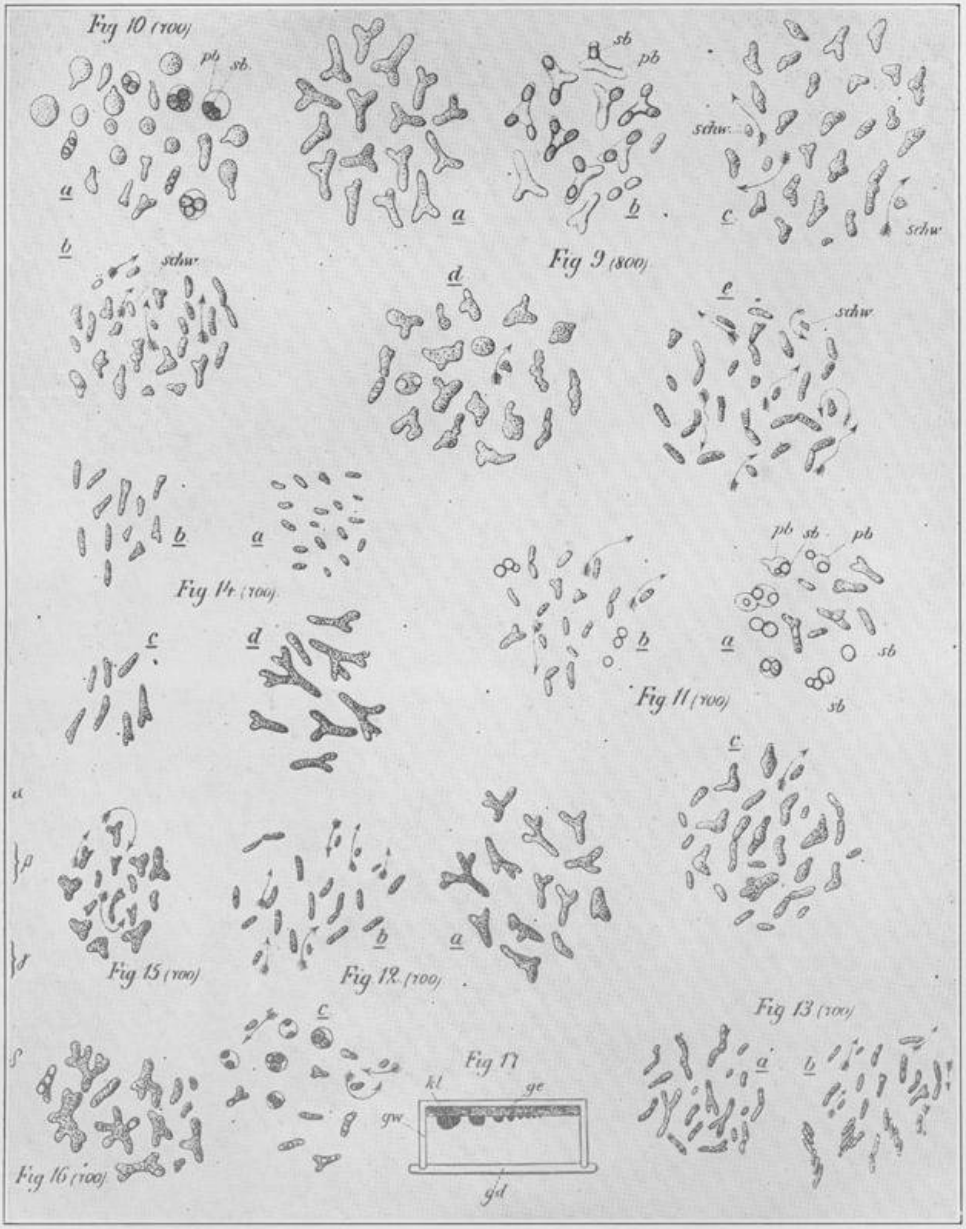
Tschirch, 1887, noted that the bacteroids as they occur in the nodules vary in size and shape with the various plant species. He described them as being mostly rod forms in *Phaseolus;* curved rods with thick round ends in *Robinia;* and small, oval or curved branched forms in *Lupinus, Lathyrus, Orobus,* and *Genista.*

Beijerinck's, 1888, observations on the shape of the organisms are of considerable interest not only because he was the first to isolate and study these organisms in pure culture, but also because of the accuracy of his observations on the occurrence of the different forms. He recognized three different forms of bacteroids dependent upon the plant from which they are secured (Plate 12): (1) the X and Y shaped forms from *Vicia* (Fig. 9), *Pisum* (Fig. 12), and *Lathyrus* (Fig. 15 and 16); (2) the rod-shaped forms from *Phaseolus, Ornithopus,* and *Lotus;* (3) the pear-shaped or spherical forms from *Trifolium* (Fig. 10).

Prazmowski, 1890, agreed with Beijerinck as to the general shape of the bacteroids. He stated that the bacteroids of the pea and various other plants are commonly branched, whereas in the lupine branched forms seldom occur and in *Phaseolus vulgaris* the bacteroids are always simple rods (see Plate 14).

Morck, 1891, a student of Frank's, made a careful study of bacteroid morphology. One of his plates showing the bacteroids of *Medicago sativa, M. lupulina, Trigonella foenum-graecum, Melilotus officinalis, M. coerulea,* and *Trifolium pratense* is reproduced in Plate 13. The bacteroids from clover are represented as spherical or pear-shaped, whereas those from the other plants are rods, sometimes swollen or forked at one end. The development of branched forms has been studied in hanging-drop preparations by Prazmowski, 1890, and Dawson, 1900b. The figures of Prazmowski are included in Plate 14 and those of Miss Dawson in Plate 11.

Practically all of the investigators who have studied the formation of bacteroids in artificial media have likewise reported variations in bacteroid morphology. Stutzer, 1901, found specific differences in the form of bacteroids from each of the 12 plant species which he studied. While most of these differences are of a minor character, two broad general groups can be clearly established. The lupine, serradella, and soybean organisms, on the one hand, form a group with rod-shaped bacteroids, while the organisms from the other plant species are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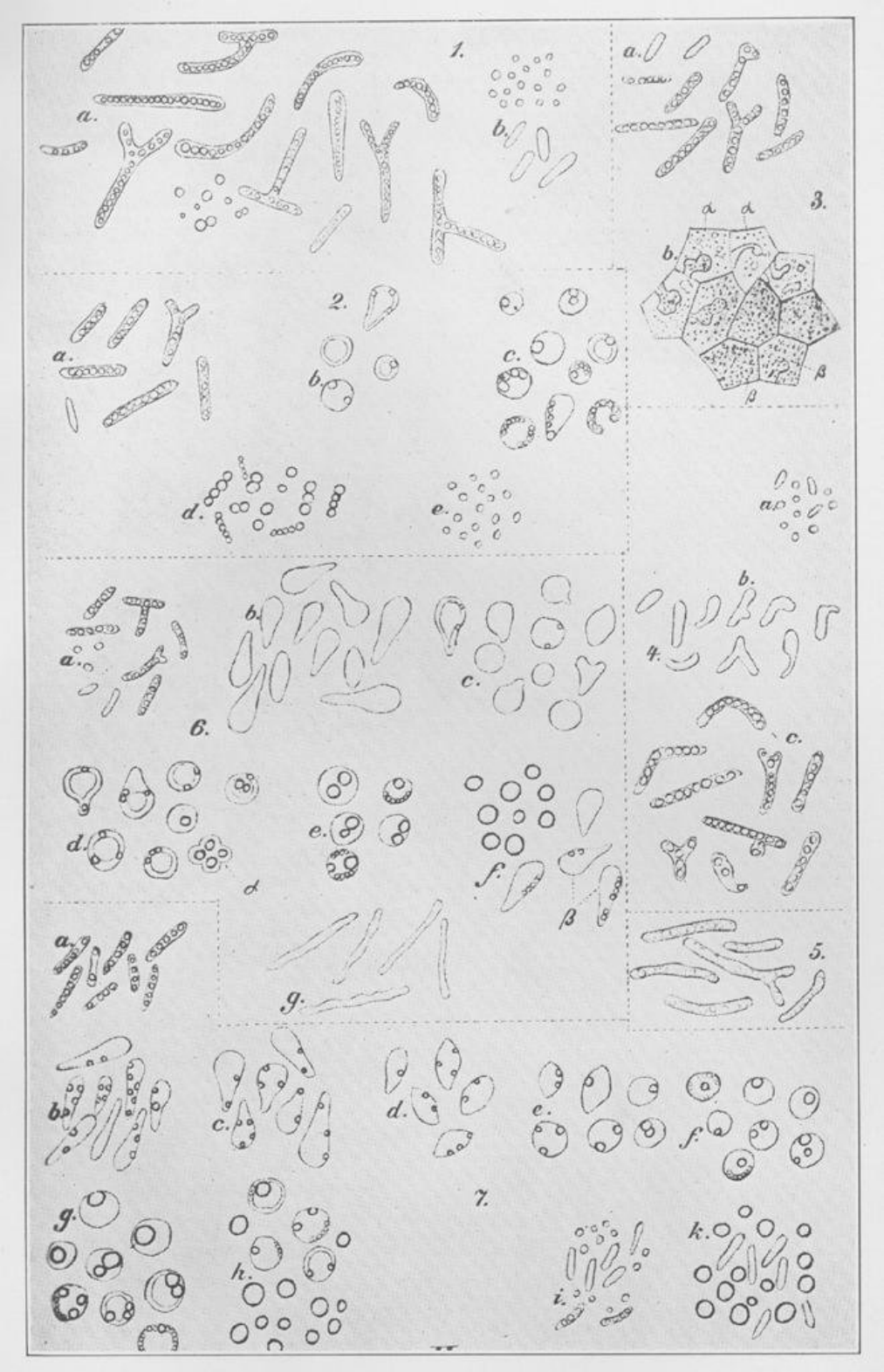
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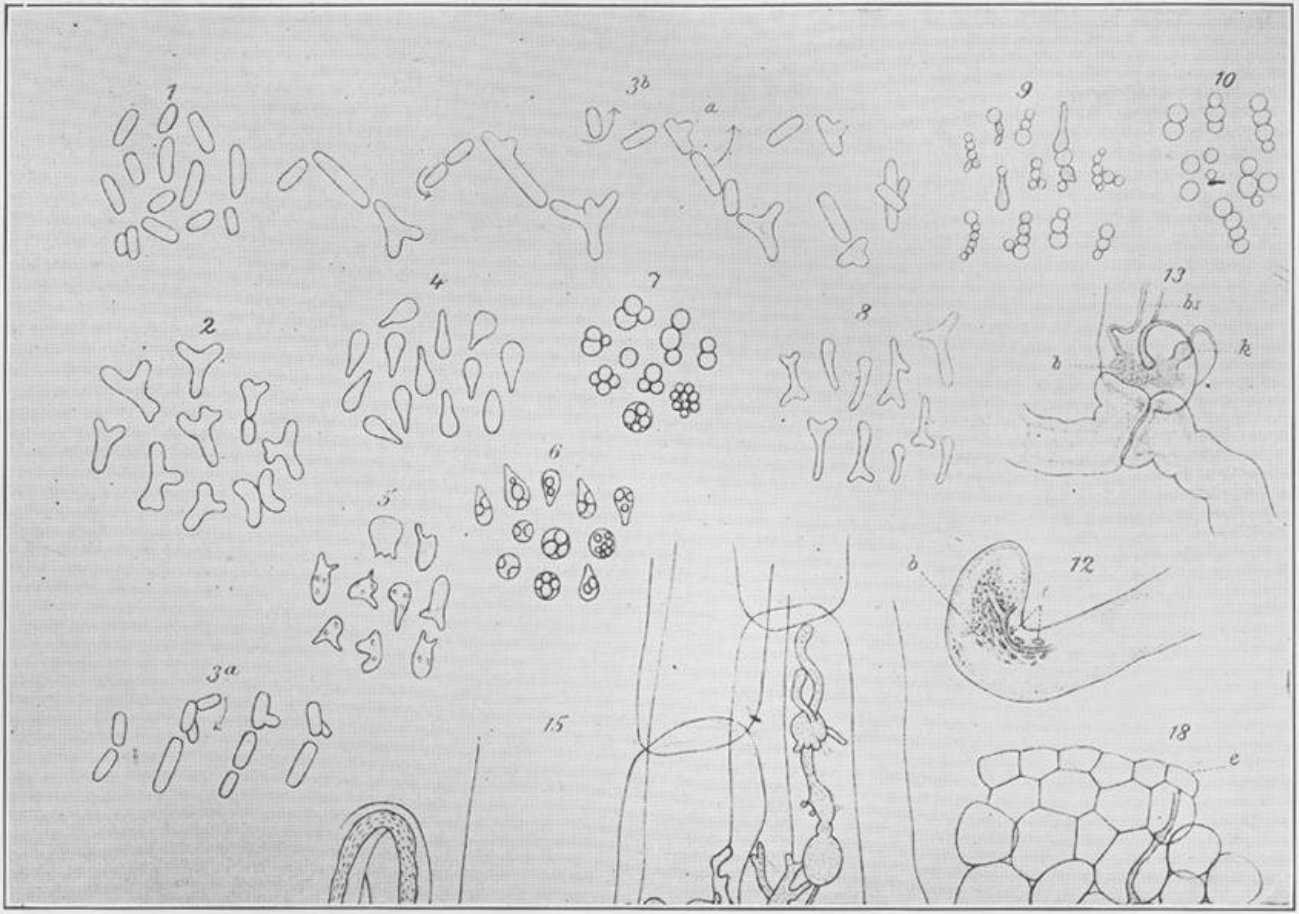
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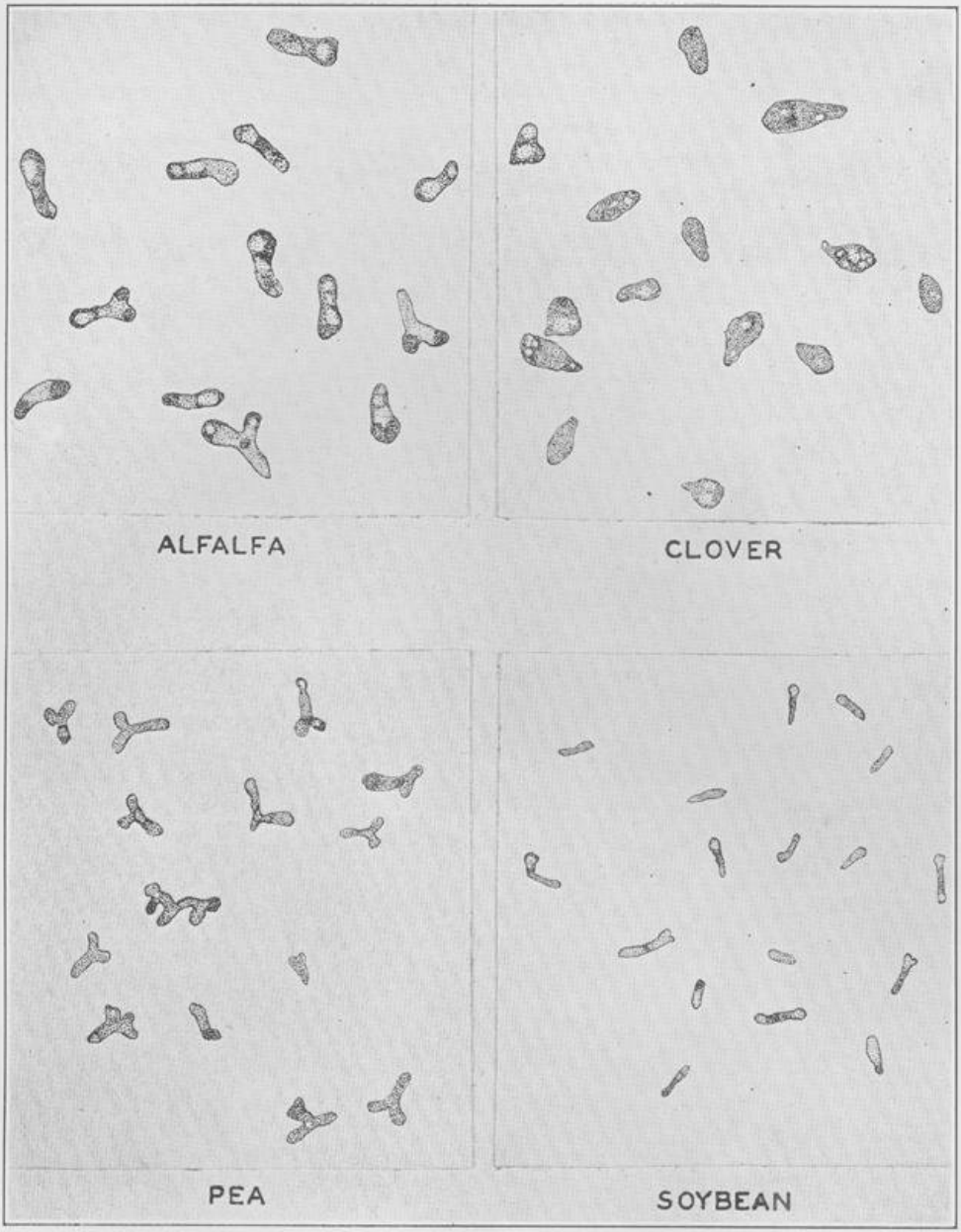
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CLOVER
PEA
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larger and more inclined to be swollen, branched, and irregular in shape. Buchanan, 1909b, studied the relationship between the bacteroids of the nodules and those produced on artificial media and concluded: "A comparison of the bacteroids to be found in the nodules of various species of legumes with those that are to be found in artificial media clearly shows that in each case we are dealing with phenomena of the same kind. The bacteria from the various legume nodules differ greatly among themselves; the same is true of the bacteroids from artificial media."

There have been one or two attempts to use the characteristic bacteroid form as one of the distinguishing features in the species identification of the organism. Schneider, 1892, proposed five species of the rhizobia with two varieties of one, based largely on the morphology of the organism as it occurred in the nodule.

Several years later Hiltner, 1900b, and Hiltner and Störmer, 1903a, utilized the bacteroid shape as one of the criteria for separating the nodule bacteria into two species. In one group comprising the organisms from *Pisum, Vicia, Lathyrus, Phaseolus, Trifolium, Medicago, Anthyllis, Onobrychis,* and *Robinia* there are bacteroids enlarged and broadened at the end. The second group contains the organisms from *Lupinus, Ornithopus, Soja,* and (*Genista?* and *Sarothamnus?*). In this group the bacteroids are definitely of rod form. Organisms of the first group were called *Rhizobium radicicola* and of the second *Rhizobium Beijerinckii* by Hiltner and Störmer.

Some twenty years later three papers appeared dealing with the separation of the root nodule bacteria into species or subgroups. In each case the shapes of the bacteroid forms were utilized as one of the important distinguishing characteristics. Müller and Stapp, 1925, formed 11 subgroups of the root nodule bacteria. They used the bacteroid form occurring on a caesium-chloride carrot agar as one of the essential differences. Dangeard, 1926, recognized and named ten species of the genus *Rhizobium.* His differentiation was based largely on careful cytological studies of the nodule and the organisms within.

Baldwin and Fred, 1929b, in a discussion of the nomenclature and species identification of the root nodule bacteria, gave the following descriptions of the bacteroid form for the five species then proposed:

1. *Rhizobium leguminosarum* Frank. (Pea cross-inoculation group) The bacteroids from nodules are commonly irregular, with many X- and Y-shaped forms. Vacuolated forms predominate.

2. *Rhizobium trifolii* Dangeard. (Clover cross-inoculation group) Bacteroids from nodules are pear-shaped, swollen, and vacuolated. Rarely X- and Y-shaped forms.

3. *Rhizobium phaseoli* Dangeard. (Bean cross-inoculation group) Bacteroids from nodules are usually rods with few branched forms. They are usually smaller than in *Rh. leguminosarum* and *Rh. trifolii,* and often vacuolated.

4. *Rhizobium meliloti* Dangeard. (Alfalfa cross-inoculation group) Bacteroids from nodules include both club-shaped and branched forms.

5. *Rhizobium japonicum* Kirchner. (Soybean cross-inoculation group) Bacteroids of nodules are long, slender rods with only occasional branched and swollen forms.

Plate 15 illustrates typical bacteroids of four of the above species.

LIFE CYCLE

The pleomorphic nature of the root nodule organism was the subject of many controversies among the early investigators, and many theories as to the nature and function of the different forms were advanced.

Beijerinck, 1888, in his first paper describing the isolation of the organism, recognized three definite forms; the swarmers or small motile coccoid bodies, typical rods, and bacteroids or swollen vacuolated forms. While he stated that these forms are derived one from the other, he made no attempt to trace in detail the manner in which the change in form occurs.

Frank, 1890b, was probably the first to study carefully the mode of reproduction of this organism. He placed the organism among the Schizomycetes, but also described several supplementary methods of reproduction in addition to the ordinary binary fission. Much of his work on the description of the organism was carried on with hanging drop cultures made directly from the nodules. In addition to this, however, he isolated the organism on gelatin plates. While his cultures may not have been pure and many of his deductions are faulty, his observations on the pleomorphism of the organism and the various methods of reproduction are extremely interesting in the light of recent investigations on the life cycle of the root nodule organism.

Frank recognized not only the three forms which Beijerinck had described, but he also added several other forms. He thought that the bacteroids were a synthetic product of the plant cell with a coccus-like stage of the microörganism enclosed within. The bacteroids were described as 3μ to 5.5μ long and containing 2, 3, 4, or more swarmers of 0.9μ to 1.3μ in length. Under suitable conditions the bacteroids were thought to decompose and liberate the swarmers. Morck, 1891, likewise saw cocci within the bacteroids (Plate 13) and suggested that they are not resorbed upon breakdown of the bacteroids but are returned to the soil. Bréal, 1888b, in studying a water mount of bacteria from nodules of lupine, pea, lentil, and acacia, noted that they often "run together and take on the form of a zoogloea." And also, "the zoogloea generally remains in company with some bacteria," which in terms of recent investigations on the life cycle seems suggestive of symplasm and regeneration of cellular forms therefrom. Frank, 1890b, also hinted at the present day symplasm when he described masses of naked *Mykoplasm,* which he observed in hanging-drop preparations of young material. He believed it to be material which normally would have been differentiated into bacteroids, but in which the process had not been completed. After observing it for 1 or 2 days he saw the development of swarmers.

He likewise noted threadlike zoogloeal forms in hanging-drop cultures, apparently formed by the secretion of a gummy substance around the multiplying rods. This later thickened and developed into sausage-like structures in which were developed many small short coccoid bodies of about 0.2μ in size. (Gonidia and gonidangia of later observers?) These small bodies developed into typical rod forms of the organisms. Another type of zooglea he described as being formed by the dissolution of a number of bacteroids with the swarmers held in the gummy material. Finger-like threads of this gummy material extended out in all directions and enclosed rod forms of the organisms. Four days after-

wards these rods had transformed themselves into colonies of small, coccoid bodies. For a few days these colonies increased in size, and the bodies exhibited a swimming motion. Then the entire culture died. Perhaps this is the first observation of the bacteriophage phenomenon(?)

Vuillemin, 1888; Laurent, 1890; and Mazé, 1898, believed that the bacteroids were concerned in the reproduction of the organism. Laurent stated that the bacteroids give off bodies by a sort of dichotomous budding similar to yeasts, and that these bodies are able to grow and reproduce the rod and bacteroid forms. Essentially the same view was given 8 years later by Mazé.

Hiltner, 1898, referring to the position of these bodies in the life cycle of the organism, was probably the first to call the bacteroids sporangia. This idea was again advanced by Hartleb in 1900, who stated that the bacteroids liberate a number of spore-like bodies which later develop into swarmers. Stutzer, 1901; Greig-Smith, 1901 and 1906b; Neumann, 1902a; and de' Rossi, 1907, all believed that the bacteroids give rise to new organisms. Paratore, 1901a, specifically mentioned "streptococcal degeneration" of the bacteroids. Schneider, 1902, stated that the organism multiplies in three ways: biseptation, multiseptation, and budding with subsequent septation. Hiltner and Störmer, 1903a, in an extensive study of the factors responsible for bacteroid formation, again affirmed their belief in the sporangial nature of the bacteroid. Peklo, 1910, associated the bacteroids with arthrospores.

Löhnis, in 1905 and 1921; Löhnis and Smith, 1916; and Löhnis and Hansen, 1921, have contributed a great deal to the study of the various forms through which the nodule bacteria pass. With a culture of the nodule bacteria from vetch, they were able to identify nine different growth forms: symplasm, small globules and ovals, small rods and threads, slime threads with cocci, granular decomposition of the rods, regenerative bodies, budding gonidia, cells with pointed ends, and starlike growths. They did not, however, attempt to outline the conditions under which the different forms developed, nor to present any definite cycle through which the organism regularly passed.

Bewley and Hutchinson, 1920, did define a regular cycle exhibited by the nodule organism in culture media, and they described the factors responsible for the appearance of the various stages. The sequence is briefly as follows: first stage, pre-swarmer (non-motile coccus); second stage, pre-swarmer (larger non-motile coccus); swarmer stage (motile ovoid cell); rod stage (motile); and vacuolated stage (non-motile, banded, ultimately yielding coccoid pre-swarmers).

Thornton and Gangulee, 1926, followed this with a description of the life cycle of the organism in soil and its relation to the infection of the host plant. They found that in sterile soil which had been inoculated with a pure culture of the nodule organism, three forms are always present: the unbanded rods, banded or vacuolated rods, and cocci. While these forms are all present at the same time, the proportions vary at different ages of the soil culture. The rods tend to predominate at the start and the cocci in the older cultures. The appearance of large numbers of the cocci can be hastened by using milk plus 0.1 per cent calcium phosphate as a suspending fluid for the culture used to inoculate the soil. They held that the rhizobia exhibit two types of multiplication: binary fission by the rod forms and multiple fission by the banded rods, which break up

into cocci. This multiple fission of Thornton and Gangulee was reported from study of free-living rhizobia in soil under laboratory conditions. That it is not true of all banded-rod or bacteroid forms is apparent from Thornton's later, 1930a. paper on the cytology of lucerne and clover nodules. He stated, "The bacteria in the cytoplasm, which in younger tissue consist of banded rods, break up into darkly staining granules which eventually disappear or at any rate lose their staining properties." The surviving viable forms in the nodule are coccoid rods in zoogloeal masses within the cell walls and intercellular spaces.

Gangulee, 1926a and b, studied the life cycle of the nodule organisms of *Crotalaria juncea* (L.) and *Medicago sativa,* and found that essentially the forms described by Bewley and Hutchinson were present. He noted that all forms were usually present at once, and that the percentage of motile cocci was greatest under conditions favoring rapid multiplication and high total numbers.

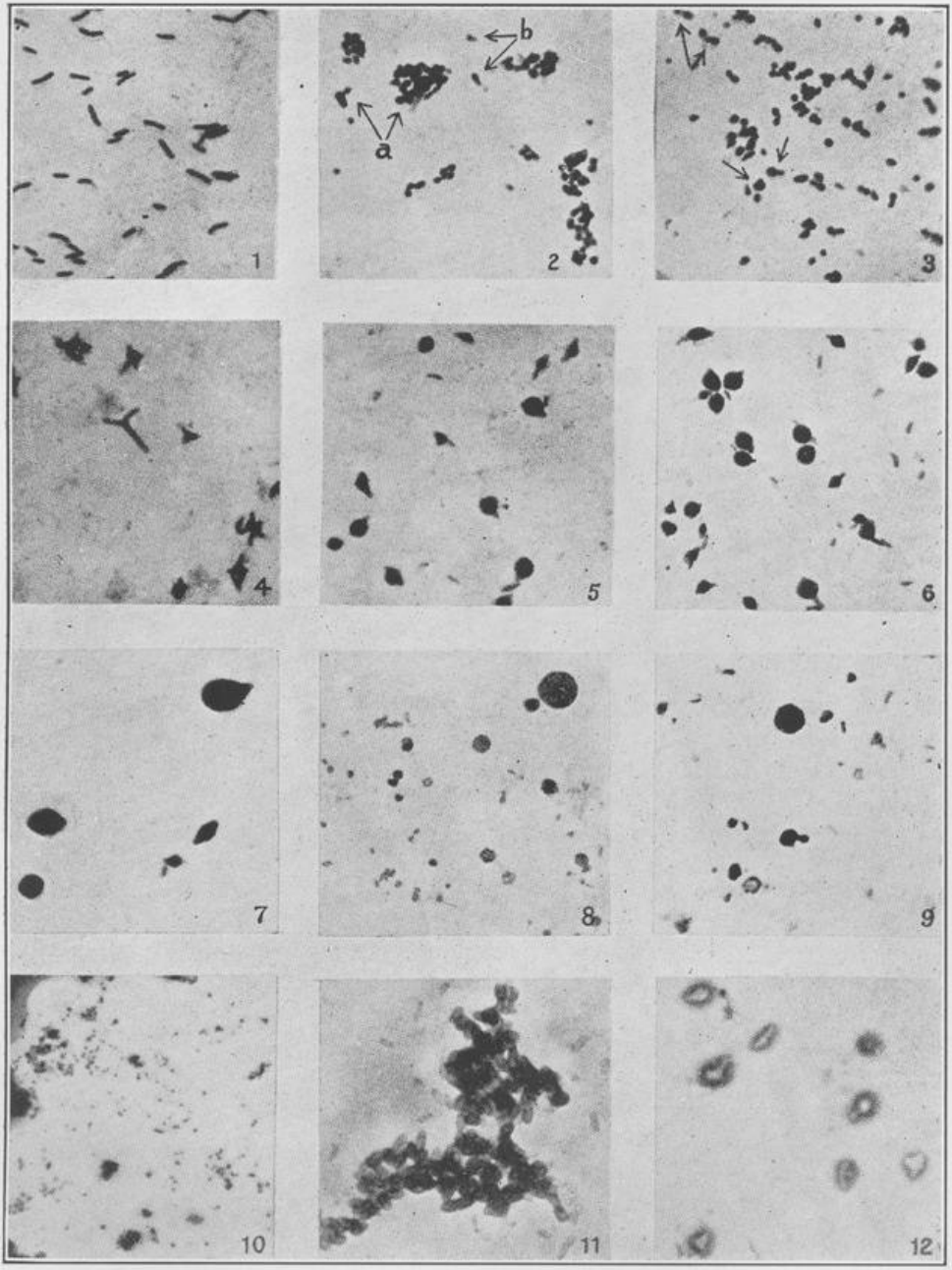
Káš, 1927, and Gibson, 1928, have presented the most complete studies of the life cycles of these organisms. Gibson recognized the following growth forms: rods, coccoids, branched forms, gonidangia, gonidia and dwarfed growth, and microcysts (Plate 16). The reproductive processes described are fission, budding, liberation of gonidia, formation of regenerative bodies, and germination. The formation of symplasm and the regeneration of cells from it was also noted. Káš described essentially the same forms, with the addition of arthrospores and exospores. Conjunction or conjugation was also noted. Káš made the interesting observation that the gonidia, 0.1μ-0.9μ, are much more resistant to drying than are the vegetative cells and that they are formed in large numbers upon the drying-down of agar or gelatin media. Gibson emphasized the fact that the greatest variability of form is observed in the early stages of growth and on culture media permitting luxuriant growth. Unfavorable conditions tended to produce the uniform small rods.

Śnieszko, 1928, and Schönberg, 1929, likewise noted the presence of several different life-cycle stages in their cultures. Plate 17 illustrates the effect of pH upon the morphology of *Rh. phaseoli* and *Rh. trifolii.*

STAINING CHARACTERS AND FLAGELLATION

Young cells of the root nodule organism stain uniformly with the ordinary anilin dyes. In older cultures, however, many of the cells are vacuolated and stain irregularly. While most of the stains ordinarily used in bacteriology can be used, a weak carbol fuchsin (Ziehl formula diluted 1-10 with water) is one of the best. Perfectly clean mounts are difficult to secure because of the interference from the gum surrounding the cells, often causing considerable precipitation of the stain.

Harrison, 1907, and Harrison and Barlow, 1907, have utilized the ability of the gum to absorb anilin dyes in a negative staining method which gives excellent results. A loopful of the growth from an agar slant culture is placed on a clean glass slide and rapidly whipped out, without addition of water, into long, thin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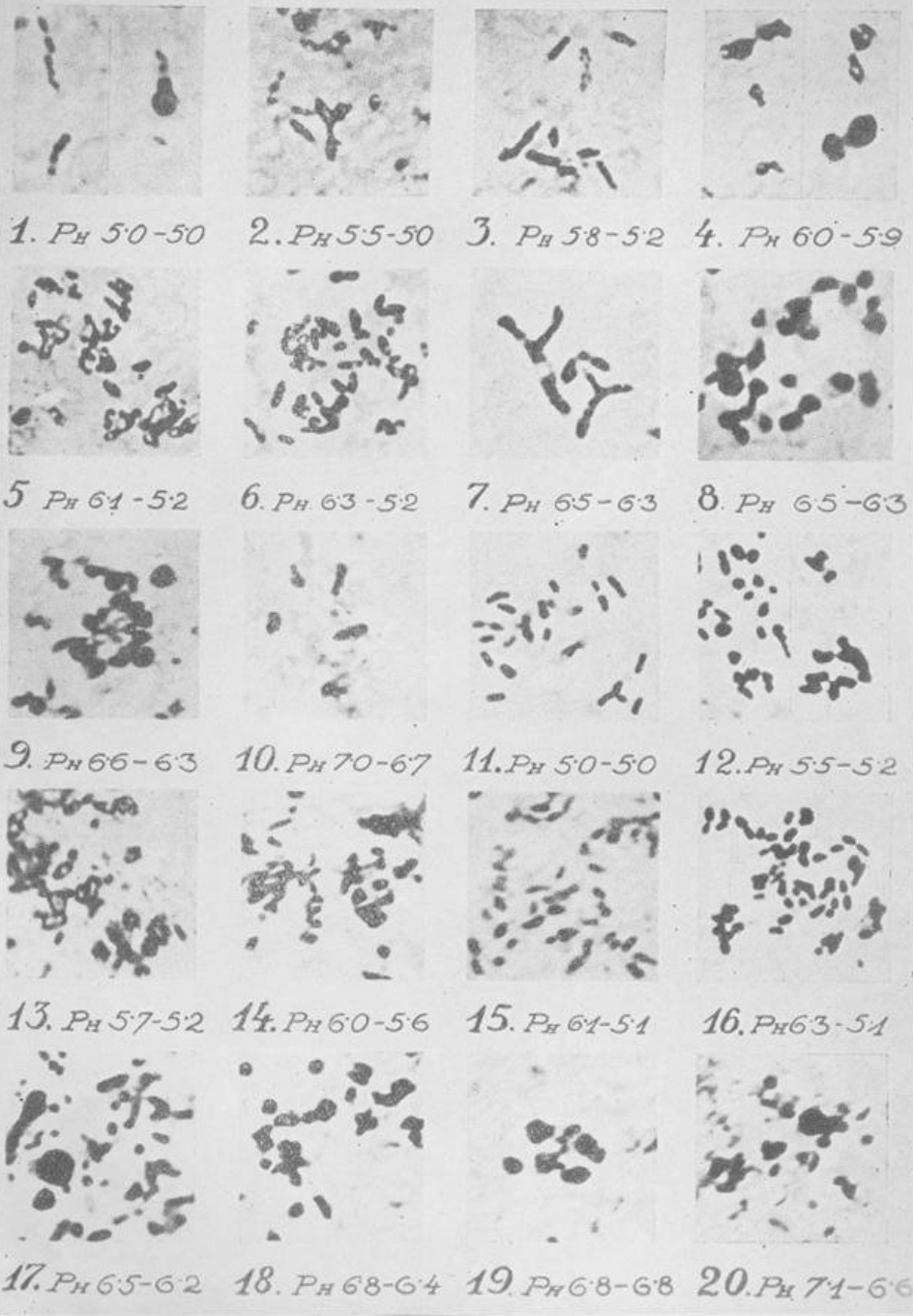
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15. PH 6·1-5·1
16. PH 6·3-5·1
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18. PH 6·8-6·4
19. PH 6·8-6·8
20. PH 7·1-6·6

streaks. Dry the slide in the air; flood with saturated alcoholic gentian violet[1] for 30 seconds to 1 minute and wash off in running water. Quickly blot off the excess water and dry with gentle heat. Saturated alcoholic solutions of methyl blue, night blue, or fuchsin may be substituted for the gentian violet.

Such preparations show the gum deeply stained, while the cells have absorbed very little of the stain. The unequal density and internal structure of the cells are often clearly portrayed. Frequently in preparations by this method there is a clear uncolored streak extending out from a cell into the deeply stained gum. This was originally considered a flagellum by Harrison and Barlow. Kellerman, 1910b and c, and others have criticized this interpretation and have shown that such appearances are produced by using this stain on an artificial mixture of a plant gum and bacteria which are known to be devoid of flagella.

Gangulee, 1926b, described a method which gives quite satisfactory results for general staining of the cells. In the hands of Gangulee the method is used also for the demonstration of flagella; however, the authors have been unable to secure satisfactory preparations with it. "A drop of suspension from a fresh culture is placed at one end of the slide with a platinum loop and the slide tilted in order to allow the drop to spread over it. The film is dried in an oven at 45° C. and fixed with absolute alcohol. After the alcohol has evaporated, the film is flooded with 5% erythrosin stain dissolved in 70% alcohol, which is allowed to act for ten minutes. After washing off the alcoholic stain, the slide is re-stained with 5% phenolic erythrosin[2] (1 gm. of stain dissolved in 5% phenol) for another ten minutes. By this method the flagella of the lucerne organism were successfully stained . . ."

A number of other flagella stains have been used by different workers. Those described by Burrill and Hansen, 1917, and Shunk, 1921, are satisfactory. Müller and Stapp, 1925, secured good preparations with Zetnow's stain and Whiting, Fred, and Helz, 1926, used the Plimmer-Paine (Casares-Gil) technique.

The number and arrangement of the flagella have been studied by many workers. Table 2 gives a summary of the results secured. As first suggested by Hansen, 1919, the organisms may be divided roughly into two broad groups on the basis of flagellation. An elaboration of Hansen's suggestion, 1919, is given in the paper by Löhnis and Hansen, 1921. Here the organisms of the cowpea and soybean cross-inoculation groups are designated as monotrichous and those of the clover, pea, alfalfa, navy bean, lupine, black locust, *Amorpha,* and *Strophostyles* as peritrichous. The careful studies of Shunk, 1921, support this classification. Müller and Stapp, 1925, did not find any of the organisms to be definitely monotrichous, although they did find that several of the organisms usually had only one flagellum.

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[1]The following modification of the original stain was given to the authors by Mr. Barlow, and is preferable to the plain alcoholic dye solution:

Glucose	50.0 gm.
Glycerol	50.0 cc.
Water, distilled	50.0 cc.
Gentian violet	3.0 gm.

Dissolve the glucose in the glycerol-water solution with heat and then add the gentian violet. Bring the mixture to a boil and allow to cool.

[2]In another place Gangulee gives, "1 per cent erythrosin dissolved in a 5 per cent phenol solution," a more accurate statement of the composition.

TABLE 2

Studies on the flagellation of the root-nodule organism

Author	Year	Type of flagellation	Organisms from	Notes
Beijerinck	1888	Monotrichous	*Vicia, Ervum, Trifolium, Pisum, Medicago, Genista,* and *Melilotus*	
Greig-Smith	1899	Monotrichous	Not stated	
Hartleb	1900	Monotrichous	Not stated	
Löhnis	1905	Probably peritrichous	Vetch or clover	No faultless preparations were obtained. The stains were of either clover or pea organisms or both
Moore	1905	"At but one end"	Leguminosae in general	Recommended *Pseudomonas*
Maassen and Müller	1906	Lophotrichous	Not definite. 27 species included in their studies	
Harrison	1907	Monotrichous	Not stated	It is doubtful whether the staining method used gives accurate results
Harrison and Barlow	1907	Monotrichous	*Lathyrus* and *Vicia* among others	It is doubtful whether the staining method used gives accurate results
de'Rossi	1909a	Peritrichous	Not definitely stated. 18 plant species included in the studies	
Zipfel	1911	Peritrichous	*Psium, Vicia, Trifolium* and *Phaseolus*	
Kellerman	1910b and c	Peritrichous	*Pisum sativum*	
Prucha	1915	Peritrichous	*Pisum arvense*	
Whiting	1915	Peritrichous	Not stated	
Burrill and Hansen	1917	Monotrichous	*Vigna, Desmodium, Genista, Mucuna, Arachis, Baptisia, Lespedeza, Acacia, Cassia, Glycine* and *Amphicarpa*	These organisms are all low gum-producers. Were unable to secure flagella stains of the heavy gum producers

TABLE 2 (Cont.)

Author	Year	Type of flagellation	Organisms from	Notes
Wilson	1917	Peritrichous	*Soja Max*	
Barthel	1918	Lophotrichous	Lupine and alfalfa	
Fred and Davenport	1918	Peritrichous Monotrichous	*Medicago sativa* *Lupinus*	
Hansen	1919	Monotrichous Peritrichous	*Vigna* and *Glycine* *Trifolium*, *Melilotus*, and *Vicia*	Suggested that the nodule organism may be divided into two definite groups on this basis
Conn and Breed	1920	Variable		Suggested that they may be monotrichous in young cultures, peritrichous in old cultures
Shunk	1921	Peritrichous Monotrichous	*Vicia, Pisum, Lathyrus, Phaseolus, Trifolium, Medicago, Melilotus* and *Robinia* *Albizzia, Cassia, Falcata, Baptisia, Cracca, Soja, Meibomia, Vigna, Arachis, Stylosanthes, Clitoria, Pueraria, Dolichos, Lespedeza* and *Stizolobium*	
Löhnis and Hansen	1921	Monotrichous Peritrichous	Cowpea, soybean Alfalfa, clover, vetch	Divide into (a) slow-growing group which is monotrichous and (b) fast-growing one which is peritrichous
Wright	1925a	Monotrichous	*Soja Max*	
Müller and Stapp	1925	2—4 flagella 2—3 flagella, occasionally 1 or 4 1 flagellum	*Pisum, Vicia, Lathyrus, Phaseolus, Trifolium, Medicago* and *Melilotus* *Lupinus* *Anthyllis, Lotus, Soja, Robinia, Onobrychis, Coronilla*	The number is not always constant, but those given are representative
Whiting, Fred, and Helz	1926	Peritrichous	*Dalea alopecuroides*	
Gangulee	1926b	More than one flagellum	*Crotalaria juncea*	

Before flagellation can be satisfactorily used as a basis for classification, however, it will be necessary to make another critical study of the problem in order to harmonize some of the conflicting data, *e.g.*:

(1) Löhnis and Hansen, 1921, classed the lupine organism as peritrichous and a fast grower. Fred and Davenport, 1918, found that their lupine strains are slow growers, and that the strain tested for flagella has only one or two flagella. Barthel, 1918, found the lupine organism to have 1 to 6 polar flagella, while Müller and Stapp, 1925, class the lupine organism as a very slow grower with 2 or 3 flagella.

(2) Shunk's, 1921, classification of the organisms into two groups agrees with Löhnis and Hansen, 1921, in every particular except with respect to the organism from *Phaseolus lunatus*. This organism belongs to the cowpea cross-inoculation group and should be monotrichous. Shunk placed it in the peritrichous class with 1 to 4 flagella.

(3) Wilson, 1917, classed the organism from *Soja Max* as peritrichous, whereas Burrill and Hansen, 1917; Löhnis and Hansen, 1921; Wright, 1925a, Shunk, 1921; and Müller and Stapp, 1925, found it monotrichous. Conn and Breed, 1920, suggested that the age of the culture may be the deciding factor, and that the organism is monotrichous in young cultures and peritrichous in old cultures. Shunk, 1921, suggested that different types of the organism may occur in different geographical regions, and that there may be both a monotrichous and a peritrichous organism forming nodules on soybean.

(4) Müller and Stapp's, 1925, failure to find a sharp distinction between monotrichous and peritrichous organisms does not harmonize with the work of Shunk, 1921, and Löhnis and Hansen, 1921.

(5) Löhnis and Hansen, 1921, suggested that there may be an actual change from peritrichous to cephalothrichous flagellation. In their opinion, however, the conflicting views are probably due to the difficulty in preparing flagella stains and the dislocation of the flagella during the process.

In a review of the morphology of the rhizobia, there are a number of points which seem well established. Perhaps the one most characteristic of the group is that of decided variations in form. Without exception, the workers in this field have found small rods, sometimes cocci, larger rods, and branched or bacteroid forms. These three types of cells are noted not only within the tissues of the nodule but also in pure cultures under laboratory conditions. These distinct changes in structure are so well known that for years the rhizobia have been the favorite objects of study for those who are interested in life cycles. Beautiful preparations of the bacteroid forms showing the various bizarre shapes are easily obtained from nodules of pea, or alfalfa, or other members of these plant-bacteria groups. Apart from change in form, the rhizobia also exhibit variations in the number of flagella. The motility of the rhizobia depends upon the age of the culture and the morphological types present.

CHAPTER 6.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF THE ROOT NODULE BACTERIA

". . . you should not so
much labour what to speak as to
find what to leave unspoken."
—BACON

A review of the voluminous literature concerning the cultural and biochemical characteristics of the nodule organisms of the Leguminosae can not but impress the reader that the time has come to stop and take thought of our knowledge of this subject; only so shall we be able to integrate our ideas in order that future work may not be vain repetition. Only so can we work intelligently to repair the weak spots in our knowledge and to prepare for further work. New ideas, new methods of study, and new findings in other fields are waiting to be applied to the nodule problem. The recent studies in the life history of the nodule bacteria, the possible rôle of the bacteriophage, and the variations between cultures of the same cross-inoculation group are but a few of the outstanding points to be given consideration. This chapter can give at best only a brief statement of some facts and ideas concerning the physiology of this group of bacteria. Much of the information has been condensed and is presented in this chapter in tabular form. Naturally, most of the work has centered around the nitrogen and carbon metabolism of the organisms. Other miscellaneous reactions are incidental and will be considered briefly at the end of the chapter.

NITROGEN NUTRITION

Although the nodule bacteria can live and multiply in a medium free or almost free from combined nitrogen, it is well known that the rate of growth is much slower than in a culture medium containing even a small amount of available nitrogen. The nature of the nitrogen compound is a very important factor and determines to a large degree the rate of growth. Of the various organic nitrogenous compounds commonly used in culture media, none has been found more beneficial for nodule bacteria than yeast, malt, or plant extracts. In Chapter 4 a more detailed discussion of this subject is presented under the title "Culture Media for Isolation."

Nitrogen fixation. The fact that leguminous plants are unable to assimilate atmospheric nitrogen in the absence of nodules would seem to indicate that the bacteria are the active agents responsible for this process. It is reasonable, there-

fore, to assume that the bacteria of the nodules when cultured in a suitable medium apart from the host plant should fix nitrogen. Unfortunately, the results of numerous tests fail to furnish conclusive evidence that the bacteria do possess the power to assimilate atmospheric nitrogen. Simple as it seems, the problem has baffled the minds of many investigators.

Some idea of the diverse results obtained by various investigators will be seen from the summary in Table 3. In a few cases appreciable increases in nitrogen were found, in others only small quantities, and in a large number no gain at all. The column designated "Reported Number of Analyses" is an approximate estimate of the total number, not including the preliminary analysis made in some cases. There are blanks in the table, as many of these reports fail to give complete information regarding the medium, the cultures used, and the results of the analyses. For the figures given in the column "Nitrogen Fixed", an effort was made to reduce all results to a common basis, milligrams of nitrogen in 100 cc., and in the last column on the right to give the author's conclusions regarding his results.

Most of these papers need not be discussed individually, since the essential data are given in the table. In some cases there is doubt about the purity of the cultures; for example, the cultures of Mazé, 1897 and 1898, are questionable, because the author himself mentioned the "cheesy" odor, and because insufficient precautions were taken to sterilize the air with which the cultures were aerated. Yet in spite of this, Mazé's experiments are often quoted as proof that the root nodule bacteria are capable of fixing nitrogen outside the host plant. A number of the papers require a further discussion.

The first work on nitrogen fixation by the root nodule bacteria was done in 1888 by Beijerinck. He found that neither bacteria from the nodules of *Cytisus laburnum* grown on agar containing nitrogen-free salts and nutrients, nor bacteria from *Vicia Faba* root nodules when grown in a salt solution with added asparagin are able to fix nitrogen. In 1891, the same author reported that bacteria from *Vicia Faba* fix only a small amount of nitrogen (0.9-1.8 mg. N in 100 cc.) when grown in bean-seedling extract to which sucrose (1.5-2 per cent) and varying amounts of KH_2PO_4 are added. Beijerinck concluded that nitrogen fixation was probable, but he was not satisfied that his experiments offered conclusive proof. In 1918, returning to the same problem, he again expressed doubt that nitrogen could be fixed by nodule bacteria in culture. Although cultures in synthetic media and plant extracts with 2 per cent sucrose gained a slight amount of nitrogen, the conclusion was drawn that the amount was too small to support the belief that nitrogen is fixed by these organisms. Beijerinck appears to have exercised unusual care in testing the purity of his cultures, so that in this regard there seems no question.

A number of other workers have reported findings similar to those of Beijerinck, *viz*: Immendorff, 1892; Gonnermann, 1894; Heinrich, 1894; Zinsser, 1897; Hiltner, 1897; Stoklasa, 1898; and Greig-Smith, 1899, 1900.

Stutzer, Burri, and Maul, 1896, used a glucose mineral-salt solution as a culture medium for alfalfa nodule bacteria. Although as high as 6 mg. of nitrogen in 100 cc. was gained in one case, no fixation was reported.

Clover and vetch nodule bacteria, as well as several unrelated organisms, were grown by Löhnis, 1905, in soil extract containing one per cent glucose and 0.05 per

TABLE 3

A summary of reports upon the fixation of atmospheric nitrogen by root nodule bacteria in culture

	Medium used	Reported number of analyses (estimated)	Nitrogen fixed in 100 cc. of culture	Nitrogen fixed
			mg.	
Beijerinck, 1888	Nitrogen-free salts, asparagin agar	----	----	No
Prazmowski, 1890, 1891	Nitrogen-free medium	----	----	Yes
Beijerinck, 1891 (1)	Bean-seedling extract + sucrose + KH_2PO_4	6	0.9–1.8	Probable
Frank, 1892a	Nitrogen-free medium	----	----	Uncertain
Immendorff, 1892	Various kinds	----	None	No
Berthelot, 1893	"Cohn's medium" + humic acid	----	5.3*	Yes
Gonnermann, 1894	Potato pulp	----	None	No
Heinrich, 1894	Potato pulp	19	None	No
Stutzer, Burri, and Maul, 1896(1)	Potassium phosphate, $MgSO_4$, NaCl, $CaCl_2$, and glucose solution	8	6.0	No
Hiltner, 1897		----	----	No
Mazé, 1897(1)	Bean-seed extract + NaCl + $NaHCO_3$ + sucrose, with and without agar	6(?)	23.4–27.1	Yes
Zinsser, 1897	Potassium phosphate, $MgSO_4$, "sugar" solution	----	None	No
Mazé, 1898(1)	Bean-seed extract + sucrose	3	24.2–30.0	Yes
Stoklasa, 1898		----	None	No
Greig-Smith, 1899	Lupine-leaf extract + KH_2PO_4 + $CaCl_2$ + agar	----	----	No
Greig-Smith, 1900	Various media, including Mazé's	----	None	No
Neumann, 1902b(1)	Plant extract and peat extract	9	4.4–49.9	Yes

*5.3 mg. gained in total culture. Volume not given.

TABLE 3 (Continued)

	Medium used	Reported number of analyses (estimated)	Nitrogen fixed in 100 cc. of culture	Nitrogen fixed
Hiltner and Störmer, 1903a	Glucose, KH_2PO_4, and peptone, asparagin or KNO_3 solutions	----	None	No
Chester, 1904	Glucose, NaCl, $CaCO_3$, $FeSO_4$, K_2HPO_4 agar.	8	0.6–2.5	Yes
Lewis and Nicholson, 1905(2)	Sucrose salt solutions and sucrose bouillon	30	0–16.2	Yes
Löhnis, 1905(3)	Soil extract + glucose + K_2HPO_4	----	2.8–3.6	Yes
Moore, 1905(1)	Maltose, $MgSO_4$, potassium phosphate solution	90	0.2–2.2	Yes
Golding, 1905-6	Bean- and pea-plant juices + sugars, K_2HPO_4, NaCl, $FeSO_4$, $MnSO_4$, $MgSO_4$ and succinic acid	10	(with pure cultures) 2.1–3.5	Yes
Greig-Smith, 1906c(4)	Glucose, sodium phosphate solution + agar	56	1.0–4.0	Yes
Budinov, 1907(1)	Bean extract + sucrose	2	4.1	Yes
Bottomley, 1909	KH_2PO_4, NaCl, $CaCO_3$, $FeSO_4$ mannitol solution	6	0.4	Yes
de'Rossi, 1909b(5)	Bean-seed and bean- and vetch-leaf extracts + sugars, NaCl, solutions or + gelatin or agar	33	–27.7 to 3.1 per 100 gm.	No
Fred, 1911b(1)	K_2HPO_4, $MgSO_4$, NaCl, $Fe_2(SO_4)_3$, $CaCl_2$, $MnSO_4$, glucose solution	----	0.18–1.68	Yes
Bottomley, 1910a	Same as Bottomley 1909	----	1.8	Yes
Golding, 1910		----	----	Yes
Fred, 1913(1)	Same salts as Fred 1911b + maltose and sucrose + agar	101	0.15–1.66	Yes
Bottomley, 1912a	$MgSO_4$, potassium phosphate, maltose solution	----	2.0	Yes
Greig-Smith, 1912		----	3–5.6	Yes
Spratt, 1912a and b(1)	$MgSO_4$, potassium phosphate, sucrose solution	8	2.5–3.5	Yes
Herke, 1913	Soil extract + K_2HPO_4 + mannitol	37	0.14–1.2	Yes

Olaru, 1915	Mazé's bean extract + Mn	22	1.5–32.1	Yes
Rocasolano, 1915–6(1)	Mannitol solution + Mn	8	2.1–9.2	Yes
Beijerinck, 1918	K_2HPO_4, lime, glucose solution + garden soil; other media	----	----	No
Hills, 1918(2)	$MgSO_4$, KH_2PO_4, NaCl, $CaSO_4$, $CaCO_3$, mannitol agar	46	0.15–3.5	Yes
Joshi, 1920	Soil extract + K_2HPO_4 + mannitol	----	0.8–2.0	Yes
Singh, 1920(1)	Soil and sucrose, K_2HPO_4 solution	42	0.25–10.75	Yes
Hutchinson, 1923		----	2.5	Yes
Voicu, 1923(1)	Bean-seed extract + sucrose and boron	45	2.4–3.5	Yes
Hutchinson, 1924		----	----	Yes
Barthel, 1926(6)	KH_2PO_4, $MgSO_4$, NaCl, $CaSO_4$, KNO_3, $FeCl_3$, mannitol solution	8	–0.21 to 0.39	No
Fred, Whiting and Hastings,1926(2)	Soil extract + sucrose	4	0.1	Yes
Allison, 1927(1)	Clover plant extract + glucose	----	None	No
Bazarewski, 1927	Synthetic medium	----	1.3–3.0	Yes
Stiehr, 1927	Lupine extract-glucose agar	5	0.6–2.8 per 100 gm.	Yes
Halversen, 1927(4)	Moore's glucose Ashby's solution	----	0.2–9.86	Yes
Skinner, 1928(4)	Ashby's agar	4(?)	None	No
Hopkins, 1929(4 and 7)	Soil extract + glucose or sucrose + K_2HPO_4	500	–0.8 to 0.4	No
Allison, 1929	Various plant extracts, soil extracts + 16 carbohydrates	600+	–2.41 to 1.09	No
Löhnis, 1930a(2 and 8)	Pea foliage, bean seed, yeast water, and soil extract + sucrose	256+	–3.66 to 4.21(2) –0.13 to 0.003(8)	No No
Pohlman, 1931a(1)	Synthetic nitrogen-free; same + peptone, urea, asparagin, yeast water; silica gel	125+	–0.36 to 0.80 –9.12 to 1.2 –0.07 to 1.06	No

When no number is given, the method of analysis is not designated.

1. Kjeldahl
2. Gunning modified to include nitrates
3. Kjeldahl—Wilfarth
4. Kjeldahl—Gunning
5. Kjeldahl—Jodlbauer
6. Bristol—Page
7. Davisson—Parsons
8. ter Meulen

cent K_2HPO_4. Although the original soil extract contained no nitrates, this author used the Kjeldahl-Wilfarth procedure of nitrogen analysis to include nitrate nitrogen, stating that it was absorbed from the air. Gains in nitrogen of 2.8 to 3.6 mg. in 100 cc. were found.

Moore, 1905, using a nitrogen-free solution of magnesium sulphate, potassium phosphate, and maltose, reported gains in nitrogen of 0.2 to 2.2 mg. in 100 cc. by cultures of root nodule bacteria from red clover, soybean, white lupine, hairy vetch, and garden pea. Chester, 1904; Greig-Smith, 1906c; Bottomley, 1909, 1910a, and 1912a; Spratt, 1912a and b; Joshi, 1920; Hutchinson, 1923, 1924; Bazarewski, 1927; and Stiehr, 1927, have reported similar gains in nitrogen.

The experiments of Golding, 1905-06, are frequently cited with those of Mazé as conclusive proof that atmospheric nitrogen is fixed by the root nodule bacteria in culture, but an examination of Golding's work seems to indicate that he has been given undue credit. He attempted to simulate the environment of the bacteria in the nodule by providing means of removing waste products during growth of the culture. This he claimed to accomplish by placing the cultures in an inverted bell jar at the bottom of which a porous filter candle was fitted, and by drawing off the medium through this candle. The whole apparatus was then covered by another bell jar in a natural position. From Golding's paper, it appears that the entire culture was put in the inverted bell jar, and culture fluid was thus not added continuously. If such is the case, there is little justification for his claim that the products of growth were continuously removed. In one experiment, unheated macerated young bean plants were placed in the inverted bell-jar with distilled water, and the apparatus was attached to the suction pump. A gain of 11.4 mg. of nitrogen in 100 cc. took place in 15 days. In later experiments the medium was sterilized and inoculated with pure cultures of root nodule bacteria (strain not mentioned). The gain in nitrogen amounted to about 2.1 and 3.5 mg. nitrogen in 100 cc. On the basis of nitrogen gained in 100 cc. of medium by pure cultures, Golding's results are not more striking than those of many other workers. De' Rossi, 1909b, attempted to duplicate the results of Mazé, but the gains of nitrogen in 100 gm. of agar medium were; +1.0 mg., —27.7 mg., +2.0 mg. Other results were +3.1 mg., —7.7 mg. He also set up an experiment similar to that of Golding, from which he obtained the following results: +4.2 mg., —3.4 mg. nitrogen in 100 cc. He concluded that the nodule bacteria fix insignificant amounts of nitrogen.

Fred, 1913, reported gains of 1.3—1.6 mg. per 100 cc. of cultures of 8 strains grown in a maltose or sucrose mineral salts agar. Olaru, 1915, added varying amounts of manganese salts to Mazé's bean-extract medium. In this medium, pea nodule bacteria fixed as much as 32.1 mg. of nitrogen in 100 cc. when 0.5 mg. of manganese was present, while without manganese salts only 1.2 mg. of nitrogen were fixed.

During 1929-30 the experiments of Golding and Olaru were repeated by workers at this station (Wilson, Hopkins, and Fred, 1932) with a view to ascertaining whether there was any substance in their claims of positive fixation under the special conditions used by them. As far as possible the details of their experiments were faithfully reproduced. Up to the present time all results have shown

that neither the crude cultures nor the pure cultures give any indication of significant gain in nitrogen.

The effect of nitrates on nitrogen fixation was studied by Hills, 1918. Calcium, potassium, and sodium nitrates were added in varying amounts to the agar. Unfortunately, the modified Gunning (salicylic acid) method to include nitrates was used for the nitrogen analyses, which showed a fixation of 0.15—3.5 mg. of nitrogen in 100 cc. This method has been shown to be unreliable for the determination of nitrate nitrogen in the presence of water, and thus Hills' results are of questionable value.

Boron is reported by Voicu, 1923, as stimulating nitrogen fixation. Varying amounts of boric acid were added to a bean-extract sucrose solution; maximum fixation of 3.5 mg. of nitrogen in 100 cc. of medium occurred when 10 mg. of boron as boric acid were present. The organisms used were from pea and vetch root nodules.

Barthel, 1926, found no nitrogen fixed by pea nodule bacteria in a nitrogen-free mineral salts solution with or without added caffein. Red-clover-plant extract, according to Allison, 1927, gave no gain in nitrogen when inoculated with red clover nodule bacteria.

Using Moore's medium and Ashby's solution with glucose as substrates for *Rh. leguminosarum,* Halversen, 1927, obtained nitrogen fixations of 0.2—9.9 mg. per 100 cc.

In 1929 Allison published an extensive study of nitrogen fixation by nodule bacteria in culture. He used many strains of the bacteria and a great variety of culture media, testing out plant extracts, soil extract, and some 16 carbohydrates. In all he analyzed over 900 cultures, of which 600 are reported in his paper. The analyses were done by the Kjeldahl method, at first with $CuSO_4$ and later with metallic mercury as catalyzer for digestion. The final conclusion reached was that "The experiments in no case gave any evidence that Rhizobia can fix atmospheric nitrogen when grown apart from the host."

Hopkins, 1929, tested the nitrogen-fixing ability of several strains of nodule bacteria when grown in soil extract cultures. The soil extract was made by heating soil with an equal volume of water, adding $CaCO_3$, and filtering. 0.05—.1 per cent K_2HPO_4 and 1 per cent sugar (glucose or sucrose) were added to the soil extract. Such soil extracts were found to contain nitrates. Davisson and Parsons, 1919, showed that the Gunning method modified to include nitrate nitrogen as given by the Association of Official Agricultural Chemists is unreliable for the determination of nitrates in the presence of water. An improved method was proposed, and Jacob and Geldard, 1922, reported favorably on its accuracy. A series of the soil extract cultures were analyzed by both the modified Gunning method and the Davisson-Parsons method. Those analyzed by the former method appeared to have made a slight gain in nitrogen content, while those analyzed by the Davisson-Parsons method showed no gain in nitrogen beyond the limit of experimental error and frequently lost nitrogen. The results are given in Table 4. The apparent fixation of nitrogen in the set analyzed by the modified Gunning method was due to the loss of nitrate nitrogen from the controls. Each figure in the column under "Total Nitrogen" represents the average of about five cultures. Diphenylamine tests for

TABLE 4

Comparison of different methods of determining nitrogen

Culture	Total nitrogen		Diphenylamine nitrate test	pH	Sugar fermented
	Modified Gunning method	Davisson-Parsons method			
	mg. per 100cc	mg. per 100cc			per cent
		Without Calcium carbonate			
Control	4.2	4.8	+	7.2	—
Red clover 205	5.3	4.7	—	6.0	25.7
Pea 310	5.6	5.0	—	6.6	36.0
Alfalfa 100	4.1	4.1	—	7.6	12.9
Soybean 504	4.2	4.4	—	7.6	None
		With Calcium carbonate			
Control	4.3	4.6	+	7.6–7.8	—
Red clover 205	5.4	4.9	—	7.6	12.1
Pea 310	5.6	5.0	—	7.5	29.8
Alfalfa 100	4.4	4.6	+	7.6	None
Soybean 504	4.2	4.2	+	7.6	1.6

Davisson-Parsons analyses made of cultures 25 days old.
Modified Gunning analyses made of cultures 52 days old.
Sugar analyses, pH and nitrate tests made on cultures 32-4 days old.

nitrates, pH of cultures, and percentage of sugar destroyed are also given in the table.

Some recent work of Dr. Marie Löhnis, 1930a, considers a new phase of this question. Using pea-leaf extract plus sucrose as a medium, and analyzing the controls at the same time as the inoculated cultures, she found as much as 3 mg. of nitrogen fixed in 100 cc. of medium. When, however, one set of controls was analyzed at the time of inoculation and another after incubation, it was found that nitrogen had been lost from the incubated controls, whereas in the inoculated medium this nitrogen had been taken up by the organisms before it could be lost. In this way, an apparent fixation resulted, and Dr. Löhnis suggests that such an effect may explain many of the cases of reported fixation. Soil extract and yeast-water mannitol solutions generally suffered a loss of nitrogen on standing, and no nitrogen was fixed in these media beyond the limit of experimental error.

What conclusions, then, can be drawn from the diversity of opinion? Were it possible to decide the question by balancing the "yeses" against the "noes," the matter would be immensely simplified, but the differences existing between the experimental conditions prevailing in each case will not admit such an easy solution. Some of the differences to be considered are: the carbon—nitrogen ratio of the medium; the amount of nitrogen initially present in the medium; reaction of the medium; the age of the inoculum; the age of the cultures on analysis; purity of the cultures; the method of analysis used; and the effect of stimulatory substances, either chemical elements or plant extracts. With such a variety of dissimilarities, it is well nigh impossible to reduce all of these results to a common basis. In the

results reported before 1900, the majority of opinion was that nitrogen was not fixed by the nodule bacteria. From 1900 until a few years ago, the majority swung in the opposite direction with only a few dissenting reports; since then the tide of opinion seems to have turned, and a number of papers indicate that nitrogen is not fixed.

The last three negative papers, the Hopkins, Allison, and Löhnis reports, are particularly interesting in that they appeared independently and almost simultaneously; all three represent extensive experiments under varied conditions and with different methods of analysis, and all three reached the conclusion that there is *no evidence of the nodule bacteria alone being able to fix atmospheric nitrogen.* Still, none of these investigators is willing to say that the bacteria cannot under any circumstances fix nitrogen outside the host. It is at least possible that sometime a new medium or new conditions of experiment may be found which will allow fixation. Neither is it disproved that nodule bacteria fix nitrogen in the soil surrounding the roots of leguminous plants. And so our conservative conclusion is very similar to that of Nobbe and Hiltner, 1893, who said, "trotz vielfacher Versuche ist es bisher nicht mit Sicherheit gelungen, durch Kultur des *Bacterium radicicola* in den verschiedensten Medien eine in Betracht kommende Zunahme des Stickstoffgehaltes zu erzielen."

Nitrogen compounds utilized by rhizobia—The behavior of the rhizobia on the inorganic nitrogenous nutrients, such as nitrates and ammonium salts in media, has not been extensively studied. It is known that the addition of nitrate to a synthetic solution such as Ashby's stimulates growth. Hills, 1918, demonstrated an appreciable reduction in the nitrate nitrogen content of media resulting from growth of the rhizobia, and since neither nitrite nor ammonia was produced, he concluded that the nitrate was assimilated as such by the rhizobia. It has long been known that the presence of nitrate has a pronounced effect upon nodule formation by the bacteria. (See Chapter 11.) Hills consequently collected some data on the effect of various concentrations of nitrate on the bacteria themselves. He found that ammonium, sodium, potassium, and calcium nitrate in sterilized soil in concentrations up to 25 mg. per 100 gm. of soil showed a distinctively stimulatory effect upon multiplication of the bacteria. Above this optimum concentration there is a progressively depressing effect until above 100-150 mg. per 100 gm. the nitrates appear to be actually toxic. This phase of the relation of the bacteria to nitrate will be further discussed in Chapter 11 under the effect of nitrates on nodule formation.

The reduction of nitrates has been investigated by Beijerinck, 1888; Orla-Jensen, 1909; Zipfel, 1911; Prucha, 1915; Wilson, 1917; Hills, 1918; Müller and Stapp, 1925; Leonard, 1929; and Pohlman, 1931c. Zipfel noted that the organisms of pea, horse bean, red clover, and garden bean nodules all reduce nitrate to nitrite but not to ammonia. Negative results are reported by Prucha for field peas, by Hills for alfalfa, and by Wilson for soybean bacteria. According to the recent report of Müller and Stapp, 1925, the reduction of potassium nitrate to nitrite varies, depending upon the strain of organism. Cultures from *Pisum, Vicia, Trifolium, Anthyllis, Lotus,* and *Coronilla* all form appreciable amounts of nitrite, whereas the organisms of *Soja, Lupinus,* and *Genista* form only a trace of nitrite.

Here again, the reduction of nitrate to nitrite and possibly to ammonia is a function of the rate of growth of the organisms, the fast-growing cultures naturally producing more than the slow-growing in a given length of time. Pohlman, 1931c, obtained reduction of nitrates by *Rh. meliloti* and *Rh. japonicum*. Nitrites accumulate in the cultures, although there is evidence of slight utilization of nitrite by both organisms. Leonard, 1929, attempted to correlate the reducing power of these bacteria with certain cultural characteristics. He found that in general the low nitrogen-fixing strains produce greater amounts of nitrite.

Ammonium salts may be used by the nodule bacteria, but all attempts to grow the nodule bacteria in media with nitrite instead of nitrate nitrogen have failed (Müller and Stapp, 1925). Pohlman, 1931c, noted that *Rh. japonicum* is able to utilize ammonium sulphate to better advantage than is *Rh. meliloti*. He suggested that the explanation lies in the fact that the soybean organism produces an alkaline reaction in culture, thereby tending to neutralize the sulphuric acid as it is liberated and prolonging the period of favorable reaction for growth.

Peptone and beef extract gelatin, the nitrogenous compounds so widely used by general bacteriologists, have not been found well adapted to the nodule bacteria. According to Müller and Stapp, 1925, the great variation in reaction of different brands of peptone is one of the reasons why peptone is not suitable. Of the five kinds of peptone investigated, Witte's peptone proved the least satisfactory for the nodule bacteria. The same authors reported that asparagin, sodium asparaginate, and uric acid may serve as sources of nitrogen, whereas glycocoll and hippuric acid are unsuitable. Alanine, leucine, and urea give only a weak growth. All of the work of Müller and Stapp was carried out in a nitrogen-free medium prepared from inorganic salts, glycero phosphate, and glucose. Pohlman, 1931c, reported the behavior of *Rh. japonicum* and *Rh. meliloti* in synthetic media containing glycocoll, dl-alanine, dl-amino-n-butyric acid, dl-valine, d-glutamic acid, l-cystine, l-tyrosine, dl-phenylalanine, p-amino benzoic acid, urea, asparagin, and peptone. In general he found that the organisms studied do utilize the nitrogenous compounds in question, usually with attack on the amino group and in some cases with liberation of free ammonia. Important differences in the behavior of *Rh. meliloti* and *Rh. japonicum* were noted in glycocoll, l-tyrosine, dl-amino-n-butyric acid, and urea media, and it was suggested that these reactions be used for differentiating. The possibility of further differentiation was found in the action of types A and B of *Rh. meliloti* on dl-alanine, dl-amino-n-butyric acid and l-tyrosine. Types A and B of *Rh. japonicum* can likewise be distinguished by testing on dl-alanine, l-tyrosine, d-glutamic acid, l-cystine, p-amino benzoic acid, and asparagin.

A study of the effect of the various nitrogenous compounds on the growth of the organisms from various cross-inoculation groups appears most promising, and it is hoped that someone will greatly extend this line of investigation. In the section devoted to factors that influence the production of bacteroids, the effect of certain alkaloids and related compounds in media for rhizobia has been discussed.

Gelatin liquefaction. Almost without exception, the early investigators, Beijerinck, 1888; Hiltner, 1900b; Kellerman and Beckwith, 1906b; Harrison and Barlow, 1907; de'Rossi, 1907; Zipfel, 1911; Prucha, 1915; Wilson, 1917; and others reported the nodule bacteria unable to liquefy gelatin. Fremlin, 1898, made

an exception of the lupine nodule bacteria, which he found to liquefy gelatin rapidly.

Burrill and Hansen, 1917, after a careful study of a large number of cultures, concluded that these organisms slowly liquefy gelatin. Somewhat similar results were reported by Fred and Davenport, 1918. Perhaps the most exhaustive study of this phase of the root nodule problem is the report of Stapp, 1924. He employed a large number of cultures, representing almost every cross-inoculation group, and without exception noted a slow liquefaction of the gelatin. Walker, 1928, also studied liquefaction of gelatin with a large number of cultures. He determined the effect of the bacteria on the gelatin by the changes in viscosity of the medium. By means of this test he found that certain strains liquefied gelatin, although the majority did not. Because of the great variation in the production of gum by different species of nodule bacteria, it seems to be writers that the viscosity method is not adapted to a study of the liquefaction of gelatin by rhizobia.

In general, it has been observed that on standard nutrient gelatin, growth of the nodule bacteria is slow, chiefly at the top of the medium, and no liquefaction is noted for one or two weeks. In older cultures, after three months or more of incubation, the gelatin shows a slow liquefaction. If soil-extract or plant-extract gelatin is used, liquefaction is somewhat faster, but never so fast as observed with many of the common soil microörganisms. According to recent findings at Wisconsin, the rhizobia of the alfalfa group are particularly active in gelatin liquefaction.

The results of Müller and Stapp, 1925, emphasized the importance of using only gelatin of the proper reaction in a medium otherwise suitable for the growth of the nodule bacteria, *e.g.*, carrot extract. They also pointed out that long incubation may be required to show liquefaction. In cultures incubated for 18 days at 15° to 18° C. a zone of liquefaction appears around the line of inoculation of cultures from *Pisum, Vicia, Phaseolus, Melilotus, Medicago,* and *Trifolium,* whereas those of *Soja* and *Lupinus* show little liquefaction or none at all.

Growth in milk. By allowing the nodule bacteria to grow in milk for 1 to 6 weeks, Löhnis and Hansen, 1921, showed that they can be divided into two clear-cut groups. The organisms of the first group form a "serum" zone in milk. They are the bacteria from alfalfa, clover, sweet clover, vetch, pea, navy bean, lupine, black locust, *Amorpha,* and *Strophostyles,* and are further characterized as growing comparatively rapidly on agar plates and having peritrichous flagella. The bacteria of the second group do not form a serum zone in milk, grow very slowly on agar plates, and have a single flagellum. The milk cultures, after several weeks incubation, show a gradual digestion. The organisms from cowpea, soybean, peanut, beggar-weed, *Acacia, Genista,* and *Cassia* belong to this group. These generalizations are not without exception, as is evident from recent results obtained at Wisconsin. Certain strains of the rhizobia from the pea, in fact, fail to produce serum zones at all.

Reaction in milk culture offers another means of distinguishing *B. radiobacter* from the nodule bacteria. It forms a serum zone on the surface, but unlike the true nodule bacteria, turns the milk brown.

The important investigations of Löhnis and Hansen were repeated by a num-

ber of investigators and in the main found to be correct. Müller and Stapp, 1925, pointed out that the lupine bacteria belong not in the first but in the second group. They also found that the organism from *Ornithopus sativus* and *Coronilla varia* belong to the second group.

Stevens, 1925b, introduced indicators into the milk and thereby was able to follow changes in reaction and reduction, as well as serum zone formation. He tried Janus green, brom thymol blue, and litmus, and found the last most satisfactory, as it is not toxic to the organisms in question, gives sharp color changes with change in reaction, and indicates reduction fully as well as Janus green. It has the further advantage that the reduction-oxidation color change is reversible.

According to serum zone and change in reaction, Stevens was able to divide the nodule bacteria into 3 groups: (1) serum zone positive and reaction acid; (2) serum zone positive and reaction alkaline; and (3) serum zone negative and reaction alkaline. These typical reactions are not immediately apparent; the cultures must be watched for several weeks in order to follow the successive stages. For example, within the first few days after inoculation, alfalfa and sweet clover organisms produce an alkaline reaction; later they begin to reduce the litmus; and still later produce enough acidity to bring out pink coloration. The acid production is more rapid with freshly isolated strains than with strains carried in the laboratory for a number of years. Cultures of the 2nd and 3rd groups develop the alkaline reaction quickly and remain alkaline to the end of the test. The final reactions of all three groups are indicated in the following table:

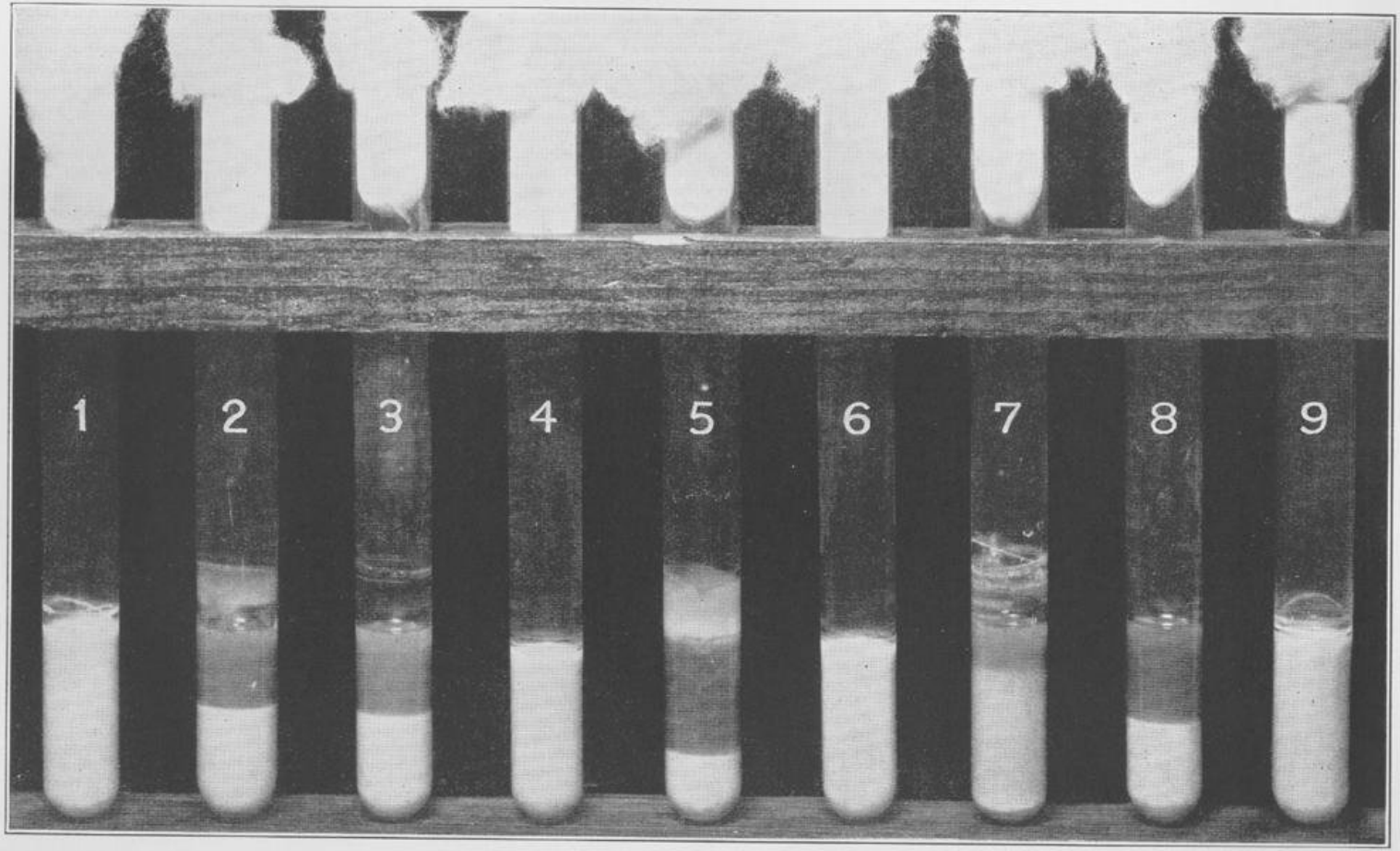
Some of the characteristics of nodule bacteria in litmus milk

Strains from	Serum zone	Reaction
Alfalfa	Present	Acid
Sweet clover	Present	Acid
Clover	Present	Alkaline
Garden pea	Present	Very Alkaline
Garden bean	Present	Very Alkaline
Vetch	Present	Very Alkaline
Cowpea	None	Very Alkaline
Lima bean	None	Very Alkaline
Soybean	None	Very Alkaline
Lupine	None	Very Alkaline

The effect of these cultures on the H-ion concentration of the milk after 47 days of incubation is shown below. The determinations reported were made by the electrometric method:

	pH
Uninoculated	6.55
Alfalfa rhizobia	5.24
Soybean rhizobia	8.06
Cowpea rhizobia	8.08
Lupine rhizobia	7.55
Lima bean rhizobia	7.85

Certain cultures in litmus milk regularly cause reduction of the litmus, while others never do. The acid-producing alfalfa and sweet clover organisms are of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8
9

the reducing type, whereas the alkali-forming cowpea, lima bean, and soybean organisms do not reduce litmus at all. Unfortunately, this division according to reduction is not clear cut, nor does it parallel that according to reaction change. Alkali-forming cultures of the serum-zone-positive type may or may not develop reduction reactions; strains of garden pea and clover are particularly variable. The nature of the serum zone is not entirely understood. It is a zone at the surface of the milk more translucent than the milk, and is due apparently to settling of the suspended constituents of the milk. It has been termed "serum" zone because of a superficial similarity to the serum zone formed in oxalated blood on standing. Plate 18 illustrates the serum zones as developed in six-week cultures of various species of rhizobia.

There is little information on the chemical changes produced in consequence of serum formation. The following figures for cultures of alfalfa and sweet clover bacteria indicate a well-defined increase in soluble nitrogen and amino acid content. The characteristic changes in skimmed milk after 450 days incubation are shown below:

Unpublished Wisconsin data

Forms of nitrogen and carbon	Control	In 100 cc. of culture	
		Alfalfa	Sweet clover
	mg.	mg.	mg.
1. Total nitrogen	526.9	526.7	502.1
2. Insoluble nitrogen (casein, albumin)	453.7	424.4	434.6
3. Not precipitated by trichloracetic acid	50.7	95.8	89.9
4. Total amino nitrogen	24.6	40.2	43.4
5. Sugar before hydrolysis	3130.0	2317.0	3496.5
6. Sugar after hydrolysis	4276.0	2340.3	3475.0

How far these data may be looked upon as typical of all serum-forming rhizobia is not known.

From a survey of the work that has been carried out on the nitrogen metabolism of rhizobia, certain features seem outstanding. The rhizobia apart from the host plant fail to show any significant fixation of nitrogen.

The more common media employed in bacteriological work are not well adapted to the growth of rhizobia. Plant extracts, preferably from members of the leguminous family, or yeast extract plus a suitable carbohydrate furnish the best substrate for the growth of rhizobia.

The nitrogen requirements of the nodule bacteria may be supplied entirely in the form of inorganic nitrogenous compounds, *viz.*, nitrate, or in the form of complex organic nitrogen compounds, plant proteins, etc. On a medium very low in combined nitrogen, these organisms make fair to good growth.

Although the rhizobia of the several plant-bacteria groups show qualitative variations, all cultures possess the power of slowly reducing nitrates to nitrites and of liquefying gelatin. In litmus milk they exhibit a slow reduction of the litmus, and in the case of certain species, a slight proteolysis with serum-zone formation. The changes in litmus milk are of value in the separation of the different species.

THE UTILIZATION OF VARIOUS CARBOHYDRATES AND RELATED COMPOUNDS

Because of the complex nitrogenous compounds required for the growth of many kinds of microörganisms, it is not always easy to limit the carbon resources of a medium and so test out the availability of carbohydrate materials for any given group of organisms. The problem is fortunately simplified in the present case by the fact that the nodule bacteria will grow in a synthetic medium with inorganic nitrogen.

A number of investigators have compared the relative value of the different carbon compounds for the growth of these bacteria. Zipfel, 1911, reported the formation of acid from both glucose and lactose. He found that the reaction developed in a medium prepared from an extract of leguminous plants, plus sugar and litmus, is sufficiently changed by the growth of these organisms to turn the litmus red. Fred, 1913, found that small amounts of acid are formed from sucrose, maltose, and mannitol in both nitrogenous and non-nitrogenous media. Four strains of alfalfa bacteria and two strains of clover bacteria were tested and no significant differences noted. Temple, 1916, noted that sucrose and glucose are superior to lactose for the culture of rhizobia. Fructose he found of no value for these organisms.

Joshi, 1920, compared the growth of the organisms from pea (*Pisum sativum*), cowpea (*Vigna catjang*), sunn-hemp (*Crotalaria juncea*), Indigo (*Indigofera arrecta*), math (*Phaseolus aconitifolius*), and val (*Dolichos lablab*) on soil-extract phosphate agar plus 1 per cent of glucose, fructose, sucrose, maltose, lactose, or mannitol. In order of their value for the growth of these various species of rhizobia, mannitol ranks first, followed by glucose, sucrose, maltose, and fructose. Lactose, on the other hand, gives comparatively poor growth regardless of the source of the nodule bacteria.

Stevens, 1925a, found that both hydrogen-ion concentration and titratable acidity are increased by the growth of the alfalfa bacteria in a peptone-sucrose broth. Eight strains were used and marked differences noted in their ability to produce acid. Quite the opposite was found to be true for the soybean bacteria. Wright, 1925a, not only failed to secure an increase in acidity but noted a decrease in both hydrogen-ion concentration and total or titratable acidity, and also an alkaline reaction in litmus milk. He used six strains of soybean bacteria and observed only slight differences in their behavior.

Müller and Stapp, 1925, brought out the important point that the various cross-inoculation groups behave differently toward the various carbon compounds. Table 5 shows the comparative values they obtained with a base medium of the following composition, to which is added 1 per cent of the desired carbon compound.

Agar	18.0 gm.
Magnesium sulphate	0.4 gm.
Potassium chloride	0.2 gm.
Potassium nitrate	2.0 gm.
Calcium glycero-phosphate	0.8 gm.
Water	1000.0 cc.

It is clear that a great variety of carbon compounds will support growth of the rhizobia. This fact appears of special significance when we consider their life in the soil and in the plant sap. In addition to the carbohydrates, the higher alcohols, mannitol, glycerol, and dulcitol are utilized by these organisms. Of all the groups of nodule bacteria, the alfalfa—sweet clover type is the most active, as judged from the number of carbon compounds attacked and the total amount of sugar consumed.

TABLE 5

Comparison of the growth of various strains of the root nodule bacteria with different sources of carbon

Glycerophosphate medium

No.	Compound	Alfalfa Sweet clover	Clover	Pea Vetch	Soybean	Bean	Lupine
1	Arabinose	+++		++	++++		++++
2	Xylose	++++		+	++++	++++	++++
3	Glucose	++++		++	+	+++	
4	Fructose	++++	+	++		+++	
5	Galactose	+++		++			
6	Sucrose	++++	++	++		++	
7	Maltose	++++	+++	+++		+++	
8	Lactose	++++	+	+		++	
9	Raffinose	++++	+++	+++		++	
10	Mannitol	++++		++		+++	
11	Glycogen	+0	+	+0			
12	Glycerol	++++		++		++	
13	Dulcitol	+++	+++	+++	+0	+0	
14	Dextrin	+0		++			
15	Inulin		+	+0			

+0Very slight growth +Slight growth ++Medium growth +++Good growth
++++Excellent or profuse growth.

Baldwin and Fred, 1927, studied the fermentation characteristics of 60 different cultures of the nodule bacteria from alfalfa, clover, pea, bean, cowpea, soybean, and Dalea. All of these cultures had been repeatedly tested for purity and ability to form nodules. For comparison, *B. radiobacter* was included in their studies.

The composition of their medium follows:

Agar	15.0 gm.
Dipotassium phosphate	0.5 gm.
Potassium nitrate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.2 gm.
Calcium carbonate[1]	3.0 gm.
Organic carbon compound	10.0 gm.
Water, distilled	1000.0 cc.

[1]Calcium carbonate was omitted from the medium to which organic acids were added.

TABLE 6

The fermentation characters of the nodule bacteria on certain sugars and glucosides

No.	Carbon compound	Alfalfa	Red clover	Pea	Bean	Cowpea	Soybean	Lupine	Dalea	B.radiobacter
1	Rhamnose	(1) +++	++	++	++	−−	−−	++ −−	++++	++++
2	Arabinose	++++	+++	++++ ++	+ ++++	+++ ++++	+++ +	++ −−−	++	++++
3	Xylose	+++	0	0 ++	+ ++	−−	−	++ −−−	−−	++
4	Fructose	+ −−−	+	+	+++ −	−−−	−−−	0 −−−	−	+++
5	Glucose	++++	+++	+++	+++ +	−−−	−−−	++ −−−	+++	+++
6	Galactose	++++	++	++	+++ +	−−−	−−−	−− −−−	0	++++
7	Mannose	+++	++	++	+++ +	−−−	−−−	+ −−	+	++++
8	Sucrose	+++	0	++	+++ +	−−	−−	−−	−	−
9	Lactose	++ −−−	+	+	++ +	−	−	−− −−−	+	+
10	Maltose	++ +	++	++	− 0	−−−	−−−	−− −−−	0	0
11	Melibiose	+++ −−	0 −	− −	0	−−	−−	0	−−−	−−−
12	Trehalose	++ −−	−	+	++ −−	−	−	−	−−−	−−−

TABLE 6—Continued

No.	Carbon compound	Alfalfa	Red clover	Pea	Bean	Cowpea	Soybean	Lupine	Dalea	B.radiobacter
13	Raffinose	++ / −−−	0	−−	++ / −−	−−	−−	−−− / −−	−	−−
14	Melezitose	++ / −	0	0	− / 0	0	0	−− / 0	−−	−−
15	Dextrin	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	++++
16	α-methyl glucoside	−−	−−	−−	−−	−−	−−	−−	−−	−−
17	Amygdalin	+++ / 0	0	0	0	−−	−−	−−	0	+++
18	Salicin	++ / −−	0	0	0	−−−	−−−	−−	0	+++

++++ strongly acid, +++ moderately acid, ++ slightly acid, + very slightly acid, −−−strongly alkaline, −−moderately alkaline, −slightly alkaline, 0 no change in reaction.

1. Variations between different cultures of the same group are indicated by double entries.

TABLE 7

The fermentation characters of the nodule bacteria on certain alcohols and organic acids

No.	Carbon compound	Alfalfa	Red clover	Pea	Bean	Cowpea	Soybean	Lupine	Dalea	B.radiobacter
1	Glycerol	(1) +++ / −−−	++ / −−	++ / −	−	−−−	−−−	−−−	0	++++
2	Erythritol	+ / −	+ / −	+	−	−	−	−	−	++
3	Adonitol	0	0	−−	−	−−−	−−−	−−	−	++++
4	Mannitol	+++ / −−	++ / −	+ / +++	++	−−−	−−	−−	−−	+++
5	Dulcitol	−−	+	0	−	−−	−−	0	−	++
6	Sorbitol	+++ / −−−	++ / 0	++	−	−−	−−	−−	−−	−−
7	Sodium tartrate	−−	−−−	−−− / 0	0	−−−	−−−	−	−	+++
8	Sodium malate	−−−	0 / −−−	0 / −−−	−−−	−−−	−−	0	−−−	−−−
9	Sodium succinate	−−−	−− / −−−	−−−	−−−	−−− / 0	0	0	−−−	−−−
10	Sodium lactate	−−−	− / −−−	− / −−	−−−	−−−	−−−	0	−−−	−−−
11	Sodium citrate	0	0 / −−	0	0	0	0	0	−−	++
12	Sodium formate	−−−	−−−	−−−	−−−	−−−	−−−	−−	−−−	−−−

++++ strongly acid, +++ moderately acid, ++ slightly acid, + very slightly acid, −−−strongly alkaline, −−moderately alkaline, −slightly alkaline, 0 no change in reaction.

1. Variations between different cultures of the same group are indicated by double entries.

Five cc. of 0.5 per cent alcoholic solution of brom thymol blue was used per liter of medium; with this internal indicator present, changes in acidity were apparent as the cultures developed.

The cultures were incubated at room temperature, 18-22° C., for 4 weeks and the visible changes in reaction recorded. Their results and some later unpublished data from the same laboratory are summarized in Tables 6 and 7.

As a rule the alfalfa bacteria were more vigorous producers of acid than any of the other organisms. This is in accord with the report of Müller and Stapp. By the use of sucrose, xylose, mannose, or galactose, the alfalfa bacteria may be separated with a fair degree of accuracy from any of the other cross-inoculation groups. There is, however, within the group of 19 strains of alfalfa bacteria studied, considerable variation with respect to the production of acid from many of the carbon compounds.

The clover group, 8 strains, and the pea group, 14 strains, react much alike. They produce acid from most of the carbohydrates in this list, but in no case is there a vigorous fermentation. In order to differentiate between these two groups, clover and pea, sucrose may be used, although the division is by no means clean cut.

The bean bacteria, 6 strains, produce variable quantities of acid. On maltose these bacteria product slight alkalinity or no change, a circumstance which may be used in part for their differentiation.

Soybean bacteria, 8 strains, and cowpea bacteria, 3 strains, react similarly. No acid is produced from any carbon compounds except arabinose, and in practically every case the medium becomes distinctly alkaline. With arabinose, however, the reaction is first strongly alkaline and later changed to acid. The same condition prevails with xylose, but the change from alkaline to acid reaction is much slower. Xylose and arabinose permit a more vigorous growth of the soybean, lupine, and cowpea bacteria than do any of the other compounds tested.

Dalea bacteria are very vigorous growers, and in almost all cases the first reaction, after 3 to 5 days, is slightly alkaline, particularly on the upper portion of the slant. Later, enough acid is produced from some of the sugars to overcome the initial alkaline reaction and to turn the balance to the acid side.

B. radiobacter behaves much the same as dalea bacteria, but with several of the carbohydrates it is a more vigorous acid producer.

In summary, we may conclude that the mono-hexoses are more easily fermented with the production of acid than are any of di- or tri-hexoses. Fructose is not utilized nearly so readily as the aldo-hexoses. None of the nodule bacteria is able to produce an acid reaction when grown on dextrin.

A similar relationship holds with the glucosides and alcohols as with the sugars. The alfalfa, clover, pea, and bean bacteria produce acid from most of these compounds, and again the alfalfa bacteria are the strongest acid producers. The weakest are the bean bacteria; the clover and pea bacteria are intermediate. The soybean and cowpea bacteria in most cases produce a strongly alkaline reaction. Dalea bacteria in every case first produce a strongly alkaline reaction and later usually an acid reaction.

With the glucosides and alcohols, as with the sugars, considerable variation

is noted among the fermentation characters of the members of each group. The polyhydric alcohols bring out these differences very clearly.

In the case of the salts of the organic acids, the utilization of the acid radical usually results in a lowered hydrogen-ion concentration.

In addition to the organic-acid salts listed in the tables, sodium oxalate, sodium acetate, and calcium pyruvate were tested. Neither the oxalate nor the acetate ions were utilized by any of the organisms tested. In the case of the pyruvate ion, slight growth with the production of a weak alkaline reaction was secured with certain cultures. The reaction of these organisms to the pyruvate ion is of interest, because normally this compound is thought to be broken down very readily by bacterial action. Only very few of the nodule bacteria, however, were able to utilize it in these tests. In this connection it should be mentioned that pyruvic acid is apparently a "normal" product in the fermentation of carbohydrates by certain root-nodule bacteria. Anderson, Peterson, and Fred, 1928, found it produced from xylose, glucose, sucrose, lactose, and mannitol by two strains of alfalfa bacteria.

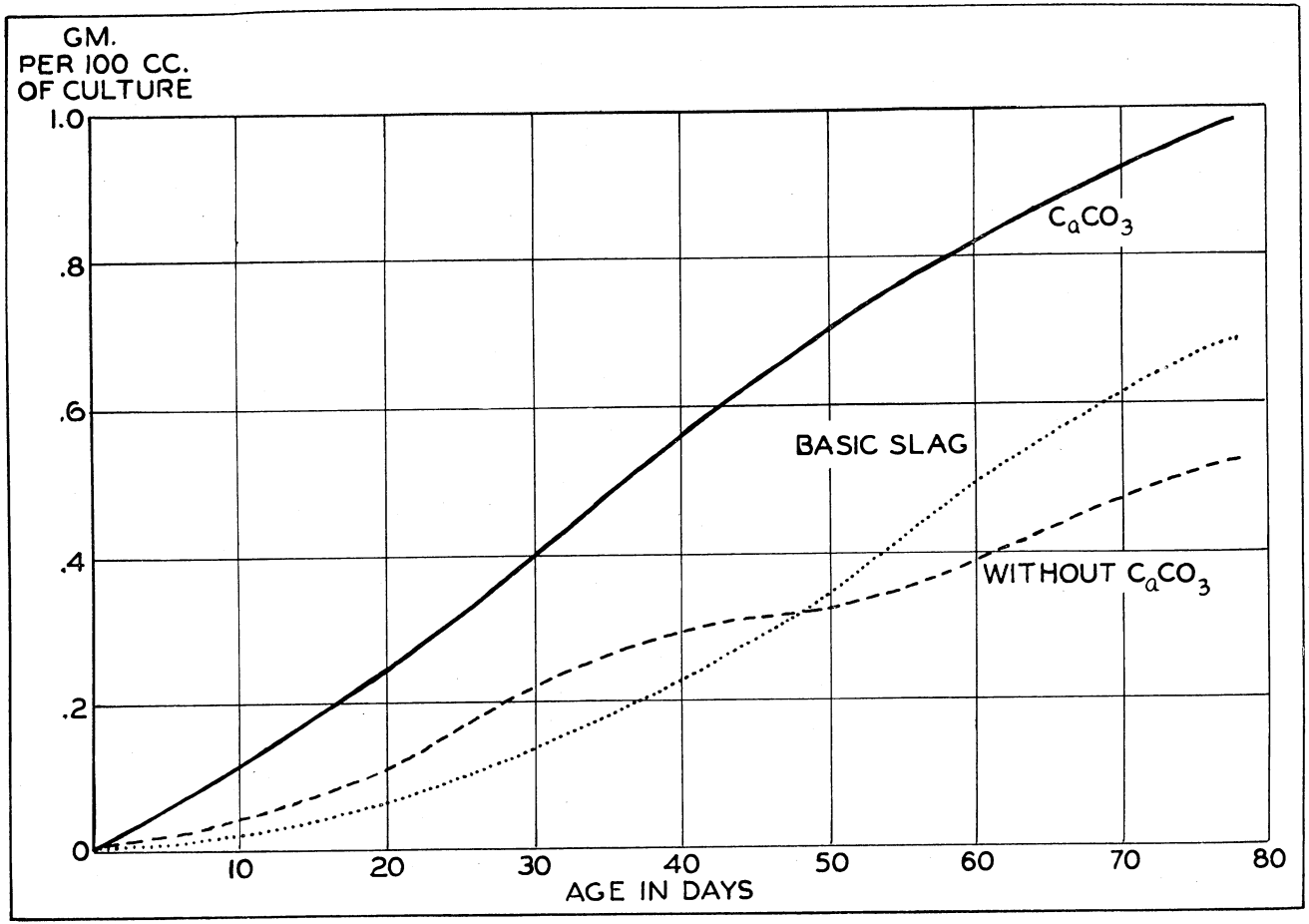
All of the other acids tested supported the growth of the organisms. In practically every case where a vigorous growth was secured, the reaction of the medium became alkaline. However, the final reaction of the medium cannot be taken as a measure of the growth of the organism. In some cases a marked lowering of the hydrogen-ion concentration occurred with a very scanty growth, whereas in others a vigorous growth was accompanied by little change in the reaction of the medium. Sodium malate, sodium succinate, and sodium lactate support better growth than do any of the other organic acids. The organic acids fail to support as good growth of the rhizobia as do the carbohydrates and polyhydric alcohols. Little difference in fermentation characters was noted among the strains of the alfalfa, soybean, and cowpea bacteria.

B. radiobacter showed the interesting phenomenon of producing acid reactions on the media containing sodium tartrate and sodium citrate.

In a recent paper, Walker, 1928, has determined the change in hydrogen-ion concentration produced by various cultures of rhizobia when grown in the presence of various carbohydrates. From a great number of tests he concluded that the fermentation reaction might be of value in the separation of cross-inoculation groups.

Miss Schönberg, 1929, made a study of the characteristics of several of the root nodule bacteria on sugar media, which she considered similar in constitution to that used by Baldwin and Fred, 1927, but she omitted NaCl, $CaCO_3$, and $MgSO_4$ and used tap water in place of distilled water. Consequently, her color reactions are not entirely comparable, for as Baldwin and Fred pointed out, the medium and the rate of growth thereon influence the observed time and degree of color change. Miss Schönberg found that all of the organisms formed acid from glucose, although the soybean cultures were first alkaline and later acid in reaction. Xylose medium gave poor fermentation results except with lupine cultures; bean organisms turned the medium distinctly alkaline. After three weeks on sucrose medium, all cultures except from *Phaseolus vulgaris* were acid.

Walker and Brown, 1930, have investigated the constancy of the fermentation


GM.
PER 100 CC.
OF CULTURE
1.0
.8
.6
.4
.2
0
10
20
30
40
50
60
70
80
AGE IN DAYS
C_aCO_3
BASIC SLAG
WITHOUT C_aCO_3

tests of various strains of *Rh. meliloti* and *Rh. japonicum* on glucose and galactose media. Their medium was the same as that of Baldwin and Fred, and their criterion of fermentation, the change in hydrogen-ion concentration. The results indicate a very large variation in the fermentation by individual strains. Some of the *Rh. meliloti* strains produced a distinctly alkaline reaction and others a strongly acid reaction. The majority of *Rh. japonicum* strains produced a more alkaline reaction than the majority of *Rh. meliloti* strains, but this is not necessarily true of individuals. Five to eight consecutive tests showed relative stability among the individual strains.

The rate of sugar fermentation. The relation between the amount of sugar fermented and the mass of cells developed is far from clear. Enough has been done, however, to demonstrate that the usual sugars and other so-called fermentable carbon compounds are utilized comparatively slowly by the nodule bacteria.

The first report is that of Mazé in 1898, made in connection with his studies of nitrogen fixation by the organisms in artificial culture. In a medium containing approximately 2 per cent of sucrose he found 59 to 68 per cent destruction of sugar in 19 to 22 days. In 1913 Fred reported a limited fermentation of maltose and sucrose by various strains of the nodule bacteria; in no case was more than 20 per cent of the sugar utilized in one month's time. In a brief report on nitrogen fixation by "non-symbiotic" organisms, Hutchinson in 1923 presented a graph of glucose destruction by *B. radicicola* (source not given). He found a relatively rapid destruction of the glucose during the first 10 days and then only a slow utilization. Of the 2 per cent in the original medium, he found about 0.4 per cent remaining after 60 days' incubation. Foote, Peterson, and Fred, 1929, followed periodically the consumption of glucose and xylose by *Rhizobium meliloti, Rh. trifolii, Rh. leguminosarum,* and *Rh. japonicum* in a yeast water medium. All cultures were grown in large flasks containing shallow layers about 2 cm. deep, both with and without a neutralizing agent present. Sugar determinations and bacterial counts by plating were made at intervals of two weeks for a period of 10 to 12 weeks.

The results of this work with glucose and *Rh. japonicum* are shown in Chart 2. A slow but continuous destruction of sugar was found. At the end of 78 days approximately 90 per cent of the sugar was destroyed in the cultures containing calcium carbonate, about 63 per cent in the presence of basic slag, and in the unneutralized culture only 50 per cent.

Change in the reaction of the medium. The growth of the nodule bacteria in liquid media with various sources of carbon is accompanied by a small increase in acidity or alkalinity depending upon the kind of bacteria and the carbohydrate used. Naturally the buffer content of the medium has a profound influence on the apparent change in reaction.

Fred and Davenport, 1918, reported that the pH of a non-nitrogenous synthetic sucrose liquid medium may be decreased slightly by the growth of the root nodule bacteria. Twenty-five different cultures, including members of the alfalfa, clover, pea, bean, soybean, cowpea, and lupine groups were tested and the change in pH found to vary from 0 to 0.4 unit from the initial.

Bialosuknia and Klott, 1923, and Bialosuknia, 1923, observed that there is a considerable variation in the degree of acidity formed by the different groups of the nodule bacteria. The initial reaction of the medium, kind of carbohydrate,

and the particular strain of nodule organism decidedly influence the amount of acid formed. Although classed as acid-sensitive, alfalfa nodule bacteria are among the highest acid producers.

TABLE 8

Increase in titratable acidity resulting from the growth of alfalfa and sweet clover bacteria after 7 weeks at 28° C.

cc. 0.1 N acid per 100 cc.

Strains from	Initial reaction				
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Alfalfa	1.50	1.50	2.75	3.50	4.75
Alfalfa	1.00	1.25	3.75	4.00	4.25
Alfalfa	1.00	2.50	5.75	5.25	6.75
Sweet clover	1.00	2.00	5.75	4.50	5.25
Alfalfa	1.00	2.00	3.75	2.50	1.75
Alfalfa	0.00	1.00	2.75	4.00	2.75
Alfalfa	1.50	2.00	3.75	3.50	2.25
Alfalfa	0.50	1.50	1.75	3.50	2.75

No growth at pH 5.5

TABLE 9

The change in pH *resulting from the growth of alfalfa, pea, and soybean bacteria*

Age	Source of carbon	Control	Alfalfa	Pea	Soybean
Days		pH	pH	pH	pH
42	Sucrose	7.2	7.2	7.2	7.2
63	Sucrose	7.2	6.5	7.2	7.3
84	Sucrose	7.2	6.7	7.2	7.5
42	Glucose	6.9	6.9	6.9	6.9
63	Glucose	6.9	6.6	6.9	7.0
84	Glucose	6.9	6.6	6.9	7.1
42	Mannitol	7.2	7.2	7.2	7.2
63	Mannitol	7.2	7.2	7.2	7.4
84	Mannitol	7.2	7.2	7.2	7.5

The results of Stevens', 1925a, study of alfalfa and sweet clover bacteria in peptone-sucrose medium are given in Table 8. The culture medium contained 2 per cent of sucrose, 0.1 per cent of peptone, 0.2 per cent of KH_2PO_4, and 0.01 per cent of $MgSO_4$, and was adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The results show that wherever there is appreciable growth, there is appreciable titratable acidity; the most acidity is produced when the initial range of reaction is pH 7.0—8.0, or about optimum for growth of the rhizobia. In general it is found that the more alkaline the medium at the time of inoculation, the greater the total amount of acid produced.

Additional evidence of the power of the alfalfa nodule bacteria to produce an acid reaction is shown in Table 9. The organisms from pea nodules produce no change in reaction, while the soybean cultures actually show a slight gain in alkalinity.

Aso and Murai, 1926, found that in the change of reaction of the culture medium, the organisms of soybean, lupine, and serradella were distinct from all others of the rhizobia group. The cultures from soybean, lupine, and serradella produce an alkaline reaction, whereas the other cultures produce an acid reaction.

ENZYMES OF THE BACTERIA

It is well known that a pure culture of bacteria may secrete a variety of enzymes, and that to a certain extent the amount and kind of enzyme may be influenced by the nature of the nutrient substrate. Enzymes are spoken of as endo- or exo-enzymes according as they act within the cell body or diffuse out into the medium and act externally. For a comprehensive review of the enzymes produced by microörganisms the reader is referred to Waksman and Davison, 1926.[2]

Beijerinck, 1888, was the first to study enzyme production by root nodule bacteria. He pointed out that these bacteria do not produce cytase, diastase, or invertase, but do produce reducing and hydrogen peroxide-splitting enzymes. Twelve years later, Hiltner, 1900a, found in the extract of nodule cultures passed through a porcelain filter a substance which caused root hairs to swell and curl, but did not dissolve the cell walls. An extensive study of the oxidizing enzymes of rhizobia was carried out by Stapp in 1923. In order to measure the production of tyrosinase by the bacteria, Stapp made use of cultures grown on two kinds of media—carrot extract with and without agar plus 0.15 per cent of tyrosin, and asparagin-mannitol plus tyrosin. The occurrence of a brown to dark brown pigment is taken as a measure of tyrosinase production. Usually this decided dark color is formed in the bacterial cultures within a few days after inoculation. Stapp stated that a more rapid test for tyrosinase can be made by incubating a suspension of bacteria in water containing tyrosin and a few drops of chloroform. A temperature of 50-60° C. and pH 8.0 appear to be optimum for the demonstration of tyrosinase. Because the test is more rapid when killed bacteria are used, Stapp believes that this enzyme should be classed as an endo-enzyme.

Stapp tested in all 76 cultures of bacteria, of which 52 were from the nodules of leguminous plants. A positive tyrosinase test was obtained with the organisms from the nodules of *Soja hispida* (5 cultures), *Lupinus* (6 cultures), *Sarothamnus scoparius, Coronilla varia, Genista tinctoria,* and *Tetragonolobus purpureus.* Thirty-seven cultures, representing such cross-inoculation groups as the clover, alfalfa, pea, and bean, failed to show positive test for tyrosinase. It may be recalled in this connection that the organisms often found with *Rhizobium,* such as *Azotobacter* and *B. tumefaciens,* are tyrosinase negative. In a general way these positive tyrosinase cultures from the nodules of leguminous plants fit into the slow-growing, non-serum-zone-forming group described by Löhnis and others.

Further work on tyrosinase production has been done by Miss L. Almon (unpublished data of the Department of Agricultural Bacteriology, the University of Wisconsin). She used a mannitol-nitrate-asparagin medium containing 1.5 gm. of tyrosin per liter. Of 153 cultures, representing 8 cross-inoculation groups, she found only 22 cultures capable of producing dark brown or black coloration and 71 cultures producing light brown color after 4 weeks incubation. Inasmuch as the tyrosinose-producing cultures were found irregularly distributed through the cross-inoculation groups, the test does not appear to be of differential value.

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[2]Waksman, S. A., and Davison, W. C. Enzymes, properties, distribution, methods and applications. Williams and Wilkins, Baltimore, 364 pp., 1926.

Müller and Stapp, 1925, called attention to the oxidizing power of various cultures of nodule bacteria, as shown by the formation of a pink color in a medium consisting of:

Potassium chinate	50.0 gm.
Dipotassium phosphate	0.5 gm.
Ammonium chloride	0.5 gm.
Ferrous ammonium citrate	0.5 gm.
Agar	20.0 gm.
Water, tap	1000.0 cc.

Adjust the reaction to pH 7.0

According to Beijerinck, 1911, the oxidation of the chinic acid to protocatechetic acid in the presence of iron produces the color. By the addition of aloin to a water solution of the gum from cultures of rhizobia, Fred, 1913, found a slight test for oxidase formation.

Just 35 years after his first publication concerning rhizobia, Beijerinck, 1923, noted the production of urease by root nodule bacteria, especially by the organisms from clover, pea, and vetch nodules. The bacteria from *Ornithopodis* and *Lupini* he found are but feeble urease-producers. His directions for demonstrating the presence of urease follow: From a colony of the desired organism remove a portion, and place on the surface of yeast-urea-malate-gelatin. "After a few minutes the 'Iris-phenomenon' becomes visible" (Beijerinck, 1901). The phenomenon is apparently due to the deposition of calcium carbonate or calcium phosphate crystals, resulting from reaction of the ammonium carbonate produced from the urea of the medium under the influence of urease. In this connection attention is called to the report of Werner, 1923, who obtained urease from leguminous roots bearing nodules, while those devoid of nodules were free of urease. The author suggests that this enzyme test might prove of value as a means of measuring the presence or absence of rhizobia in roots. In 1924 Hutchinson also found urease in cultures of rhizobia grown on a solid medium. Incidentally it may be mentioned that the rhizobia may encounter urea in the leguminous plant, for urea has been reported by Fosse, 1913, as present in clover, pea, and a bean (of genus *Faba*).

Müller and Stapp, 1925, were unable to detect the presence of lipase, amylase, or any glucoside-splitting enzyme. The occurrence of the hydrogen-peroxide splitting enzyme, catalase, in nodule bacteria has been reported by various investigators.

The nature of the enzymes, their activity, and the factors which influence their production are among the many problems connected with the nodule bacteria that deserve careful study.

Reduction of indigo carmin, methylene blue, and sodium selenite and tellurite. In general the nodule bacteria exhibit only a weak reducing power. Beijerinck in his original paper in 1888 reported these organisms unable to reduce indigo blue or "Bleu Coupier." Zipfel, 1911; Fred, 1913; Müller and Stapp, 1925; and others have noted a slow reduction depending upon the strain of the organism, the indicator used, and the nature of the culture medium. Indigo carmin, according to

Müller and Stapp, 1925, is reduced by all strains of bacteria, while methylene blue is reduced only by the fast-growing strains. Fred, 1913, found that in a soil extract plus 1 per cent of glucose, 48 hour old cultures reduced methylene blue in a concentration of 1 to 250,000. Some of his results are given:

Time required for reduction of methylene blue

Bacteria from alfalfa	about 2.5 hours
Bacteria from clover	about 2.5 hours
Bacteria from pea	about 4 hours
Bacteria from cowpea	about 2 hours
Bacteria from soybean	about 6.5 hours

He also noted a reduction of Schultze's strain, 1910,[3] a mixture of α-Naphthol and p-Nitrosodi-methylaniline. The fast-growing cultures generally produce a deeper blue color than the slow-growing strains; *e.g.*, soybean nodule bacteria.

Sodium selenite and tellurite are reduced by the rhizobia with the liberation of free selenium and tellurium (Müller and Stapp, 1925). In cultures these reductions are indicated by appearance of red and black colors respectively for the selenium and tellurium.

Indol, phenol, hydrogen sulphide, and ammonia production. According to the findings of various investigators, the nodule bacteria of the leguminous plants do not produce indol (Kellerman and Beckwith, 1906b; and Zipfel, 1911), phenol (Müller and Stapp, 1925), hydrogen sulphide (Prucha, 1915), or appreciable amounts of ammonia. Joshi, 1920, however, reported the formation of very small amounts of ammonia from a peptone medium. His results follow:

	Nitrogen as ammonia	
	After 3 days	After 7 days
	mg. in 100 cc.	mg. in 100 cc.
Organism from pea nodule (*Pisum sativum*)	4.2—4.9	6.3—4.9
Organism from math nodule (*Phaseolus aconitifolius*)	4.9—3.5	5.6—4.2
Organism from gram nodule (*Cicer arietinum*)	4.2—3.5	5.6—4.9

Should there be a like production of ammonia in the nodule, Joshi suggested that it might be the form of nitrogen absorbed by the plant. Recently also Pohlman, 1931c, has noted ammonia production by *Rhizobium meliloti*.

In closing this section upon enzyme production by the rhizobia, it is well to summarize our present knowledge of their enzyme system. They are known to possess the following: gelatinase, catalase and other reductases, tyrosinase and other oxidases, urease, and various sugar-splitting enzymes (see the fermentation characters); they are reported devoid of lipase, amylase or diastase, cytase, and glucoside-splitting enzymes.

[3]Schultze, W. H., Ueber eine neue Methode zum Nachweis von Reduktions- und Oxydationswirkungen der Bakterien. Centbl. Bakt. (etc.) 1 Abt. 56: 544-551, 1910.

BACTERIOPHAGE

The phenomenon of bacteriophagy is coming to be recognized as worthy of careful consideration in a study of the physiology of bacteria. In this respect the rhizobia have received their share of attention through the papers of Gerretsen, Gryns, Sack, and Söhngen, 1923; Grijns, 1927b; Hitchner, 1930; and Laird, 1932. There is also a hint of bacteriolytic activity of rhizobia in an early paper of Loew and Aso, 1908, who cited the partial clearing of a culture in pea-leaf extract medium, upon standing for six weeks. Upon such slight evidence the Japanese investigators suggested that the bacteria within the nodule may be dissolved by their own bacteriolytic enzyme. Of the recent papers dealing with authentic bacteriophage, that of Laird is most extensive and most exact. Experiments showing virulence of the phage to 1 x 10^8 dilution and demonstrating that the lytic agent is transmissible in series are reported in full. The finding of Hitchner that gum production retards or masks bacteriophagy is confirmed; an inorganic salt medium, containing as the organic nutrient 200 cc. of yeast water per liter, is shown to be most suitable for demonstrating lysis, either in fluid culture or by plaque formation on agar. The optimum pH for the action of the phage is 7.6 to 8.0. The phage may be isolated readily from *young nodules* and with difficulty or not at all from old nodules. It has been obtained also from a laboratory culture of *Rh. trifolii*. There is great variation in sensitivity of the laboratory strains of rhizobia, due to variation in the proportion of sensitive and resistant individuals which they contain. By suitable plating it is possible to separate the types, and by determination of the ratio of sensitive to resistant sub-strains to express the degree of sensitivity of the original culture. In other words, the original *Rh. trifolii* exists as a heterogeneous culture.

OXYGEN CONSUMPTION AND GROWTH IN THE PRESENCE OF DIFFERENT GASES

The gas exchange of cultures of the nodule bacteria has received very little attention. It is commonly stated that the organisms are aerobic, an opinion probably empirically derived from culturing the organisms. There is, however, the early work of Mazé, 1898, presenting some actual experimental evidence on oxygen consumption by cultures. His method was essentially the culture of the organisms on solid medium in an apparatus designed to supply CO_2-free air and to trap the atmosphere in the apparatus during definite periods of incubation. Analyses were then made of samples of air withdrawn at intervals. His figures are summarized below:

Percentage analyses of atmosphere over cultures of nodule organisms

Nature of gas	8 days	11 days	12 days
Carbon dioxide	20.70	18.70	8.04
Oxygen	1.80	3.16	13.41
Nitrogen + Argon	77.50	78.14	78.55
Carbon dioxide + Oxygen	22.50	21.86	21.45

The first two figures for oxygen indicate the enormous oxygen demand of a vigorous young culture. The reduction of oxygen concentrations from the normal 21 per cent of the air to 1.8 and 3.16 per cent in the 8 and 11 day analyses is truly remarkable when one considers that Mazé's procedure was to refill the apparatus with CO_2-free air after each analysis. Thus in the 3 days between the first and second analyses, the culture reduced the oxygen content of the surrounding atmosphere from 21 to 3.16 per cent and again the following 24 hours from 21 to 13.4 per cent. In tangible figures for the amount of O_2 absorbed, Mazé makes the following statement, although he does not give the data for his calculation: "In 24 hours a culture with a total surface of about 27 square decimeters absorbs a third of the volume of O_2 contained in a confined atmosphere of 5 liters, or 350 cc."

The carbon dioxide evolution from his culture was correspondingly high—20.7 and 18.7 per cent in the atmosphere over the culture, found upon analysis on the 8th and 11th days respectively. This CO_2 liberated accounts for a large part of the total carbon converted into products of metabolism. Mazé calculated that a 12-day culture which had utilized 1347.7 mg. of carbon as sucrose, liberated 1055.6 mg. of carbon as carbon dioxide, that is, 78.4 per cent. This would seem to be a large evolution of CO_2, but it must be remembered that the Mazé calculations are based upon cultures of large surface exposure. In liquid cultures the evolution of gas is not rapid enough to form gas bubbles.

COMPOSITION OF THE CELLS

An excellent review of work on the composition of bacteria in general is given by Buchanan and Fulmer, 1928.[4] Since the greater portion of this work bears little relation to the organisms under consideration, only a few of the papers dealing with nitrogen-fixing organisms such as *Azotobacter* will be considered.

Omeliansky and Sieber, 1913,[5] grew mass cultures of *Azotobacter chroococcum,* and analyzed the dried cells for carbon, hydrogen, and nitrogen. The nitrogen distribution in the protein was determined by the Van Slyke procedure. Some of their results follow:

Carbon	22.42 per cent
Hydrogen	6.41 per cent
Nitrogen	2.07 per cent
Protein	12.93 per cent

They found the lysine content of these bacteria to be high, much higher than that in animal and plant proteins. The arginine content was about the same as that of ordinary proteins. No cystine was found. Tests for purine bases were negative, so that nucleoproteins did not seem to be present. Stoklasa *et al.,* 1908,[6]

[4]Buchanan, R. E., and Fulmer, E. I. Physiology and biochemistry of bacteria. Williams and Wilkins, Baltimore, 516 pp., 1928.

[5]Omeliansky, W. L., and Sieber, N. O., Zur Frage nach der chemischen Zusammensetzung der Bacterienkörper des *Azotobacter chroococcum.* Ztschr. Phys. Chem., **88**: 445-459, 1913.

[6]Stoklasa, J., *et al.* Beitrag zur Kenntnis der chemischen Vorgänge bei der Assimilation des elementaren Stickstoffs durch Azotobacter und Radiobacter. Centbl. Bakt. (etc.), **2 Abt. 21**: 484-509; 620-632, 1908.

however, reported the presence of nucleoproteins in these same organisms and obtained evidence of the presence of adenine, guanine, and hypoxanthine after the hydrolysis of these nucleoproteins.

Perhaps the first to pronounce on the chemical nature of the nodule bacteria was Brunchorst, 1885a, who spoke of the bacteroids as protein bodies. Prazmowski, 1890, recognized within the bacteroids certain highly refractile corpuscles. He found them soluble in sulphuric acid and staining reddish brown with iodine. He assumed them to be albuminous granules. Frank, 1892b, discovered two types of nodules on the pea plant, the one containing normal bacteroids and rod bacteria and the other being full of large bacteroids containing highly refractive, iodine-absorbing granules of what he took to be amylodextrin. The presence of these peculiar granules in the bacteroids naturally reduced their nitrogen content and likewise that of the nodule as a whole. For example, the total N content of nodules containing this type of bacteroid was found to be 4.836 per cent, while that of normal nodules was given as 6.936 per cent. Moeller, 1892a, studied the iodine-absorbing granules in the nodules of *Trifolium* and on the basis of their solubility reactions, namely in chloroform, acetone, acetic acid, clove oil, and benzene, disagreed with Frank on the nature of the substance composing them. He concluded that it could not be carbohydrate, but rather a fatty or waxy substance, probably cholesterin. Frank, 1892c, answered that the granules in the pea did have all of the properties claimed by Moeller and were therefore of one and the same substance in the two species. He agreed that it was fatty and probably cholesterin. Hiltner and Störmer, 1903a, studied some preserved nodules supposed to have been material collected by Frank himself, and finding fungal hyphae in their specimens, considered the whole phenomenon as due to plant disease. Heinze, 1905, reported a high glycogen content in certain nodules; he considered the condition due to environmental factors affecting the plant and not necessarily pathological. Hiltner and Störmer, 1903a, were able to demonstrate the iodine-absorbing substance in bacteroids of the soybean organism in pure culture. More recently Müller and Stapp, 1925, have confirmed Hiltner and Störmer, have been able to isolate the substance, and have characterized it as follows: it consists "mainly of fatty acids or esters of fatty acid and glycerol or perhaps of a mixture of these, but contains, moreover, wax-like substances or fatty alcohols (perhaps cholesterin)."

Miss Löhnis, 1930b, re-investigated the iodine-absorbing substance in bacteroids. She attributed the following properties to it: solubility in conc. sulphuric acid, chloralhydrate, chloroform, acetone, acetic acid, and benzene, and insolubility in 20 per cent potassium hydroxide, ether, gasoline, carbon disulphide, and xylol. The granules possess double refraction, which is stable through boiling and treatment with 20 per cent potassium hydroxide.

A paper by Parisi and Masetti-Zannini, 1926, gives a report of the nitrogen distribution in the root nodule bacteria. Their results are presented below:

	Total nitrogen per cent
Amide N	18.80
Amino N	12.53
Diamino acid N	16.91

Monoamino acid N	50.87
Non amino N	9.18
Total	99.11

Hopkins, Peterson, and Fred, 1929, made carbon and nitrogen analyses of *Rhizobium meliloti,* the root nodule bacteria of alfalfa. Mass cultures of the organisms were grown on modified Ashby's solution with or without agar. The solution cultures were diluted, while the growth on the agar was suspended in water and then run through a supercentrifuge. The bacterial mass was dried, ground, and dried to constant weight. Analyses were made for carbon, total nitrogen, and fat of bacteria from cultures of different ages. The results are given in Table 10. It will be noted that the nitrogen content of the organisms increases with the age of the culture, and that the C:N ratio decreases. Table 11 gives the results of the analyses of the composition of the bacteria as calculated from the carbon content. The carbon in the proteins and fats was subtracted from the total carbon, and the remainder calculated as carbohydrate. Virtanen and Hausen, 1931c, found somewhat higher nitrogen content of pea bacteria grown on nutrient gelatin. He noted also some differences depending upon the pH of the medium; for example,

TABLE 10

The relation of age to composition of Rh. meliloti — *Dry basis*

Sample number	Age of culture	Carbon (ash-free)	Nitrogen (ash-free)	C:N ratio	Fat	
					Ether soluble	Chloroform soluble
	days	per cent	per cent		per cent	per cent
1	15	52.8	4.4	12.0	1.2	10.2
2	23	54.6	4.6	11.9	0.6	14.6
3	31	53.5	4.9	10.9	0.6	14.9
4	21	54.2	4.9	11.1	0.9	21.7

TABLE 11

The composition of Rh. meliloti — *Wet basis*

Sample number	Moisture	Ash	Protein	Fat	Carbohydrates	Total
	per cent	per cent	per cent	per cent	per cent	per cent
1	71.9	0.8	7.6	3.2	17.7	101.2
2	71.9	0.8	7.9	4.3	16.6	101.5
3	71.9	0.8	8.4	4.4	15.1	100.6
4	71.9	0.5	8.4	6.4	12.6	99.8

Reaction	Nitrogen in dry substance per cent
pH	
7	12.04
6	10.27
5	8.63

If the proximate composition of alfalfa nodule bacteria be compared with the results tabulated by Buchanan and Fulmer, 1928, for bacteria in general, certain similarities as well as certain differences will be apparent. The figures given by Buchanan and Fulmer for analyses of the dry material were corrected to their approximate equivalents for the wet bacteria by dividing the figures given by four. They are as follows: moisture 73.3 to 86.9 per cent, ash 0.5 to 4 per cent, protein 1 to 15 per cent, ether extract 0.1 to 7 per cent, carbohydrate 3 to 7 per cent. The moisture content of the rhizobia analyzed was somewhat lower than the range of the figures given by Buchanan and Fulmer. The ash content, too, is near the lower limit reported. The amount of protein present is about midway in the range. Ether extract is generally taken as representing the true fat. However, since most of the fat of the rhizobia is insoluble in ether but soluble in chloroform, the figures obtained for total fat should not be compared with ether extract of other bacteria. It will be noted that the combined ether and chloroform-soluble fractions indicate an unusually high content of fatty substance. The carbohydrate content is much higher than that given for any other organisms.

The fat of alfalfa rhizobia was further examined (unpublished data). Sterol tests performed on both the ether-soluble and chloroform-soluble fats are negative. The chloroform-soluble material on saponification yields acetic acid and a monohydroxy fatty acid of the order of oxybutyric or oxyvaleric acid. The fatty material extracted by chloroform is not a true glyceride, but is probably a polymer of the hydroxy-acid.

COMPOSITION OF THE GUM

The viscid consistency of cultures of the nodule bacteria was first observed by Beijerinck in 1888. Atkinson, 1893; Mazé, 1897; Hiltner, 1900b; and Dawson, 1900b, remarked upon this zoogloea-like type of growth.

The nature of this gum has been investigated by several workers. Mazé, 1898, considered it to be a nitrogenous by-product of bacterial action, and as such to constitute the nitrogenous material supplied to the plant. In 1906a and 1911 Greig-Smith reported that the gum of white lupine, pea, and broad bean nodule bacteria contains a small amount of nitrogen and gives glucose and galactose on hydrolysis. Buchanan, 1909a, prepared a nitrogen-free gum from cultures of bean nodule bacteria, and from its chemical reactions thought it closely related to the dextrans. Clover nodule bacteria were found by Gage, 1910, to produce a nitrogen-free gum which gave reducing sugar on hydrolysis. Fred, 1913, also obtained a nitrogen-free gum from nodule bacteria. Beijerinck, 1912, reported the gum to be cellulan on the grounds that it may be formed from any sugar, and is not fermentable by organisms of the *Granulobacter saccharobutyricum* type. Dextrans and levulans would be fermentable by these organisms and would be formed only from glucose or cane sugar. Kramár, 1921-22, classified the gum of *B. radicicola* as a dextran, since it yields glucose on hydrolysis.

Hopkins, Peterson, and Fred, 1930, working with *Rh. meliloti, Rh. trifolii,* and *Rh. leguminosarum* have also found that the gum is nitrogen-free. Glucose was crystallized from the hydrolyzed gum of *Rh. meliloti* and identified by its specific rotation. Phenylglucosazone was prepared from the hydrolyzed gums of *Rh.*

trifolii and *Rh. leguminosarum*. Qualitative tests on the sugar obtained by hydrolysis of the gums indicated that neither mannose nor fructose was present. Quantitative fermentation tests by pure cultures of yeast and lactic-acid bacteria on the gum sugar and on known mixtures of sugars showed that galactose and pentoses were absent, and that the gum sugar was glucose. The gums of *Rh. meliloti, Rh. trifolii,* and *Rh. leguminosarum* give strong napthoresorcinol tests for uronic acid, and a quantitative uronic acid determination on these gums shows that uronic acid is present in amounts from 4.1 to 25.3 per cent on the ash-free basis. In a subsequent investigation (Hopkins, Peterson, and Fred, 1931) this uronic acid was identified as glucuronic by the properties of its derivative, p-bromophenylosazone of barium glucuronate.

From these reports it is clear that the composition of the cells and gum of rhizobia offers a complex problem which deserves most careful study. No doubt the general question of the nitrogen nutrition of leguminous plants is closely associated with the nature and composition of the bacterial cell. The key to this fundamental problem has not yet been found.

CHAPTER 7

SOME FACTORS WHICH INFLUENCE THE GROWTH AND LONGEVITY OF THE NODULE BACTERIA

"Growth is the only evidence of life."
—SCOTT

CONDITIONS AFFECTING THE GROWTH OF RHIZOBIA

Our knowledge of the cultural conditions influencing the growth of rhizobia apart from the host plant has been derived from many sources, some more reliable than others. Many of the earlier studies included observations upon cultures of questionable purity, and in some cases no mention was made of the host plant from which the culture was secured. Thus it is often difficult to evaluate the various findings and to express them in terms comparable to later and more exact work.

Effect of air supply. The nodule bacteria of leguminous plants are accustomed to live in soil or in the tissues of the roots and thus are exposed to an environment of at least reduced oxygen-tension. In the laboratory, however, it is common experience that colonies on the surface of agar, as in a petri dish, are large and spread out over the surface as the culture grows older; the deep colonies are small, often lens-shaped, and do not increase in size with longer incubation. The profuse surface growth on stab cultures with but little growth in the deeper layers likewise seems to indicate the aerobic nature of these organisms. It has been mentioned in the preceding chapter that the nodule bacteria give a strongly positive catalase reaction. On this basis also they should be considered aerobic, and in the main, experiments confirm this hypothesis.

Almost every report concerned with the physiology of these organisms has mentioned their aerobic nature. Beijerinck, as early as 1888, spoke of the need for oxygen in the culture of nodule bacteria and suggested that they be grown on a solid medium according to the Koch plate method. He considered an oxygen tension somewhat lower than that of the atmosphere to be best for the growth of these bacteria.

Fourteen years later Schneider, 1902, stated that rhizobia of sweet clover will grow under aerobic conditions, although he thought the organism might be a facultative anaerobe. Laurent, 1891, observed that colonies of *Rhizobium* on lupine-extract gelatin develop in an atmosphere of pure nitrogen, although not so fast as in free air. It is probable that his so-called pure nitrogen carried oxygen sufficient for slight growth.

Mazé, 1898, reported that when incubated in an atmosphere of nitrogen, the

nodule organism shows a tendency to produce coccus forms. Maassen and Schönewald, 1910, found that in an atmosphere of nitrous oxide the nodule bacteria of *Pisum sativum* and *Phaseolus vulgaris* remain alive but do not grow. The effect of this gas they found to be about the same as that of hydrogen. When removed from the N_2O atmosphere to air, the organisms showed a good growth. They also observed growth of the nodule bacteria, but not of *Azotobacter,* in a mixture of N_2O and air. According to Wilson, 1917, the nodule bacteria of soybeans are facultative anaerobes. Peirce, 1902; de'Rossi, 1907; Harrison and Barlow, 1907; Zipfel, 1911; Whiting, 1915; and Burrill and Hansen, 1917, all regarded the rhizobia as aerobic.

Bewley and Hutchinson, 1920, studied the behavior of the nodule organisms from four cross-inoculation groups—clover, pea, alfalfa, and lupine—exposed to different atmospheres. Tubes of soil-extract mannitol agar were inoculated and placed in a glass bottle with alkaline pyrogallol. The bottles were then evacuated, and normal pressure was restored by allowing a slow current of air, free of oxygen, to pass through. They found that organisms from clover and alfalfa failed to grow, while those from horse bean and lupine made a slight growth. After long incubation in an atmosphere of nitrogen, they observed a decrease in the mucilaginous growth and the production of coccoid forms. Somewhat similar changes in growth and form of the cells were noted in the cultures confined in an atmosphere of hydrogen. They found that exposure to free ammonia or to coal gas reacts unfavorably on the growth of the organisms.

Ockerblad, 1918, compared the survival of rhizobia of several cross-inoculation groups on both solid and liquid media in containers with varying degrees of aeration. At intervals, he made bacterial counts with the following results:

	Viable bacteria after 30 days per cent
1. Liquid, cotton-plugged	38.5
2. Liquid, sealed with cork	18.0
3. Agar, cotton-plugged	87.0
4. Agar, sealed with cork	55.8

These results point to the superiority of the cotton-plugged container for the preservation of the rhizobia. According to Ockerblad, the chief factors responsible for a decrease in the number of viable bacteria are: (a) anaerobic conditions, (b) accumulation of metabolic or toxic products, (c) plasmolysis caused by concentration through evaporation.

A decided reduction in the number of bacteria in a tightly-stoppered bottle has also been noted by Fred, Whiting, and Hastings, 1926.

Alicante, 1926, studied the effect of oxygen on the multiplication of pea bacteria in a liquid and a solid medium in both open and sealed containers. After 10, 20, and 30 days of incubation at 26° C., cultures sealed with rubber stoppers contained less than 10,000 cells per cc., while parallel cultures with cotton plugs showed several million cells per cc. Greenhouse experiments with peas grown in sterilized quartz sand and inoculated with a water suspension of the sealed and cotton-stoppered cultures yielded results in agreement with the plate counts. The effect of various factors on the growth of the bacteria from the nodules of *Amorpha*

fruticosa was studied by Fehér and Bokor, 1926. They found that these organisms fail to grow when placed under anaerobic conditions, except on a medium consisting of bean-extract agar rich in carbohydrate.

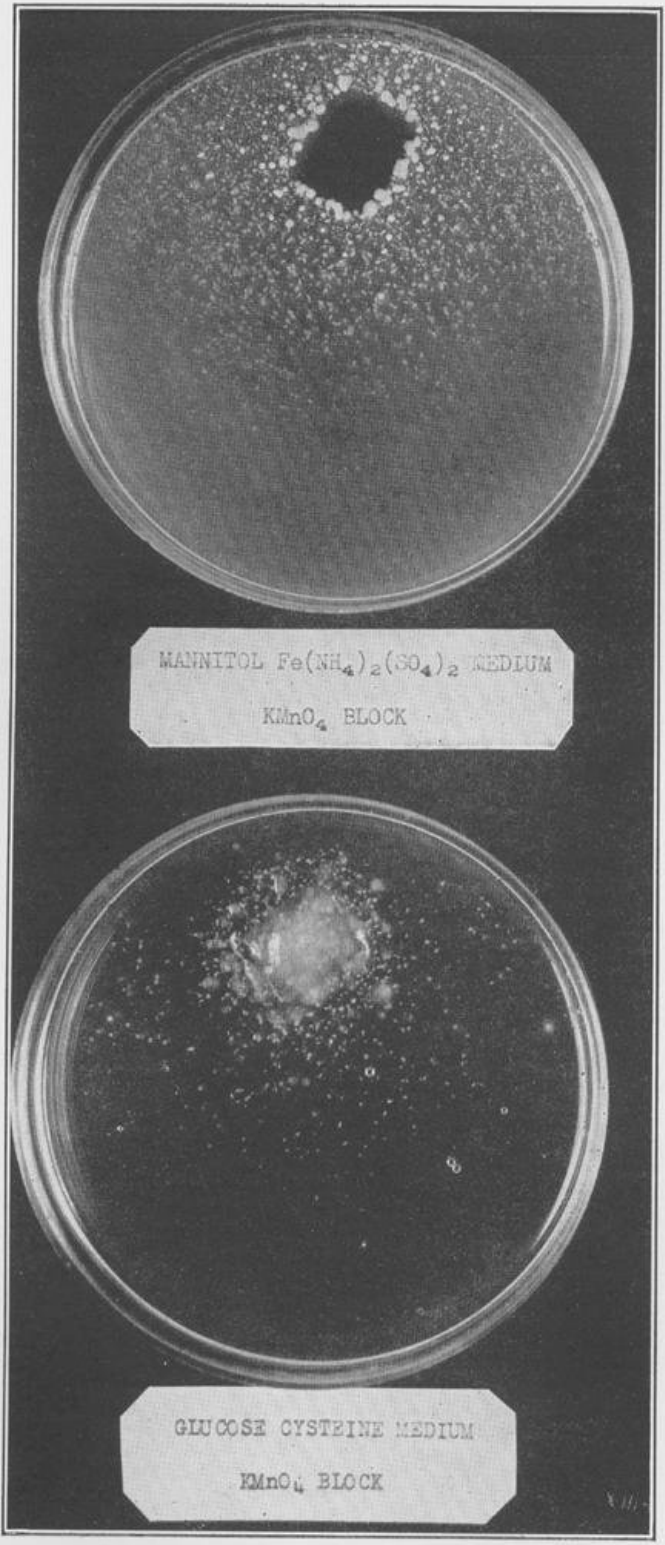
One of the most convincing experiments relating to the effect of oxygen on nodule bacteria is from Gangulee, 1926c, at Rothamsted. Sterilized soil in large test tubes was inoculated with alfalfa bacteria, incubated at 25° C., and at intervals of 3, 7, 14, and 30 days, plate counts were made. Eight of the sixteen tubes in this experiment "were sealed with paraffin wax over alkaline pyrogallol," and the rest were incubated with free access of air. After 30 days the conditions were reversed and the experiment continued for another 30 days. The results in graphic form are shown in Chart 3. The beneficial effect of free access of air on reproduction is emphasized by the majority of investigators.

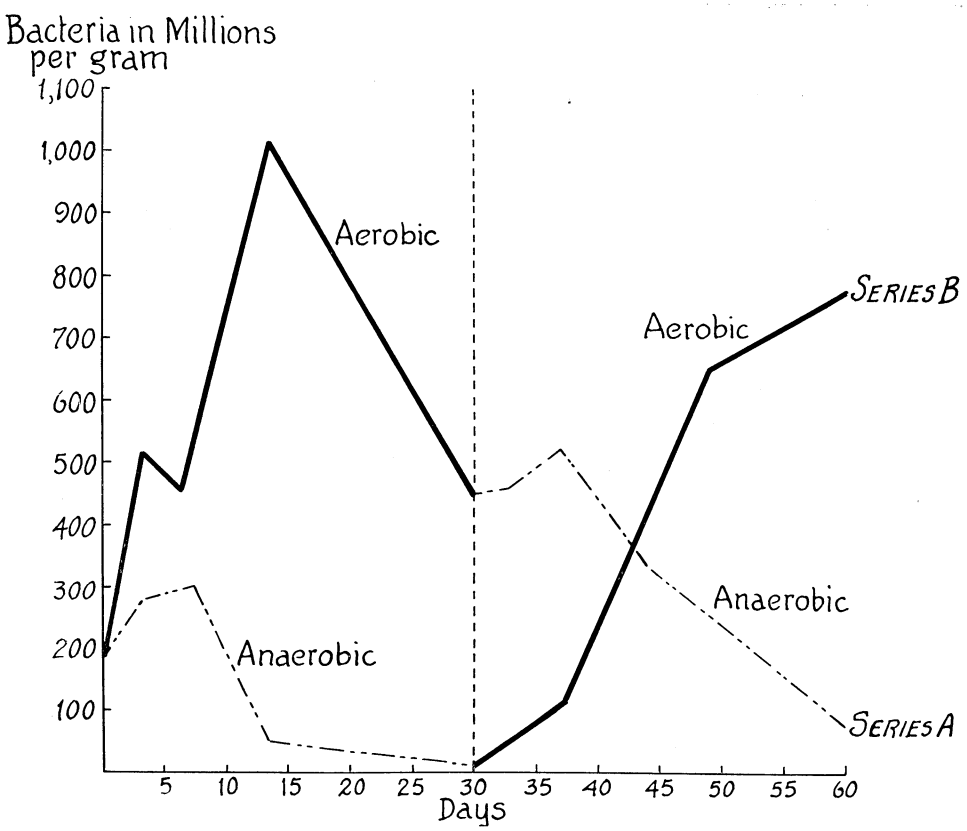
Allyn and Baldwin, 1930, have shown that the growth of rhizobia is to some extent dependent upon the oxidation-reduction character of the medium. In agar shake cultures of a highly oxidized medium—mannitol-nitrate mineral salts or ferric ammonium citrate medium, for example—the organisms establish a line of growth beneath the surface of the agar. In a yeast-water mannitol medium (somewhat more reducing) the organisms of the cowpea, soybean, and lupine groups still grow under the surface, whereas those of the alfalfa and clover groups grow at the surface only. When 0.1 per cent of cysteine is added to this medium, all cultures grow upon the surface. Additional experiments with petri dish cultures have shown that by reducing restricted areas of a medium which is slightly too oxidized for the best growth of the rhizobia, it is possible to increase greatly the number of colonies which develop within the zones affected. Similar results were secured by oxidizing restricted areas in a medium too reduced for the best growth (see Plate 19). Further experiments along these lines are in progress.

Although the evidence is not conclusive, there are some indications that the different cross-inoculation groups vary in relation to oxygen requirements. More intensive study on this factor in the growth of the root nodule bacteria seems highly desirable.

Effect of temperature. Beijerinck, 1888, gave us the thermal growth range of rhizobia as 0 to 47° C. and called special attention to the growth of these organisms at 10° C. Since then de'Rossi, 1907; Zipfel, 1911; Burrill and Hansen, 1917; Müller and Stapp, 1925; Gangulee, 1926c; and many others have studied the effect of temperature on the root nodule bacteria. Briefly, their results show growth between 0 and 50° C. with an optimum between 20 and 28° C. In 1918 Ockerblad reported the thermal death point of rhizobia in liquid media as between 59 and 61° C. with 10 minute exposure. His results are based on a study of 12 different cultures. According to Müller and Stapp, 1925, the representatives of the various cross-inoculation groups are not alike with respect to maximum and minimum temperature tolerance. The resistance of these organisms to high temperatures depends on a number of factors, such as substrate, age, and number of cells. Müller and Stapp, 1925, studied the effect of heat on a water suspension of pea bacteria containing 1,369,587,200 cells per cc. They found at 50° C.

After 5 minutes	280,000 cells alive
After 20 minutes	1,050 cells alive
After 25 minutes	all cells killed


MANNITOL $Fe(NH_4)_2(SO_4)_2$ MEDIUM
$KMnO_4$ BLOCK
GLUCOSE CYSTEINE MEDIUM
$KMnO_4$ BLOCK


Bacteria in Millions
per gram
1,100
1,000
900
800
700
600
500
400
300
200
100
Aerobic
Anaerobic
Aerobic
Anaerobic
SERIES B
SERIES A
5
10
15
20
25
30
35
40
45
50
55
60
Days

From the various reports it appears that about 2 to 3 minutes are required to kill the bacteria in a water suspension at 60° C. The fact must not be overlooked, however, that in soil and certain other media they may be much more resistant. Gangulee, 1926c, observed that alfalfa bacteria in soil remained alive after exposure to 50° C. for 7 days.

Although sensitive to high temperatures, the nodule bacteria are very resistant to low temperatures. Bréal, 1889a, noted that the bacteria of peas remain alive all winter in frozen water and the next spring are able to produce nodules. Fred and Frazier, 1920, also found no evidence that freezing of the substrate caused injury to rhizobia. Vass, 1919, exposed cultures of the nodule organism of field pea to temperatures ranging from —15° to —190° C. for 3 minutes to 6 hours and found that they were alive at the end of the experiment. These tests were made by inoculating three kinds of media, a mannitol liquid, sand, and soil, with suspensions of the pea bacteria and after one week of incubation subjecting small samples of these cultures to low temperatures. After this, the samples were diluted and the number of bacteria measured by means of plate counts. Some of the results obtained by Vass are shown below:

	Bacteria in 1 cc. of solution	Bacteria in 1 gm.	
		Sand	Soil
1 Normal soil	3,000,000	3,700,000	400,000,000
2 Frozen at −15 °C., 3 minutes	1,400,000	5,200,000	470,000,000
3 Frozen at −15 °C., 6 hours	600,000	2,800,000	400,000,000
4 Frozen at −190 °C., 30 minutes	1,000,000	2,700,000	470,000,000
5 Frozen at −190 °C., 6 hours	450,000	2,100,000	400,000,000

These figures show a gradual decrease in the number of bacteria in the mannitol solution as a result of freezing. In the sand and soil there is no marked effect, even in the long exposure at —190° C. Vass further noted that an increase in concentration of the medium by the addition of sugar or some similar substance exerts a decidedly protective effect. The concentration, however, has no effect when the temperature is lowered to a point where the substance begins to crystallize out.

The findings of Vass are of decided economic importance in relation to the general problem of the existence of rhizobia in soils. It seems safe to conclude from his results that rhizobia in soil are able to withstand the effect of temperatures far below any which might be encountered in nature. The effect of season on the numbers of free-living rhizobia of the soil may well be considered under temperature, although other factors, such as availability of water and food, may be equally important. Wilson, 1930, made the interesting observation that the numbers of *Rh. trifolii* and *Rh. leguminosarum* in field soils decrease as the winter season advances and increase during spring and summer.

Effect of drying. Like all non-spore-forming bacteria, rhizobia are sensitive to excessive drying. The effect of desiccation, however, depends on several conditions: the nature of the substrate, reaction, temperature, etc. Harding and Prucha as early as 1905 reported that rhizobia cannot survive on cotton under or-

dinary atmospheric conditions. In thin films dried on the surface of glass, they die rapidly, according to Chester, 1907; whereas on cotton in the open air they may live from 11 to 16 days, but if dried quickly and kept in sealed bottles they may survive considerably longer. Edwards and Barlow, 1909, experimented on the desiccation of rhizobia on various substrates, such as seeds, glass, paper, and nodule tissue. They found that most bacteria on glass or filter paper die rapidly, but that a few cells are able to withstand desiccation for a long time.

Giltner and Langworthy, 1916, carried out extensive studies on the longevity of rhizobia under various conditions. They found that rhizobia survive a long time in air-dry soil, probably because of the films of hygroscopic moisture which surround the soil particles. Protective colloids in the soil may also play a part in the survival phenomenon, as the bacteria appear to be much more resistant in a clay loam soil than in sand. The gum produced by the rhizobia themselves may serve also as a protective covering. Temple, 1916, and Vandecaveye, 1927a, have likewise reported upon the remarkable viability of rhizobia in air-dried soil.

Rogers, 1914, showed that a culture of sweet clover nodule bacteria grown in milk may be dried to a powder in vacuum (see Rogers' freezing method) and kept for at least six months at room temperature, perhaps much longer. At the end of six months, Rogers found more than one million viable cells in each gram of this powder.

Effect of sunlight. Directions for the use of artificial cultures and popular writings on the process of inoculating leguminous seed often include warnings about the harmful effects of sunlight on the bacteria. Actual experimental results in the literature however, do not altogether support these statements. Otis, 1898, reported that light has only a slight effect on the nodule bacteria in soil, and Simon in 1907 called attention to the relative resistance of the nodule bacteria to sunlight. Burrill and Hansen, 1917, found that exposure to direct sunlight for several months does not kill organisms grown in favorable medium with proper precautions to prevent evaporation. The paper of Nobles, 1919, is single evidence to the harmful effect of light. Nobles claimed that the nodule bacteria are quite sensitive to direct sunlight, though the compactness of the soil offers some protection. For example, he found that 80 per cent of the bacteria in a sandy soil are destroyed by 15 minutes exposure, and about 54 per cent are so destroyed in a more compact soil. It is difficult to correlate this work with the findings of Albrecht, 1921; Erdman and Wilkins, 1928; and Albrecht and Turk, 1930, all of whom agree with the earlier reports that the rhizobia in soils are singularly resistant to light. Albrecht, 1921, allowed soil with nodule bacteria to dry in sunlight and then stored it for three years. At the end of this time he found enough viable bacteria to produce good infection. In a later paper, 1922, he reported a further study of the effect of sunlight on nodule bacteria of red clover and soybeans. In this test, one-half of the soil containing the nodule bacteria was spread out in a thin layer about ¼ inch deep in direct sunlight until dry, then exposed for 2 months on a green-house bench, then stored in a closed container. The other half was dried in the dark and similarly stored. Tests of the sun-dried soil and of that dried in the dark showed survival of nodule bacteria in both. His conclusion that there is no evidence that the nodule bacteria are especially sensitive to sunlight seems justified.

Two still more recent reports present further evidence in the same direction. According to Erdman and Wilkins, 1928, exposure of either inoculated soybean seeds or inoculated soil does not seriously injure the "inoculating efficiency" of the nodule bacteria. An even more severe test is reported by Albrecht and Turk, 1930. Knowing that it is the ultra-violet fraction of sunlight which is fatal to most bacteria, Albrecht and Turk exposed soil containing the nodule bacteria to strong radiation with ultra-violet light from a mercury arc lamp and found no detrimental effect. The absorptive capacity of soil apparently prevents the penetration of the destructive rays.

In this connection, attention may be called to the results of Leonard and Marsh, 1928, concerning exposure of culture media to sunlight. They worked with four kinds of culture media and the bacteria from the nodules of alfalfa, clover, vetch, and soybean. A culture medium exposed to direct sunlight for 270 hours and then seeded with bacteria will still support growth, though the appearance of the culture may be somewhat altered. An exposure of 10 hours induced no apparent ill effects.

Effect of reaction. Field observations have shown that leguminous plants bearing nodules are to be found in soils of widely different reactions. This tolerance on the part of the host plant suggests that the root nodule bacteria may be equally tolerant of change in reaction. In fact, Moore, 1905, reported that the nodule bacteria will stand any degree of acidity or alkalinity of the soil that will permit the growth of its particular host plant. Similar observations have been reported by various investigators. Maassen and Müller, 1906, on the other hand, consider the nodule bacteria very sensitive to the reaction of the medium.

One of the first to emphasize the importance of reaction in the separation of the nodule bacteria was Mazé, 1899, who divided these organisms into two groups, those accustomed to acid soils and those accustomed to alkaline soils. Zipfel, 1911, observed that the growth of bacteria from pea, horse bean, red clover, and garden bean nodules is not influenced by small amounts of acid or alkali. Richmond, 1926b, reported that rhizobia from cowpea and soybean are killed in acid soil within 3½ years.

Prucha, 1915, showed that growth of the nodule bacteria of alfalfa on agar slants is injured by the presence of small amounts of mineral acid like HCl, while equivalent amounts of a base like NaOH have no harmful effect.

Salter, 1916, studied the behavior of rhizobia from alfalfa and clover in Ashby's mannitol medium of varying reactions. A neutral or slightly acid reaction is favorable for the reproduction of the clover bacteria, whereas a slightly alkaline or neutral reaction is best for alfalfa bacteria. Fred and Loomis, 1917, also reported a neutral or slightly alkaline medium most favorable for the alfalfa bacteria. The relation between hydrogen-ion concentrations and limits of growth of the different cross-inoculation groups is reported in the paper by Fred and Davenport, 1918. They found that the nodule bacteria from different plants behave differently toward acidity. The alfalfa organism is most sensitive and, conversely, the lupine organism most resistant to acidity. The critical acidity or pH for the bacteria of several cross-inoculation groups follows:

Rhizobia from	*Critical pH*
Alfalfa and sweet clover	4.9
Garden pea and vetch	4.7
Red clover and beans	4.2
Soybeans	4.2
Lupine	3.2

These values are similar to those limiting growth of the plants. The limit of alkalinity for growth is approximately the same for all the groups of nodule bacteria, about pH 9.6. Bryan, 1923b, tested for the survival of alfalfa, red clover, and soybean bacteria in soils of varying reaction. After 75 days, heavy suspensions from each soil tested were used to inoculate sand pots of the appropriate host plants. Nodule production was taken as evidence of survival of the original bacteria. The following critical pH values were found: alfalfa bacteria killed at about pH 5.0, red clover at pH 4.5-4.7, and soybean bacteria at pH 3.5-3.9.

Experiments carried out by Virtanen, 1927, 1928a, on the influence of acidity of soil upon the higher plant and the bacteria indicate that the organisms of red clover are more resistant to soil acidity than those of peas. In general he found the nodule bacteria more sensitive to acidity than the higher plant. The optimum pH value for growth of the nodule bacteria is 6.5 to 7.5; the optimum for plant culture is pH 6.5, according to Virtanen and Hausen, 1931c.

Effect of various inorganic substances. The growth of bacteria, like that of higher plants, is complicated by the fact that they may take from the air some of the raw materials for their synthesis. The substances needed in quantity are water, carbon as complex carbon compounds or perhaps CO_2, nitrogen, oxygen either in the elemental form or suitable compounds, phosphorus, sulphur, potassium, calcium, magnesium, and perhaps iron. Apparently sodium and chlorine are non-essential or need be present only in traces. Other elements generally present in very small amounts in nutrient media, especially in soil and water, are manganese, aluminum, and boron. Whether these last-named elements function in the building up of cell substance or have simply a beneficial effect as catalytic agents is not known. It is generally assumed that the nodule bacteria take all of their carbon from the complex organic carbon compounds. Much has been said in the preceding chapter concerning the nitrogen requirements of the rhizobia. One is apparently justified in stating that under the usual cultural conditions, at least, the rhizobia are not able to assimilate nitrogen from the air. They can, however, use combined nitrogen in the form of inorganic salts or more complex organic compounds.

The influence of a great number of salts such as chlorides, nitrates, and sulphates on the vitality of soybean bacteria was studied by Wilson, 1917. His procedure was to expose the bacteria to the action of the salts of Pfeffer's solution in various dilutions and combinations in distilled water. After 4-6 weeks he tested for survival, or the ability of the organism to effect nodulation. In 61 of the 77 cases studied, nodules were produced, indicating that the bacteria had not been seriously injured by the presence of the salts. In another experiment, in which were tested nitrates and sulphates in soil in quantities to prevent nodulation, it was found that the bacteria were alive and capable of forming nodules on re-

turn to favorable conditions. The nitrates of mercury, uranium, and nickel, and the sulphate of mercury, however, are exceptionally toxic and apparently kill the organisms. Somewhat similar results are reported by Ohkawara, 1928, with the nodule bacteria from Genge, lupine, and serradella. He found these three strains of the nodule bacteria retain their vitality for more than 40 days in water solutions of 0.1 per cent of KNO_3, $NaNO_3$, and $Ca(NO_3)_2$ but are destroyed in water solutions of the same concentration of $(NH_4)_2SO_4$. Hills, also, has studied the effect of nitrates on the rhizobia apart from the host plant. In 1918 he found that approximately 25 mg. of nitrate in 100 gm. of soil actually stimulate the multiplication of *Rh. meliloti*. Higher concentrations have a depressing effect, and above 100-150 mg. per 100 gm. appear to be definitely toxic.

Pitz, 1916, noted a beneficial effect of calcium sulphate on the multiplication of *Rh. trifolii* both in synthetic and soil extract media. Truesdell, 1917, obtained an increase in the number of alfalfa nodule bacteria in sterilized soils to which a small amount of phosphate was added. Fulmer, 1918, observed the rapid multiplication of rhizobia from alfalfa and lupine in sterilized acid Colby silt loam soil to which small amounts of calcium carbonate, magnesium carbonate, or limestone were added. Somewhat similar results were obtained by Alicante, 1926, from the use of $CaCO_3$. Singh, 1920, studied the gain in nitrogen in sterilized and unsterilized soil plus 5 per cent of glucose, to which pure cultures of the nodule bacteria were added. In the unsterilized soil he found that $CaCO_3$ and $CaSO_4$ alone have no effect; however, these inorganic substances, when combined, enhance nitrogen fixation. In general, large applications of $CaSO_4$ to soil increase nitrogen fixation, while in liquid cultures they have no effect.

Bewley and Hutchinson, 1920, studied the effect of various salts on the morphology of the nodule organism. Magnesium or calcium carbonate induces the formation of pre-swarmers, while the phosphates, particularly calcium phosphate, favor the development of swarmers. Other salts such as chlorides, sulphates, and nitrates are ineffective. Thornton and Gangulee, 1926, further studied the effect of phosphate and found that a combination of phosphate and milk hastens the formation of motile cocci in soil cultures, and thereby brings about a more rapid spreading of the bacteria through the soil. The Rothamsted method of inoculation takes advantage of this observation and recommends the use of phosphate and milk with the proper culture. The method is given in detail in Chapter 13.

Using a neutral medium consisting of calcium glycero phosphate 0.08 per cent, magnesium sulphate 0.06 per cent, sodium chloride 0.02 per cent, potassium chloride 0.02 per cent, and glucose 2.0 per cent, Müller and Stapp, 1925, studied the effect of a number of inorganic compounds. The presence of potassium, sodium, or ammonium nitrates in concentrations of 0.2 per cent is beneficial for the organisms of the alfalfa, bean, clover, lupine, pea, soybean, and locust cross-inoculation groups. The compounds of ammonia, as NH_4NO_3, NH_4Cl, and $(NH_4)_2SO_4$, also favor the growth of the organisms of these groups.

They also observed that higher concentration of nitrate up to 1 per cent retards growth, and that 2 per cent entirely prevents growth. Potassium nitrite, on the other hand, is toxic in concentrations of 0.2 per cent. Chlorides of sodium, potassium, lithium, and caesium were also tested by Müller and Stapp, 1925. They found that 2.4 per cent of KCl and 3.5 per cent of NaCl check the growth

of the rhizobia, but in lower concentrations merely induce the formation of many branched and coccoid forms. Lithium chloride at the rate of 0.1 per cent is harmful, while caesium chloride is less toxic than lithium chloride and in the concentration of about 0.125 per cent is actually stimulatory to the formation of the swollen vacuolate forms. Calcium chloride exerts no special effect on the morphology of the nodule bacteria. It is injurious in amounts of 1.5 to 2.1 per cent, and toxic at 2.4 per cent. Scanlan, 1928, has shown that one part of calcium chloride to 1500 parts water exerts a decidedly beneficial effect on the longevity of rhizobia from soybeans. Calcium salts, according to this investigator, tend to keep the bacteria in an active state.

Magnesium chloride, added to carrot agar at the rate of 2 per cent, was found by Müller and Stapp greatly to favor the formation of banded and swollen forms. The various phosphate salts used, monopotassium and dipotassium phosphate, and disodium phosphate (Sörensen), appeared to vary decidedly in effect upon the bacteria. In general, the monobasic potassium phosphate is most suitable for the growth of these bacteria, and the Sörensen disodium phosphate least suitable. Concentration of either is to be considered, however.

Certain compounds which in small amounts have been found favorable for the growth of nodule bacteria should be mentioned. Olaru, 1915, asserted that manganese sulphate in concentrations of 1:50,000 to 1:200,000 greatly favors the assimilation of nitrogen by the nodule bacteria. Whiting, 1923, studied the effect of aluminum salts on the growth of nodule bacteria in an agar medium with and without calcium carbonate. He worked with aluminum hydroxide, phosphate, acetate, nitrate, chloride, and sulphate, and also with metallic aluminum, and found that rhizobia make normal growth in presence of aluminum salts except as influenced by the acidity of the salts. Voicu, 1923, recommended boron as boric acid in concentrations of the order of 1:10,000 for stimulating nitrogen fixation. On the contrary, Brenchley and Thornton, 1925, were unable to show any beneficial effect of boron on the multiplication of *Vicia Faba* bacteria in a synthetic medium.

In summary, then, we may conclude that calcium, potassium, and phosphorus salts favor the development of the nodule bacteria, especially in culture solutions and certain soils; the phosphorus compounds especially accelerate the activity of the nodule bacteria. The so-called stimulants, boron, manganese, etc., in the proper concentrations may exert a favorable effect, although evidence in their favor is not extensive.

Vitamin B and the multiplication of rhizobia. Following the lead of biological chemists, a number of microbiologists have investigated the relation of vitamins to the multiplication of microörganisms. In general, these substances are present in aqueous extracts of plants and in very small amounts are claimed to stimulate growth. For a detailed review of this question of growth-promoting substances, especially the bios question, the reader is referred to Tanner, 1925[1] and Miller, 1930.[2] In a paper on "Vitamin effects in the physiology of microörganisms," Werkman, 1927, warned against confusion of the effect of addition of small quantities of easily available food and the addition of vitamin B. From the results of numerous

[1]Tanner, F. W., The "bios" question. Chem. Rev., 1: 397-472, 1925.
[2]Miller, W. L., Bios. Jour. Chem. Ed., 7: 257-267, 1930.

tests with rhizobia grown in Ashby's medium to which various amounts of a vitamin B *concentrate* were added, Werkman concluded that vitamin B exerts no stimulation on the rate of reproduction of the nodule bacteria. The transient stimulation following the addition of a vitamin B *concentrate* (Harris Laboratory preparation) he ascribed to the small quantity of food substances and not to vitamin B.

Slanetz in 1923 fed cultures of *Azotobacter chroococcum,* rhizobia, and other soil bacteria to mice, and concluded that these microörganisms do not produce vitamins A or B. A recent paper by Sunderlin and Werkman, 1928, however, presents results showing the synthesis of vitamin B by *Rhizobium meliloti* in Ashby's culture solution.

Effect of dyes. Following Churchman's work, 1912, 1926,[3] on the bacteriostatic effect of certain aniline dyes on bacteria, there have appeared a number of papers on the relation of rhizobia to dyes. It will be remembered that Churchman has found Gram-positive bacteria particularly sensitive to basic tri-phenyl methane dyes such as crystal violet. All strains of the root nodule bacteria have been found to be Gram negative and consequently are comparatively resistant. The use of tri-phenyl methane dyes in culture media for the isolation of these bacteria from nodule material is therefore of value in excluding all of the Gram-positive soil contaminants. It has also been claimed that the dyes have a selective action in eliminating *B. radiobacter* (Ruehle, 1928; Anderson, 1929). Ruehle's method for securing pure cultures of the nodule bacteria has been tested repeatedly by Wright and Baldwin of Wisconsin. Their results, however, fail to show any marked superiority over other well-known methods. The crown-gall and hairy-root types of *Ph. tumefaciens* and the colon-aerogenes bacteria are likewise too nearly related to the rhizobia for the dye method to be of value in separation.

The relation of the growth of the root nodule bacteria to crystal violet has been studied by Stevens, 1925a; Wright, 1925a; Wright and Simington, 1927; Vandecaveye, 1924; Burke and Burkey, 1925; Anderson, 1929; and Pohlman, 1931b. The results of these investigators show a great range in concentrations of crystal violet which the various groups will tolerate. As was to be expected, all strains are crystal violet resistant, although the limiting concentrations of dye are reported to vary from 1:5000 to 1:100,000. Burke and Burkey were able to adapt a strain to increased concentrations of crystal violet, but found that the culture reverted to the more sensitive condition after cultivation away from the dye for a short time. This is to be expected, as Churchman, 1912,[4] has shown that the usual pure culture of even a Gram-negative organism contains both crystal-violet-positive and crystal-violet-negative cells.

Antagonism and symbiosis. Like all other living organisms, the nodule bacteria are required to make adjustments to a two-fold environment. The factors so far considered in this chapter belong to the class of inanimate physical and chemical agents. Perhaps equally important are the complex biological relations between the rhizobia and their plant, animal, and microörganic neighbors. Probably in the

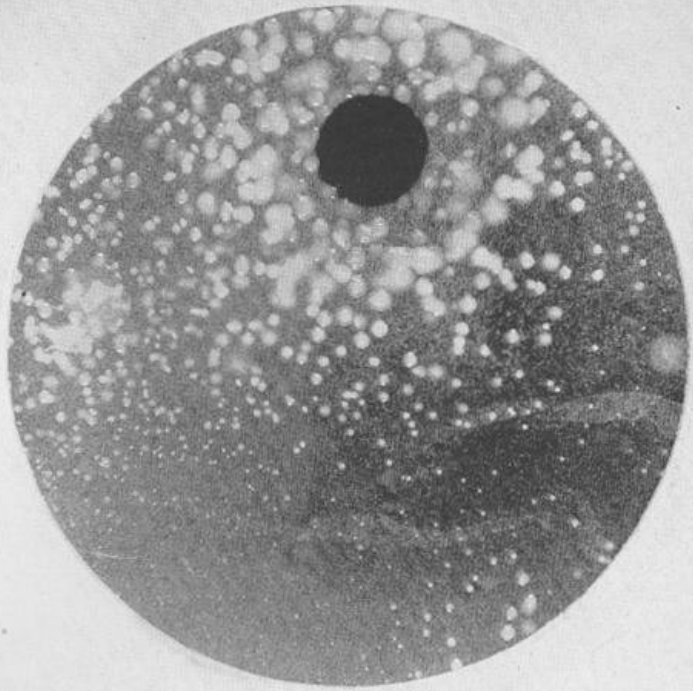
[3]Churchman, J. W., and Michael, W. H. The selective action of gentian violet on closely related bacterial strains. Jour. Expt. Med., **16**: 822-830, 1912.
Churchman, J. W. Purification of cultures of bacteria by means of reverse selective bacteriostatic properties of aniline dyes. Soc. Expt. Biol. and Med., Proc., **23**: 530-534, 1926.

[4]Churchman, 1912 *loc cit.*

last analysis, the effect will be explainable in terms of physics and chemistry. But for the present we cover our ignorance by speaking of symbiosis and antagonism, according as the net effect of the association of organisms is beneficial or harmful to the rhizobia. The nodule bacteria have undoubtedly existed in the soil and tissues of the Leguminosae for ages. Why are they not more abundant and more widely distributed? Perhaps the answer is that the antagonism of other soil microörganisms holds them in check. As early as 1899b Nobbe and Hiltner suggested that the competition between rhizobia and the rest of the soil flora results in harm to the rhizobia; they implicated both bacteria and fungi in the opposition. Sackett, 1906, in a laboratory study of associated growth of *B. ramosus* and *Ps. radicicola,* observed that multiplication of the latter is greatly inhibited. Simon, 1907, apparently the first to study the rhizobia in natural soil, reported that the soil flora is definitely antagonistic to the nodule organisms, and in a later paper, 1911, asserted that the presence of fungi is particularly harmful. From the fact that rhizobia grow better in sterilized soil than in natural unsterilized soil, Duggar and Prucha, 1912, argued that there is an antagonistic action of the general soil flora toward the rhizobia. Kühn, 1911, on finding poor results with certain commercial cultures on seeds in rich humus soil, reasoned that competing microörganisms in such a soil destroy the rhizobia. Other investigators, however, are only willing to grant that certain bacteria of the soil flora are harmful. Kellerman and Robinson, 1906, reported an organism resembling *B. coli* to be inhibitory to the rhizobia. A year later Kellerman and Fawcett, 1907, found that *B. ochraceus* in a liquid medium has no effect upon nodule bacteria, whereas an organism like *B. coli* is distinctly harmful. In soil, however, no harmful effect is noted. Kalantarov, 1914, showed that *Bacillus mycoides* and *Bacterium fluorescens liquefaciens* neither in soil nor in culture media influence the nodule bacteria. On the other hand, he observed that *Bacillus mesentericus vulgatus* and *Bacterium coli communis* are strongly antagonistic. Alicante, 1926, found a number of organisms non-injurious to the rhizobia; namely, *B. radiobacter, Azotobacter chroococcum, B. prodigiosus, B. capsulatus, B. subtilis, B. mesentericus,* a pink yeast, and some molds. A mixture of nodule bacteria from pea, clover, cowpea, and soybean nodules are likewise not mutually antagonistic.

While at Wisconsin, Konishi, 1931, obtained some interesting data on the relation of certain soil bacteria to the rhizobia. He isolated several unidentified organisms from soil and tested them out with rhizobia in liquid medium, with and without $CaCO_3$, and also in soil. He found inherent differences in the degree of antagonism exhibited by the cultures and also differences according to the conditions of the experiment. In general, the effects were most severe in liquid media without $CaCO_3$ and least severe in soil. Certain known cultures of soil bacteria were tested and also found most injurious in liquid. For example, *B. subtilis* inhibits *Rh. meliloti, Rh. leguminosarum,* and *Rh. japonicum* in liquid cultures, but not in soil. *B. coli* likewise is without effect on *Rh. meliloti* in soil. The other organisms tested, *B. mycoides, B. megatherium, B. prodigiosus, B. fluorescens,* and *B. aerogenes,* are also harmless in soil.

So much for the antagonistic or neutral side to the competition. There remain to be considered a few reports on the beneficial effects of certain organisms upon the rhizobia. Hiltner, 1907, stated that the presence of another organism



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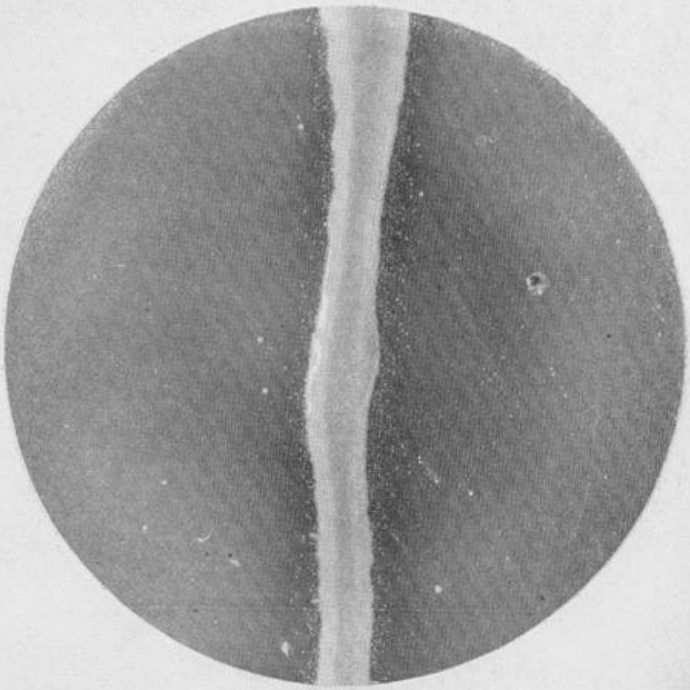

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stimulates rhizobia. Bottomley, 1910a, reported enhanced nitrogen fixation by mixed cultures of nodule bacteria and *Azotobacter*. Manns and Goheen, 1916, and Fellers, 1918a, referred to the beneficial effect of association of rhizobia and *Azotobacter* upon colony formation in petri plates. Löhnis and Hansen, 1921, observed that *B. radiobacter* on a plate stimulates colonial growth of the cowpea-soybean group of rhizobia. Burrill and Hansen, 1917, noted stimulation by *Penicillium glaucum,* and Fellers, 1918a, also spoke of profuse growth of nodule bacteria "entirely covering the fungus mycelium." Somewhat similar stimulation has been noted at Wisconsin; Plate 20 is a clear example of stimulation of *Rh. japonicum* by a colony of *Penicillium,* and of the cowpea organism in the vicinity of a streak of *B. radiobacter*. Konishi, 1931, however, found no evidence of benefit induced by any of the soil organisms or stock cultures which he tested.

The reader will perhaps notice some discrepancies in the above listing of harmful, neutral, and beneficial organisms. These may be accounted for by the different conditions under which the tests were made, since several reports claim complete reversal of the relation as, for example, in soil *vs.* liquid substrate. Generally the soil is reported to have a protective action; how successful the protection is in nature or, for that matter, how real is the antagonism is not known. The whole subject deserves further study.

Another element in the biological environment of the rhizobia in soil is the activity of protozoa. In a study of the food requirements of soil amoebae, Severtzova, 1928, has shown that on artificial media these organisms destroy large numbers of bacteria. Strangely enough, Severtzova observed a pronounced selective action. Of the 26 species tested, 21 were eaten readily and 5 species very little or not at all. The organisms from the nodules of leguminous plants are listed among the edible species. The importance of Severtzova's findings is obvious, if it be assumed that the amoebae behave in the same way in soil; it is thus possible that amoebae play an important rôle in the destruction of rhizobia in soil. From some preliminary unpublished data of E. W. Hopkins at Wisconsin it appears that not all nodule bacteria are acceptable to the amoebae. Of the 41 strains tested on a nitrogen-free agar containing 1:100,000 crystal violet, only 12 strains were fed upon. The availability appears to depend upon the rate of growth of the bacteria and the type of gum produced. Strains producing heavy gum are not attacked. Losina-Losinsky and Martinov, 1930, reported an investigation of the diffusion of pure cultures of *Rh. trifolii,* an amoeba (*Vahlkampfia*), and a ciliate (*Colpoda steini*) planted together in the center of a petri dish of sterile soil. All three types of microörganisms multiply rapidly and spread outward at a rate seemingly controlled by the moisture content and mechanical structure of the soil. The rhizobia always precede the protozoa and serve as food for them. Hino, 1930, presented another side to the picture. He found soil microörganisms roughly of two types: those promoting the growth of soil protozoa and those inhibiting their growth. Strangely enough, he lists the rhizobia as of the inhibitory type.

LONGEVITY OF THE RHIZOBIA OUTSIDE OF THE PLANT.

In view of the many factors, physical, chemical, and biological, which influence the nodule bacteria, it is useless to make a definite statement concerning

their possible or probable longevity. The reader will appreciate that any of the numerous factors discussed in this chapter may cause a rapid dying off of at least the majority of cells in a culture. Under some circumstances or sets of conditions, on the other hand, some hardy individuals may survive for astonishing periods of time. It will be appropriate to record here some of the cases of long survival recorded in the literature.

It is usually recommended that inoculated seed be planted as soon as possible in order that the bacteria may not die from exposure on the seed coats. Fellers, 1919, studied the viability of rhizobia on seeds and discovered that soon after inoculation the majority of the bacteria die. A small percentage of the cells which are apparently better able to withstand desiccation survive as long as 6 to 9 months. Practically it is the few resistant cells which bring about nodule production. Lochhead, 1927b and c, obtained somewhat similar results. Thornton, 1929b, also observed the greatest loss from death on the seed coat during the first 7 days after inoculation. After 14 days of storage enough organisms were left to produce numerous nodules, 61 per cent of the number on a parallel set of plants sown after one day of storage.

Porges, 1931b, has found that the medium in which inoculum is applied to seed is of some significance in the longevity of the rhizobia on the dried seed coats. Plant sap added to the bacterial suspension provides some protective material which favors longevity of the bacteria after drying, being superior to milk in this respect. Refrigeration at 5° C. also tends to protect the bacteria from death by drying. The investigation was undertaken to test the feasibility of inoculation of leguminous seed by the dealer rather than by the farmer immediately before use. The data are suggestive but not sufficient to recommend the use of dry inoculated seed.

There are on record 3 or 4 remarkable reports of survival of rhizobia in laboratory cultures; the maximum time now known extends into years, and who knows what might be the limit? Edwards, 1923, reported that he had been able to sesure excellent nodule production with cultures from red clover, white clover, and alfalfa, respectively 16, 10, and 10 years old. These cultures had been kept at room temperature in Freudenreich flasks sealed with wax. The culture medium consisted of wood ash, 10 gm.; maltose, 10 gm.; agar, 15 gm.; and tap water, 1000 cc.

Stapp, 1924, also reported a 16-year survival of 3 cultures. These 3 were found among 65 cultures sealed in glass tubes and stored at room temperature. Cultures of alfalfa, clover, beans, peas, soybeans, lupines, and locust were represented among the stocks, but only the cultures from *Vicia* were found viable. These were in a neutral carrot-agar medium rather than gelatin, a fact which Stapp considers significant.

Perhaps the most exhaustive study of the viability of the nodule bacteria is that of the Canadian bacteriologist, Jones, 1927. He stored stock cultures of a large number of strains of rhizobia for 11 to 15 years in small Freudenreich flasks, containing a modified Ashby's agar. The flasks were plugged with cotton, sealed with wax and kept in the laboratory at room temperature. After 11 years the cultures were plated out on the same modified Ashby's agar. Strangely enough, all 12 of the cultures representing the red clover, white clover, crimson clover, sain-

foin, alfalfa, sweet clover, field pea, sweet pea, hairy vetch, field bean, and cowpea groups showed typical rhizobium colonies. Even more striking are the results of viability tests on an alfalfa culture kept in a two ounce Blake bottle and tightly corked. Periodic counts of viable cells showed:

After 2 months	5,000,000,000	cells
After 12 months	1,400,000,000	cells
After 15 years	110,000,000	cells

The longevity of rhizobia in soil under varying conditions both in laboratory and field has been reported by a number of investigators. It should be said that unless killed off by unfavorable soil conditions, the rhizobia may survive in a soil *in situ* for a number of years even in the absence of a leguminous crop. A general summary of this question will be found in the paper by Albrecht and Turk, 1930. They found that in dried soils the rhizobia gradually lose their power to produce nodules. For three years these soil cultures retained their efficiency, showing a pronounced loss after four years.

CHAPTER 8

SPECIES RELATIONSHIPS

"The species name has only the value of a name on a package: we must always be ready to change it or to do away with it."

—DUCLAUX

Relation of the rhizobia to other bacteria. Because the science of bacteriology is comparatively new, the questions of species relationships and nomenclature of the bacteria have not been settled. This is particularly true of the root nodule organisms. Some students would place these bacteria close to the *Azotobacter* group; others believe them nearly related to the nitrifying bacteria; while a third group of bacteriologists would link them with the colon-aerogenes group. The latter position is strongly urged by Löhnis, 1925; Löhnis and Pillai, 1907 and 1908; Löhnis and Hansen, 1921; and Skinner, 1928; who have shown that the rhizobia possess many characteristics in common with *B. radiobacter, B. pneumoniae,* and members of the colon-aerogenes groups.

Of considerable practical importance is the relationship existing between *Phytomonas tumefaciens,* the organism of crown gall on various plants; *Rhizobium,* the root-nodule organism of leguminous plants; and *B. radiobacter,* a soil organism morphologically and physiologically very similar to the other two, but lacking the ability to attack living plants. In his first paper dealing with the rhizobia, Beijerinck, 1888, described *B. radiobacter* and remarked upon its surprising similarity to the root nodule organism. Löhnis, 1905, and Löhnis and Hansen, 1921, in careful studies of these organisms also pointed out their close relationship. Similar likeness was shown in the original description of *Ph. tumefaciens* by Smith and Townsend, 1907. Kellerman, 1911b, also called attention to the fact that crown gall lesions on certain leguminous plants might be confused with the tubercles or nodules induced by the true root nodule organism. He suggested the use of Congo red in the isolating medium as an indicator for the separation of *Ph. tumefaciens* from the root nodule organism. Later investigators have failed to substantiate the value of this test, however, as certain pure cultures of the two organisms often have nearly equal capacity to absorb Congo red.

More recently Lieske, 1927; Riker, Banfield, Wright, Keitt, and Sagen, 1930; and Israilsky, 1929, have again pointed out the similarity in morphology and physiology of these organisms. The only tests which Israilsky, 1929, found of value in separating the organisms were their reaction toward the bacteriophage and their specificity toward appropriate host plants. Stapp and Bortels, 1931, in their recent study of the production of "Bakteriensterne" with *Ph. tumefaciens,* have again

called attention to the apparent relationship between these organisms. The crown-gall organisms have been divided into two species by Riker, Banfield, Wright, Keitt, and Sagen, 1930: *Ph. tumefaciens,* which produces typical crown-gall lesions, and *Ph. rhizogenes,* which produces the hairy-root type of lesion on appropriate host plants. Their bacteriological studies have shown that from a morphological and physiological standpoint *Ph. tumefaciens* resembles *B. radiobacter* more than *Ph. rhizogenes.* The root nodule organisms, as has been pointed out, may be divided into two broad groups, the peritrichous forms of clover, alfalfa, peas, beans, etc., and the monotrichous forms of soybeans and cowpeas. Here also the relationship between *B. radiobacter* and the peritrichous forms of rhizobia seems closer than the relation of the peritrichous forms of rhizobia to the monotrichous forms of the same genus.

It seems certain that the rhizobia, *B. radiobacter,* and *Ph. tumefaciens* are closely related and should be placed near to each other in taxonomic grouping. While the relationship of these organisms to those of the colon-aerogenes group is less evident, certainly the rhizobia are more closely related to the colon-aerogenes organisms than to either *Azotobacter* or the autotrophic nitrifying bacteria.

Differentiation of the rhizobia. It is almost unanimously agreed that the ability to cause the formation of root nodules on the Leguminosae is an important distinguishing character of the members of the genus *Rhizobium.* Any organism possessing this ability is placed in this group, whose members are generally believed to attack only the Leguminosae. The relationship of the organisms causing nodulation of certain non-leguminous plants to the true leguminous rhizobia is still uncertain. In some instances it has been claimed that the organisms forming non-leguminous nodules are very similar to those of the leguminous root nodules. It is probable, however, that further study will bring out more differences than have yet been recognized. Chapter 3 gives a detailed account of the organisms associated with the non-leguminous plants.

The early students of the nodule bacteria considered them to constitute a single species adaptable to various host plants, but as the study continued, it was recognized that the organisms from different plant species are not strictly alike. The question of the extent and permanence of the differences observed is still a disputed point. Some believe that the differences are only adaptations resulting from the prolonged growth of the bacteria with a certain host or in a certain medium; others, that the differences are reasonably constant, but not great enough to justify specific differentiation; and still others, that several species are involved in nodule formation on different Leguminosae.

A number of different characters have been used as a basis for species differentiation. The most important are nodule form; ability of the bacteria to produce nodules on various plant species; and morphological, cultural, biochemical, and serological characters of the bacteria themselves.

Nodule form and structure. Even before the rhizobia had been isolated and studied in pure culture, certain characteristic differences were noted in both the external and internal structure of the nodules occurring on different host plants. It is very doubtful, however, whether differences in the structure and form of the nodules can be taken as necessarily indicating differences in the bacteria respon-

sible for nodule formation. The nodule is the resultant of the interaction of the host plant and the bacteria, and it is probable that the form and internal structure of the nodule are determined as much by the host plant as by the invading microörganism, but the presence or absence of infection threads may be a result of inherent properties of the bacteria. The questions of nodule form and structure will be discussed in detail in Chapter 9, and since their value as indicators of differences in the microörganisms is doubtful, it will not be necessary to treat the subject extensively at this point. It is interesting to note, however, that probably the first separation of the organism into two species by Schroeter, 1886, was based entirely on nodule structure. He recognized two species, *Phytomyxa leguminosarum* (Frank) Schroeter and *Phytomyxa lupini* Schroeter. The first of these according to Schroeter's experiment shows infection threads in the nodular tissue and forms nodules on most leguminous plants, *e.g. Trifolium repens, Lotus corniculatus,* and *Orobus vernus,* etc.; the second forms no infection threads in the nodules and only infects *Lupinus luteus* and *Lupinus angustifolius.* The characteristics of nodule form and structure are still used by some writers as criteria for differentiation of the organism into species. Dangeard, 1926, in a comprehensive survey of the question of species relationships, considered that at least ten species should be recognized, and he used the nodule structure, together with the morphology of the organism within the nodule, as the principal characteristic for species differentiation.

Cross-inoculation groups. Most of the early workers were of the opinion that there was only one organism, and that it could form nodules on any of the leguminous plants. In order to explain the difficulties which they often encountered in transferring the organism from one plant to another, the theory was evolved that long-continued association of the bacteria with one host plant produced an "adaptation form" which would attack other leguminous plants only with great difficulty. As the study continued, however, it became evident that the ability of a given organism to pass from one plant to another was limited to a certain group of plants. This resulted in the establishment of a series of plant groups known as "cross-inoculation groups," *i.e.,* groups of plants within which the root nodule organisms are mutually interchangeable.

The establishment of these cross-inoculation groups proceeded very slowly for a number of years. Many of the earlier tests on cross inoculation gave results indicating that practically all leguminous plants would develop nodules in response to any culture of rhizobia. In most cases the necessity of adequate seed and soil sterilization was not recognized; control plants were not carried under similar conditions to be sure that the nodules resulted only from the inoculum; and technique had not been developed which enabled the investigators to be sure of the purity of their cultures.

Certain early reports on cross inoculation are interesting from an historical standpoint, although their results cannot be confirmed in all cases by present workers. Bréal, 1888b, was probably the first to make cross inoculation experiments. He reported nodulation of pea, lupine, and alfalfa plants with alfalfa bacteria. Ward in 1889 secured cross inoculation between peas and broad beans, and Laurent, 1890 and 1891, stated that he had been able to produce nodules on peas with cultures isolated from 36 different plant species.

Prazmowski's failure, in 1889, to secure nodulation of lupine with pea organisms is the first definite indication that all of the organisms are not interchangeable. Hellriegel and Wilfarth, 1889, also indicated that neither lupine nor serradella becomes nodulated in soil upon which peas have been previously grown. Nobbe, Schmid, Hiltner, and Hotter, 1891, confirmed and extended Prazmowski's tests. They were unable to obtain nodules on lupines with rhizobia isolated from either *Pisum, Robinia, Cytisus,* or *Gleditsia* soil. They found that nodules were formed on *Phaseolus* by either pea or bean bacteria but not by the organisms from lupine or *Robinia.*

Atkinson, 1893, working with a pure culture from vetch, was able to produce nodules on vetch but not on *Dolichos sinensis.* Nobbe, Hiltner, and Schmid, 1895, found that a culture from *Pisum sativum* would induce nodules upon both *Vicia sativa* and *V. Faba,* but not upon *Trifolium, Medicago, Robinia, Anthyllis,* or *Ornithopus* spp. In addition they reported several other cross inoculations, which were probably the result of faulty technique. Kirchner, 1895, noted that soybeans did not bear nodules in the garden at Hohenheim even though grown with a number of other leguminous plants, each of which bore nodules. On the other hand, nodulated plants were successfully produced in soil from Japan.

Nobbe and Hiltner, 1896d, were granted a patent on the claim of having shown differences among the organisms. "It has been established by us as an entirely new fact that the tubercle bacteria of the various Papilionaceae are of full strength (*i.e.,* in the production of efficient nodules or tubercles) only with that species from whose root-tubercles they were themselves obtained. With nearly-allied species they are of weaker strength, and with systematically different species they are useless. Bacterial cultures from pea roots, for example, are quite useless for *Robinia* plants, while they promote the growth of peas in an extremely energetic manner, and that of the allied vetches somewhat more feebly; and on the other hand, the bacteria from *Robinia* nodules or tubercles are quite efficient with *Robinia* plants, but in a lesser degree with *Colutea,* and are absolutely useless with peas."

During the next decade a number of field observations were reported on the cross-inoculation problem. Duggar, 1897 and 1898, noted the failure of cross inoculations between alfalfa, clover, lespedeza, and vetch; the pea nodule organisms, on the other hand, produce nodules on vetch. Duggar's views, 1897, are similar to those in the patent of Nobbe and Hiltner, "As a rule, to which there are exceptions, the germ which induces the growth of tubercles on one legume is unable, at least temporarily, to produce tubercles on plants belonging to other genera . . ." Dodson, 1897, used an infusion from nodules as an inoculating agent and decided that each genus demands a specific microörganism. Munson, 1899, carried out a series of cross-inoculation tests but failed to secure reliable data because of nodules on his control plants. Mazé, 1899, divided the organisms into two groups on the basis of the reaction of their host plants toward lime. One group was supposed to be effective in an acid and the other in an alkaline soil. This classification was soon shown to be erroneous. Otis, 1900, secured nodulation of alfalfa, clover, field peas, and soybeans with a sample of soil from Massachusetts, which failed to produce nodules on adzuki beans and cowpeas. Judging from her cross-inoculations in the *Vicia* group, Miss Dawson, 1900a and b, be-

lieved that there was only one species of rhizobia with "adaptation forms." Buhlert, 1902a and b, using supposedly pure cultures, secured cross inoculation with *Pisum sativum, Phaseolus,* and *Vicia Faba.* Evidence of the cross inoculation between sweet clover and alfalfa was given in 1903 and 1904 by Hopkins, and by Russell and Moore in 1905.

During the next few years a large number of observations on cross inoculation were reported. Unfortunately, however, few of them were carried out under adequately controlled conditions. The opinions of the workers during this period may be grouped into three general divisions.

1. The root nodule bacteria constitute a single species, which is capable of forming nodules on any of the Leguminosae. After association with a certain host plant, the bacteria become adapted to that plant and are more effective with it than with other species of the Leguminosae.

The ideas of this group are expressed in the words of Moore, 1905, "The most that can be maintained is that there is a slight physiological difference due to the long association with a plant of particular reaction, which enables the bacteria more easily to penetrate the host plant upon which they have been accustomed to grow. These slight racial characteristics can readily be broken down by cultivation in the laboratory, and it is entirely possible to secure a universal organism capable of producing a limited number of nodules upon all the legumes which now possess these outgrowths." Again in 1905 Moore and Robinson stated, " . . . any difference in the infective power of bacteria from different host plants is due to slight physiological variations which can be broken down readily by artificial cultivation." Similar views were held by Starnes, 1905; Sheldon, 1906; Bottomley, 1906; Kellerman, 1910a and 1912; Ewart and Thomson, 1912.

2. Each species of leguminous plant requires a special and particular nodule-forming organism. Dodson, 1897; Norton and Walls, 1905; and Harrison and Barlow, 1907, held this view. The experiments of Harrison and Barlow, 1907, are unusual because of their failure to secure nodulation of vetch with pea bacteria, a case of cross inoculation which many investigators have shown to be common. According to Harrison and Barlow's results, even when the two plants were grown in the same flask and inoculated with an organism from peas, only the pea plant bore nodules.

3. Within limits the nodule organisms may be transferred satisfactorily from one species of host plant to another. Not all plants, however, may be placed in the same group. This finding has led to the classification of the host plants into cross-inoculation or bacteria-plant groups, the boundaries of which have been fairly well established by a long series of careful investigations. Lewis and Nicholson, 1905; Maassen and Müller, 1907; Simon, 1907, 1911, and 1914; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918; Joshi, 1920; Whiting and Hansen, 1920; Hansen, 1921b; Hutchinson, 1922; Müller and Stapp, 1925; Whiting, Fred, and Helz, 1926; have carried out experiments in which certain cross inoculations are proved or disproved under carefully controlled conditions.

The following list gives the cross inoculations which are now recognized, together with the authorities for their occurrence. In the main, only references

to original work, in which the technique and data are given, are included. In a few instances, other references are used, either because of the proven accuracy of early workers, *e.g.,* Maassen and Müller, 1907, or because of the fact that certain cross inoculations which appear logical have not been otherwise listed, *e.g.,* Löhnis and Leonard, 1926. In some cases, data which are at variance with those of the majority of workers have been discarded, even though secured under what appear to be carefully controlled conditions, *e.g.,* Joshi, 1920, and Hutchinson, 1922. Where a cross inoculation appears probable, but is supported only by a reference of doubtful value, the species name is preceded by a question mark. Within the groups reciprocal crosses have not been attempted with all members of the group. If the organism from one plant species causes nodule formation on another species of the group, it is assumed that it will act similarly with all members of the group. There is some evidence that this may not be true in all cases; see, for example, Whiting and Hansen, 1920; Jardine, 1924 and 1926; Leonard, 1923a; Sears and Carroll, 1927; and Sears and Clark, 1930. However, in most cases there is no deviation from the rule.

For the plant species the nomenclature of Bailey, *Manual of Cultivated Plants,* 1924; and Britton and Brown, *An Illustrated Flora of Northern United States and Canada,* 1913, has been followed for the cultivated leguminous plants. In cases of conflict between these authorities, Bailey has been followed. Gray's *New Manual of Botany,* 1908, has been used for the wild forms. For species not otherwise listed, *Index Kewensis* has been consulted. The use of two or more scientific names for the same plant species and the use of the same common name for different species has occasioned some difficulty in correctly ascribing certain species to their proper groups.

I. ALFALFA GROUP

Bacteria—Rhizobium meliloti

1. *Medicago sativa* L. Alfalfa, lucerne, purple medic, snail clover, Burgundy, Chilian, or Brazilian clover

   Hopkins, 1904; Russell and Moore, 1905; Lewis and Nicholson, 1905; Simon, 1907, 1914; Maassen and Müller, 1907; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

2. *Medicago lupulina* L. Black or hop medic, blackseed, hop clover, nonesuch

   Maassen and Müller, 1907; Simon, 1907, 1914; Krüger, 1914; Garman, and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

3. *Medicago hispida* Gaertn. (*M. denticulata* Willd.) Bur clover, toothed medic, toothed bur clover

   Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

4. *Medicago falcata* L. Horned medic, Swiss lucerne, yellow lucerne, yellow medic, horned clover

Burrill and Hansen, 1917.

5. *Medicago orbicularis* All. Snail medic, button clover (small-flowered yellow annual species of S. Europe)

Löhnis and Leonard, 1926.

6. *Medicago arabica* Huds. (*M. maculata* Sibth.) Southern bur clover, spotted medic, heart clover, purple grass, St. Mawes clover, spotted clover, spotted bur clover, heart trefoil

Löhnis and Leonard, 1926.

7. *Melilotus alba* Desv. White sweet clover, honey clover, white melilot

Hopkins, 1904; Russell and Moore, 1905; Lewis and Nicholson, 1905; Simon, 1914; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

8. *Melilotus officinalis* (L.) Lam. Yellow sweet clover, yellow melilot, yellow millet

Maassen and Müller, 1907; Burrill and Hansen, 1917.

9. *Melilotus indica* (L.) All. Yellow sweet clover

Burrill and Hansen, 1917.

10. *Trigonella Foenum-Graecum* L. Fenugreek

Simon, 1914; Krüger, 1914; Burrill and Hansen, 1917.

II. CLOVER GROUP

Bacteria—Rhizobium trifolii

1. *Trifolium pratense* L. Red, purple, or meadow clover

Lewis and Nicholson, 1905; Simon, 1907, 1914; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

2. *Trifolium repens* L. White clover, white Dutch or honeysuckle clover, shamrock, white trefoil, purple grass, purple wort

Lewis and Nicholson, 1905; Simon, 1907, 1914; Maassen and Müller, 1907; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

3. *Trifolium hybridum* L. Alsike or Alsatian clover
Lewis and Nicholson, 1905; Simon, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

4. *Trifolium incarnatum* L. Crimson, carnation, French, or Italian clover
Lewis and Nicholson, 1905; Simon, 1907, 1914; Maassen and Müller, 1907; Garman and Didlake, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

5. *Trifolium alexandrinum* L. Berseem, Egyptian clover
Burrill and Hansen, 1917.

6. *Trifolium medium* L. Zigzag or mammoth clover, cow or marl grass, cow clover
Burrill and Hansen, 1917.

7. *Trifolium agrarium* L. Yellow or hop clover
Löhnis and Leonard, 1926.

8. *Trifolium dubium* Sibth. Least hop clover, hop trefoil
Löhnis and Leonard, 1926.

9. *Trifolium pratense* L. Mammoth clover
var. *perenne* Host.
Löhnis and Leonard, 1926.

10. *Trifolium arvense* L. Rabbit-foot, old field or stone clover
Löhnis and Leonard, 1926.

11. *?Trifolium resupinatum* L. Annual strawberry clover, reversed clover
(*T. suaveolens* Willd.)
Pieters, 1927.

12. *?Trifolium carolinianum* Carolina clover
Michx.
Pieters, 1927.

13. *?Trifolium parviflorum* Small-flowered clover
Perreym.
Pieters, 1927.

14. *?Trifolium fragiferum* L. Trefoil, strawberry clover, strawberry-headed clover
Pieters, 1927.

15. *?Trifolium pannonicum* Hungarian clover
Jacq.
Pieters, 1927.

16. *?Trifolium reflexum* L. Buffalo clover
Pieters, 1927.

III. PEA GROUP

Bacteria—Rhizobium leguminosarum

1. *Pisum sativum* L. Garden pea
Buhlert, 1902a and b; Simon, 1907, 1914; Maassen and Müller, 1907; Burrill and Hansen, 1917; Koch and Butler, 1918.

2. *Pisum sativum* L. Field pea
var. *arvense* Poir.
Simon, 1907, 1914; Krüger, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

3. *Lathyrus odoratus* L. Sweet pea
Maassen and Müller, 1907; Simon, 1914; Burrill and Hansen, 1917; Koch and Butler, 1918.

4. *Lathyrus latifolius* L. Everlasting pea, perennial pea
Nobbe, Hiltner, and Schmid, 1895; Burrill and Hansen, 1917.

5. *?Lathyrus tingitanus* L. Tangier pea
Pieters, 1927.

6. *Lathyrus sylvestris* L. Flat pea
Maassen and Müller, 1907; Simon, 1914.

7. *?Lathyrus venosus* Muhl. Veiny pea
Pieters, 1927.

8. *Lathyrus sativus* L. Grass pea
Maassen and Müller, 1907.

9. *Vicia sativa* L. Spring vetch, common vetch, tare, pebble vetch
Lewis and Nicholson, 1905; Simon, 1907, 1914; Maassen and Müller, 1907; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917.

10. *Vicia villosa* Roth. Hairy or winter vetch
Simon, 1907, 1914; Maassen and Müller, 1907; Garman and Didlake, 1914; Burrill and Hansen, 1917.

11. *Vicia hirsuta* (L.) S. F. Gray Hairy vetch, tare, tineweed
Nobbe, Hiltner, and Schmid, 1895.

12. *Vicia sepium* L. — Bush vetch, wild tare, hedge vetch, crow pea
Nobbe, Hiltner, and Schmid, 1895.

13. *Vicia Faba* L. (*Faba vulgaris* Moench.) — Broad bean, horse bean, Windsor bean
Maassen and Müller, 1907; Simon, 1914; Burrill and Hansen, 1917.

14. *Vicia angustifolia* L. — Smaller common vetch, narrow-leaved vetch
Burrill and Hansen, 1917.

15. *Vicia dasycarpa* Ten. — Vetch
Burrill and Hansen, 1917.

16. *Vicia atropurpurea* Desf. — Purple vetch
Löhnis and Leonard, 1926.

17. *Vicia monantha* — Monantha vetch
McKee, Schoth, and Stephens, 1931.

18. *Lens esculenta* Moench. (*Ervum Lens* L.) — Common lentil
Maassen and Müller, 1907; Burrill and Hansen, 1917.

19. *Cicer arietinum* L.[1] — Chick pea, garbanzo, coffee pea, gram
Simon, 1914.

IV. BEAN GROUP

Bacteria—Rhizobium phaseoli.

1. *Phaseolus vulgaris* L. — Garden bean (many varietal names), kidney bean, navy bean, haricot bean
Simon, 1907; Maassen and Müller, 1907; Krüger, 1914; Garman and Didlake, 1914; Burrill and Hansen, 1917.

2. *Phaseolus angustifolia* R. Roxb.
Burrill and Hansen, 1917.

3. *Phaseolus coccineus* L. (*P. multiflorus* Willd.) — Scarlet runner bean, Spanish bean, Dutch case knife bean, multiflora bean.
Burrill and Hansen, 1917.

[1]Recent studies in this laboratory indicate that *Cicer arietinum* does not belong in this group.

V. LUPINE GROUP

Bacteria—Rhizobium lupini

1. *Lupinus luteus* L. Yellow lupine
   Simon, 1907; Maassen and Müller, 1907; Nobbe, Richter, and Simon, 1908; Krüger, 1914.

2. *Lupinus angustifolius* L. Blue-flowered lupine, narrow-leaved blue lupine
   Simon, 1907, 1914; Maassen and Müller, 1907; Krüger, 1914.

3. *Lupinus polyphyllus* Lindl. Many-leaved lupine
   Simon, 1907.

4. *Lupinus perennis* L. Wild or perennial lupine
   Simon, 1914; Krüger, 1914; Burrill and Hansen, 1917.

5. *Lupinus albus* L. White lupine
   Pieters, 1927

6. *Ornithopus sativus* Brot. Serradella, seratella, serratella, serradilla
   Simon, 1907, 1914; Maassen and Müller, 1907; Nobbe, Richter, and Simon, 1908; Krüger 1914; Burrill and Hansen, 1917.

VI. SOYBEAN GROUP

Bacteria—Rhizobium japonicum

Glycine Max Merr. Soybean
(*G. Soja* Sieb. and Zucc.)
(*Soja Max* Piper)
(*G. hispida* Maxim.)
(*Phaseolus Max* L.)
Simon, 1907; Maassen and Müller, 1907; Garman and Didlake, 1914; Krüger, 1914; Burrill and Hansen, 1917.

VII. COWPEA GROUP

Bacteria—Rhizobium sp.?

1. *Vigna sinensis* Endl. Cowpea, black-eyed bean, China bean
   (*Dolichos sinensis* Stickm.)
   Garman and Didlake, 1914; Burrill and Hansen, 1917; Whiting and Hansen, 1920.

2. *Cassia Chamaecrista* L. Partridge pea, prairie senna
Burrill and Hansen, 1917.

3. *Arachis hypogaea* L. Peanut, goober, groundnut
Burrill and Hansen, 1917; Whiting and Hansen, 1920.

4. *Lespedeza sericea* Benth. Bush clover
Pieters, 1927.

5. *Lespedeza striata* Japan clover
Hook. and Arn.
Burrill and Hansen, 1917; Tracy and Coe, 1918; Whiting and Hansen, 1920.

6. *Lespedeza virginica* (L.) Slender bush clover
Britton
Burrill and Hansen, 1917.

7. *Stizolobium utile* (Wall.) Velvet bean
n. comb. (*Mucuna utilis* Wight)
Burrill and Hansen, 1917; Tracy and Coe, 1918.

8. *Baptisia tinctoria* R. Br. Wild indigo; yellow, indigo, or clover broom; horsefly weed
Burrill and Hansen, 1917.

9. *Desmodium canescens* (L) Tick Trefoil
DC.
(*Meibomia canescens* (Ktze.)
Burrill and Hansen, 1917.

10. *Desmodium illinoense* Gray Tick Trefoil
(*Meibomia illinoense* (Ktze.)
Burrill and Hansen, 1917.

11. *Desmodium tortuosum* DC. Beggarweed
(*Meibomia tortuosum* DC.)
Whiting and Hansen, 1920.

12. *Desmodium purpureum* Florida beggarweed
Hook. and Arn.
(*D. polycarpum* Wight and Arn.)
Löhnis and Leonard, 1926.

13. *Acacia armata* R. Br. Kangaroo-thorn
Burrill and Hansen, 1917.

14. *Acacia longifolia* Willd. and *Acacia longifolia* Willd. var. *floribunda* F. Muell. Sidney golden wattle
Burrill and Hansen, 1917.

15. *Acacia linifolia* Willd. Acacia
Burrill and Hansen, 1917.

16. *Acacia melanoxylon* R. Br. Blackwood acacia
Burrill and Hansen, 1917.

17. *Acacia semperflora* Everflowering acacia
Burrill and Hansen, 1917.

18. *Albizzia lophantha* Benth. (*Acacia lophantha* Willd.)
Maassen and Müller, 1907.

19. *Genista tinctoria* L. Dyer's greenweed, woad-waxen, dyeweed, greenweed, whin, base broom
Burrill and Hansen, 1917.

20. *?Cytisus scoparius* Link. Scotch broom, green broom, hagweed
(*Sarothamnus scoparius* Wimm.)
(*Spartium scoparium* L.)
(*Genista scoparia* Hort.)
Pieters, 1927. Listed by Maassen and Müller, 1907, as a separate group.

21. *Phaseolus lunatus* L. var. *macrocarpus* Benth. Lima bean, Sieva bean
(*Ph. limensis* Macf.)
Tracy and Coe, 1918; Whiting and Hansen, 1920.

22. *Phaseolus angularis* Wight Adzuki or adsuki bean
Leonard, 1923a; Richmond, 1926a.

23. *Phaseolus aconitifolius* Jacq. Moth bean, mat bean; various Indian names —math, banmunga, kheri, bir mung, mattikalai, tulka, mout
Joshi, 1920; Richmond, 1926a.

24. *Phaseolus aureus* Roxb. Mung bean, green or golden gram
Richmond, 1926a.

25. *Phaseolus calcaratus* Roxb. Rice bean
Richmond, 1926a.

26. *Phaseolus Mungo* L. Urd bean, urid, black gram
Richmond, 1926a.

27. *Phaseolus radiatus* L. Mung
Richmond, 1926a.

28. *Phaseolus acutifolius* Gray var. *latifolius* Freem. Tepary bean
Löhnis and Leonard, 1926.

29. *Vigna sesquipedalis* Wight (*Dolichos sesquipedalis* L.) (*Vigna sinensis* var. *sesquipedalis* Koern.) Asparagus bean, yard-long bean
Richmond, 1926a; Walker, 1928.

30. *Canavalia ensiformis* DC. Jack bean
Löhnis and Leonard, 1926; Richmond, 1926a.

31. *Canavalia gladiata* DC. Sword bean
Pieters, 1927.

32. *Pueraria hirsuta* Schneid. (*P. Thunbergiana* Benth.) (*Dolichos japonica* Hort.) Kudzu-vine
Löhnis and Leonard, 1926.

33. *Cajanus Cajan* Millsp. (*C. indicus* Spreng.) Pigeon pea, Congo pea
Joshi, 1920; Whiting and Hansen, 1921; Löhnis and Leonard, 1926; Richmond, 1926a.

34. *Stizolobium Deeringianum* Bort. (*Dolichos multiflorus* Hort.) (*Mucuna Deeringiana* Small) Deering velvet bean, Florida velvet bean
Tracy and Coe, 1918; Löhnis and Leonard, 1926. Classified as a separate group by Walker, 1928.

35. *Cyamopsis tetragonoloba* (L.) Schinz. (*Cy. psoraloides* DC.) (*Psoralea tetragonoloba* L.) Guar bean
Richmond, 1926a.

36. *Dolichos Lablab* L. Bonavist, lablab, hyacinth or Egyptian bean
Richmond, 1926a.

37. *Dolichos biflorus* L. Kulthi bean, gram, horse grain, horse gram
Richmond, 1926a.

38. *Crotalaria retusa* L. Cherokee clover, rattle box, yellow-flowering pea
Richmond, 1926a.

39. *Crotalaria juncea* L. Sunn hemp, Bengal hemp, false hemp, Bombay hemp
Joshi, 1920. Classified by Walker, 1928, as a separate group.

40. *?Voandzeia subterranea* Thou. Juga bean, earth pea, Bambarra ground nut, Madagascar pea nut
Pieters, 1927.

41. *?Ulex europaeus* L. Furze, gorse, whin
Pieters, 1927. Classified by Simon, 1914, and Walker, 1928, as a separate group.

VIII. LOTUS GROUP

Bacteria—Rhizobium sp.?

1. *Lotus uliginosus* Hoffm.
(*L. corniculatus* L.)
Simon, 1914; Krüger, 1914.

2. *Lotus corniculatus* L. Bloom-fell, bird's-foot trefoil, ladies' fingers, ground honeysuckle, crow-toes, cross-toes, cat's clover, sheepfoot, bird's eye, devil's fingers, shoes and stockings, claver
Simon, 1914.

3. *Lotus Tetragonolobus* L. Winged pea
(*Tetragonolobus purpureus* Moench.)
Simon, 1914; Krüger, 1914.

4. *Anthyllis Vulneraria* L. Kidney vetch, lady's fingers, woundwort
Simon, 1907, 1914; Maassen and Müller, 1907; Krüger, 1914.

IX.

Dalea alopecuroides Willd. Wood's clover
(*Psoralea Dalea* L.)
(*Parosela Dalea* Britton.)
Whiting, Fred, and Helz, 1926.

x.

Onobrychis viciaefolia Scop. (*Onobrychis sativa* Lam.) (*Hedysarum onobrychis* Neck.) — Sainfoin, Saintfoin, holy clover, esparcet

Simon, 1907; Maassen and Müller, 1907; Krüger, 1914.

xi.

Strophostyles helvola (L.) Britton — Trailing wild bean

Burrill and Hansen, 1917.

xii.

Robinia Pseudo-Acacia L. — Yellow, black, false, or bastard acacia; common locust, yellow locust

Maassen and Müller, 1907; Burrill and Hansen, 1917.

xiii.

Amphicarpa monoica (L.) Ell. — Hog peanut

Burrill and Hansen, 1917.

xiv.

Amorpha canescens Nutt. (*A. canescens* Pursh.) — Lead plant, wild tea, shoestrings

Burrill and Hansen, 1917.

Amorpha fruticosa L. (*A. fragrans* Sweet.) — False indigo, bastard indigo, river locust

Maassen and Müller, 1907.

xv.

Coronilla varia L. — Crown vetch

Maassen and Müller, 1907.

xvi.

Caragana frutex Koch. (*C. frutescens* DC.) — Pea tree

Maassen and Müller, 1907.

The groups listed above are generally conceded to be accurate as far as practical purposes of inoculation are concerned.

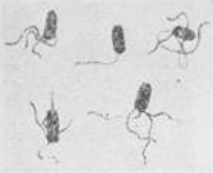
Under certain conditions, however, it seems that there may be cross inoculation between the members of different groups. The cases of cross inoculation between soybean and cowpea, Leonard, 1923a, and Sears and Carroll, 1927, and of non-reciprocal crossing between Dalea and garden bean, Sears and Clark, 1930, have been the most carefully studied. The use of single-cell cultures and adequate precautions to prevent outside contamination by Sears and Carroll, 1927, have established that the organism from soybean may under certain conditions cause the formation of nodules on cowpeas as well as on soybeans. Hansen and Tanner, 1931, have also worked with the non-reciprocal lima bean-navy bean and dalea-navy bean crosses and the reciprocal soybean-cowpea cross. It is probable that other cross inoculations which are now considered impossible may sometimes occur.

The necessary conditions have not been thoroughly studied; it seems highly desirable that such a study be made in the hope of ultimately arriving at an explanation of the factors responsible for the specificity of the action of the rhizobia on their host plants. The ability of the organism to gain an entrance and multiply within the host plant seems to depend upon the character of the organism and the defensive forces of the host. Changes in the physiology of either the organism or the higher plant may make it possible for infection to occur on plants not normally susceptible.

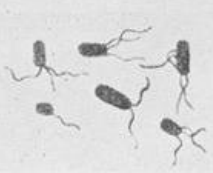
In spite of the few known exceptions to the ordinary list of cross-inoculations, it seems true that the ability of the organism to infect certain plants and not others is as fixed and definite as any phase of the physiology of the organism. In view of its relative stability and practical importance, we feel justified in regarding it as the prime character in species differentiation.

Possible explanation for cross-inoculation specificity. Several theories have been advanced as possible explanations for the specificity which normally obtains. Mazé, 1899, divided the root nodule organisms into two groups, one of which was capable of infecting the lime-loving leguminous plants and a second which infected only the acid-loving plants. Fred and Davenport, 1918, established limiting hydrogen-ion concentrations for the growth of the nodule bacteria from several plants. As they pointed out, these acidities roughly correspond to the acidity of the juice expressed from the corresponding plants. Other investigators, Stevens, 1925a, and Wright, 1925a, have since pointed out that considerable variation exists in the limiting hydrogen-ion concentration for different strains of the same rhizobium.

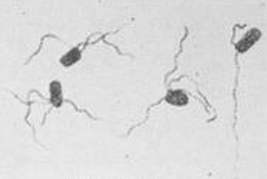
Prazmowski, 1890 and 1891, demonstrated that the bacteria enter the root by way of the root hair and that a characteristic curling of the tip of the root hair occurs when it is attacked by the organisms. Hiltner, 1900a, demonstrated that the action is probably enzymatic, since this curling of the root hair occurs when a bacteria-free filtrate of the organism is placed on the root. It is conceivable that failure to secrete the proper enzyme to gain entrance into foreign root hairs is the reason for the specificity of the rhizobia. McDougall, 1921, has claimed this in the case of *Gleditsia,* the root hairs of which are exceptionally thick-walled and



1. *Pisa. arv.*



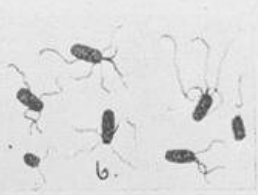
2 *Vicia sat.*



3. *Lathyr. sat.*

4. *Trif. incar.*

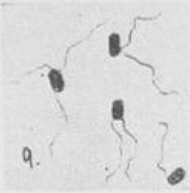
5. *Trif.-prat.*



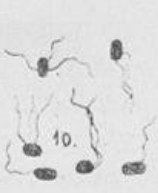
6. *Phaseol. ruby.*

7. *Melilot.-offic.*

8. *Medic.-sat.*

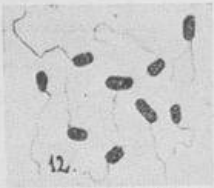


9. *Lup. mutab.*



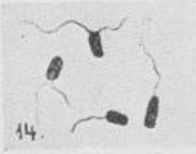
10. *Lup. hirsut.*

11. *Ornithop. sat.*



12. *Soja hisp.* (schw. Var.)

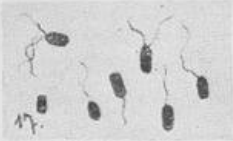
13. *Onobrych. sat.*



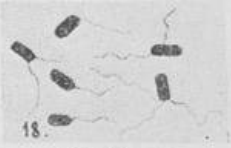
14. *Genista tinct.*

15. *Anthyll. vul.*

16. *Lot. cornic.*



17. *Rob. Pseud-Ac.*



18. *Coronilla var.*

19. *Pis. sat.*

20. *Lathyr. odorat.*

Geißelfärbungen von Knöllchenbakterien nach Zettnow.

Vergr. 2400 fach.

lignified. *Gleditsia,* it will be recalled, is one of the few Leguminosae which bear no true nodules.

Baldwin, Fred, and Hastings, 1927, classified the leguminous plants on the basis of the serological reactions of their seed proteins and showed that such a classification corresponds very closely to the groupings made on the basis of cross-inoculation tests. The protein characteristics of the host plant may be the factor which determines whether an organism will infect a given host plant, or it may be merely an expression of the total physiological complex of the host plant. Some other associated factor may then determine whether infection occurs. As a matter of fact certain exceptions have already been found to the theory of infection based upon protein compatibility. For example, Hansen and Tanner, 1931, cite the non-reciprocal lima bean-navy bean and dalea-navy bean crosses and the reciprocal soybean-cowpea cross.

None of the theories so far advanced to account for the observed specificity of cross inoculation fits all of the facts. This points to the need for further studies of the complex physiological relationships to definitely establish the various factors involved.

Morphology and flagellation. Since flagellation, life cycle changes, and other features of morphology of the rhizobia have been treated in detail in Chapter 5, only a brief characterization will be given here. It was noted by Wigand, 1887; Beijerinck, 1888; and Morck, 1891, that the enlarged forms which Brunchorst, 1885a, had called bacteroids are somewhat varied and characteristic of the plant species in which they occur. Careful studies have shown that essentially the same type of bacteroids tends to occur throughout each cross-inoculation group, Dangeard, 1926, and Müller and Stapp, 1925. Buchanan, 1909b, also demonstrated that the characteristic shapes of bacteroids occur in pure cultures on artificial media as in the nodules. Flagellation studies on the organisms have shown that two general types occur; see Löhnis and Hansen, 1921, and Shunk, 1921. The organisms from the alfalfa, clover, pea, and bean cross-inoculation groups are peritrichous, whereas those of the soybean and cowpea groups are monotrichous. The studies of Müller and Stapp, 1925, indicate that no absolute division into monotrichous and peritrichous forms can be made. In a general way, however, their findings agree with those of the earlier workers. Plate 21 is reproduced from Müller and Stapp. In summary it may be said that the morphology and flagellation of the rhizobia, while not in themselves sufficient, are of some value in separation of the rhizobia into species.

Physiology and cultural characters. Since a complete discussion of the physiology and cultural characters of the rhizobia has been given in Chapter 6, only the items which are useful in separating the species will be considered here. Many of the early workers, Beijerinck, 1888 and 1890; Gonnermann, 1894; Kirchner, 1895; Burrage, 1901; Schneider, 1902 and 1903; and Chiarizia, 1903, showed that the organisms from various leguminous plants are not alike culturally. Hiltner and Störmer in 1903a divided the rhizobia into two groups on the basis of cultural characteristics, particularly their use of carbohydrates and growth on gelatin. Later work by Burrill and Hansen, 1917, and Löhnis and Hansen, 1921, has established the so-called fast-growing group, which is characterized by rapid, profuse, slimy growth and appreciable acid formation; and a second group which

exhibits slower and less profuse growth and an alkaline reaction, rarely, if ever, an acid reaction. Associated with these characters is a difference in the growth of the organisms in milk. The first group produces a serum zone without coagulation, while the second group gives no macroscopic change in the milk. To the first group belong the organisms from alfalfa, clover, pea, bean, and Dalea, and to the second, the organisms of soybean, cowpea, and lupine.

As the study of these organisms has continued, it has become increasingly apparent that these two large groups can be further subdivided on the basis of cultural and physiological characters. These groupings follow the lines of the cross-inoculation groups; and although the differences are not as distinct as those separating the two main groups, it is evident that the organisms of the various cross-inoculation groups are not identical, either culturally or physiologically. The work of Müller and Stapp, 1925, is the most thorough and exhaustive yet published, but the reports of Simon, 1911, and of Prucha, 1915, with the organism from pea; of Wilson, 1917, and Wright, 1925a, with the organism from soybean; of Stevens, 1925a, with the organism from alfalfa; of Gangulee, 1926a and b, with the organism from *Crotalaria juncea;* of Whiting, Fred, and Helz, 1926, with the organism from Dalea; and of Eckhardt, Baldwin, and Fred, 1931, with the organism from *Lupinus* deserve mention.

Studies on the fermentative characters and gelatin liquefaction by the various rhizobia have been carried out by a number of workers recently. It has been found that significant differences do exist; these have been stated fully in Chapter 6 and will be introduced wherever useful in the species characterization which will be given later in this chapter. In some cases the variation between the reaction of known strains of the same rhizobium is so great that apparent differences between cross-inoculation groups cannot be urged too far. One would hesitate to identify an unknown culture of rhizobia entirely on the basis of fermentative characters; yet the authors feel that the fermentation reactions are valuable aids in species characterization and should be used as far as may be.

Serological reactions. With the development of serological reactions by workers with animal pathogens and their general adoption as an aid in species identification, a number of studies were made on the antigenic specificity of the rhizobia. The early results of Zipfel, 1911; Krüger, 1914; Klimmer and Krüger, 1914; Simon, 1914; Vogel and Zipfel, 1921; Klimmer, 1922; and Asō and Ohkawara, 1926; as well as the more recent studies of Walker, 1928, indicated that certain serological reactions, particularly the agglutination test, might be used to separate the various species of rhizobia. In each instance one, or at most a very few, strains of each species were tested. The results with such a limited number of strains render of little value the sweeping conclusions which were often drawn. The fact that in no instance was cross agglutination observed between different species is probably satisfactory evidence for the conclusion that the species are serologically distinct. On the other hand, the assumption by several investigators that all strains of a species are serologically identical has little basis in fact.

Several serological groups have been recognized within the species by a number of workers: Bialosuknia, 1923; Bialosuknia and Klott, 1923; Stevens, 1923 and 1925a; Wright, 1925a; Ohkawara and Yoshida, 1925 (see Jimbo, 1930); Israilsky, 1929; and Jimbo, 1930. The work of Stevens, 1923, is the most exten-

sive on this subject, and the following Table 12 illustrating the behavior of various strains of *Rhizobium japonicum* is typical of the results secured with other species.

Of the eight strains of *Rh. japonicum* studied, four definitely belong in one serological group and two in another, while the two remaining strains are somewhat related, but far from identical.

TABLE 12

The grouping of strains of Rhizobium japonicum by means of immune sera

Antigens	Antisera and titers							
	161	164	166	168	163	162	160	165
161	1:5000	1:2000	1:1000	1:7500	0	0	0	0
164	1:2000	1:2000	1:1000	1:2000	0	0	0	0
166	1:2000	1:2000	1:1000	1:7500	0	0	0	0
168	0	1:2000	1:2000	1:7500	0	0	0	0
163	0	0	0	0	1:20000	1:5000	0	0
162	0	0	0	0	1:10000	1:5000	0	0
160	0	0	0	0	0	0	1:20000	1:100
165	0	0	0	0	0	0	1:50	1:5000

Fifty-five strains of rhizobia, representing seven cross-inoculation groups, were studied by Stevens, 1925a, and in each cross-inoculation group where more than one strain was studied, at least two serological groups were recognized. Four such subgroups were found with *Rh. trifolii, Rh. leguminosarum,* and *Rh. japonicum.* With *Rh. meliloti, Rh. phaseoli,* and the *Rhizobium* from cowpea, two serological subgroups were established. Only one strain of *Rh. lupini* was studied.

Agglutination of the rhizobia with the homologous serum often occurs in dilutions as high as 1:20,000, in spite of the interference of the heavy gum produced by the organism on artificial media. Differences between the various serological groups are usually wide and unmistakable. The serological properties also seem to be quite constant regardless of the medium upon which the rhizobia are cultivated; they are also stable after passage of the culture through an appropriate host plant, Wright, 1925a.

Although the serological reactions have only a limited value in species identification, they are of the utmost value in strain identification and characterization. Such reactions have made possible demonstration of the simultaneous presence of different strains of the organisms in the various nodules of a single host plant, Bialosuknia and Klott, 1923, Ohkawara and Yoshida, 1925, and Jimbo, 1930. Dunham and Baldwin, 1931, have used this reaction to advantage as one of the criteria of strain identification in their studies of the resistance of the host plant and of the infective power of various strains of the rhizobia.

Nomenclature. The question of the nomenclature for the bacteria causing the formation of root nodules on the Leguminosae has occasioned much discussion. Scientific workers have not agreed either as to the number of species which should be recognized or the terminology which should be applied to them. Consequently, both American and foreign investigators are at present using several different names. To avoid confusion, many investigators have abandoned the use of scientific terminology and have substituted such terms as "root nodule bacteria" of

leguminous plants or, when more specific designations are desired, "alfalfa nodule bacteria" or "soybean nodule bacteria." At best this can only be regarded as a temporary expedient and not as a solution of the problem.

Most of the early students in this field were of the opinion that all of the organisms producing nodules on the roots of leguminous plants should be considered as a single species, although some investigators did recognize that there are certain important differences among the bacteria from the different species of plants. Frank, in 1877, was the first to carefully study the root nodules from the standpoint of the causative agent and to apply a name to the responsible organism. In this early paper he tentatively placed the nodule organism in the species, *Schinzia cellulicola.* The ascription of the organism to the genus *Schinzia* was made because of its fancied resemblance to *Schinzia alni,* a fungus found in the root nodules of the alder. In 1879 he worked with nodules from *Lathyrus, Orobus, Lupinus,* and *Genista.* While he noted certain differences between *Lupinus* and *Lathyrus* as to the type of nodule and the organisms contained therein, he still felt that all of the bacteria in the root nodules of leguminous plants belonged to a single species and applied the name *Schinzia leguminosarum.* Although Frank did not secure pure cultures of the nodule organism, an examination of his descriptions and figures can leave little doubt that he actually saw the causative agent of the leguminous root nodules.

Schroeter, 1886, in Cohn's *Kryptogamen Flora,* proposed the new generic name *Phytomyxa,* and also created a new family Phytomyxacei and a new order Phytomyxini. He felt the ascription to the genus *Schinzia* to be in error and that the organisms of the leguminous root nodules are closely allied to the slime molds. He also differed from Frank in that he proposed two species instead of one, *Phytomyxa leguminosarum* and *Phytomyxa lupini.* The first species he described as occurring in the root nodules of *Trifolium repens, Lotus corniculatus,* and *Orobus vernus*; while *Phytomyxa lupini* was described as in *Lupinus luteus* and *L. angustifolius.* The separation into two species was based largely on the lack of infection threads in the lupine nodule.

Beijerinck, 1888, who first secured the organism in pure culture, gave it the name *Bacillus radicicola.* Previously the organism responsible for the formation of root nodules on the Leguminosae had been classified with the higher fungi. Beijerinck was the first to definitely place the organisms with the true bacteria, although Woronin, 1866, and others, had stated that the nodules contained bacteria-like bodies. Beijerinck recognized that not all of the organisms are identical and listed seven varieties of *Bacillus radicicola, i.e. Fabae, Vicia hirsutae, Trifoliorum, Pisi, Lathyri, Lupini,* and *Cytisi,* and also mentioned *"Phaseolus* and *Robinia* types." Later, in 1890, he reported the differences between the root nodule bacteria of leguminous plants to be greater than he had earlier supposed, and that *Bacillus Ornithopi,* from serradella, should be recognized as distinctly different from *Bacillus Fabae,* from *Vicia Faba.* This is apparently his first use of the species designations *Bacillus Fabae* and *Bacillus Ornithopi.* In earlier papers and even in the same paper he referred to the organism from *Vicia Faba* as *Bacillus radicicola var. Fabae.*

Beijerinck was evidently aware of Frank's earlier paper, as he called attention to Frank's statement that nodules do not develop in sterile soil. He made no

statement of his reasons for discarding Frank's name, *Schinzia leguminosarum.* It is probable, however, that he discarded both the species and genus names on the grounds that Frank had ascribed the organisms to an incorrect genus. This, of course, is not a valid reason for discarding the species designation *leguminosarum.*

Vuillemin, 1888, reviewed the characteristics of the root nodule organism and decided that Frank had been in error in placing those organisms in the genus *Schinzia.* He made no mention of Schroeter's names, *Phytomyxa leguminosarum* and *Phytomyxa lupini.* He himself proposed a new designation *Cladochytrium tuberculorum.*

Prazmowski, in 1890, following de Bary's and Hüppe's characterization of the terms *Bacillus* and *Bacterium,* decided that Beijerinck was in error in calling the organism *Bacillus.* He found the organism to be a short non-sporulating rod and called it *Bacterium radicicola.*

Frank, in 1889 and 1890b, recognized his error in placing the organism in the genus *Schinzia* and proposed a new genus *Rhizobium.* The earlier species designation *leguminosarum* was retained.

Laurent, in 1890, published a preliminary paper, followed in 1891 by a more complete discussion, in which he accepted Frank's name *Rhizobium leguminosarum.* In his judgment, however, the organism is not a true bacterium, but is related to Metchnikov's *Pasteuria ramosa* and therefore one of the Pasteuriaceae.

Schneider, in 1892, described and named five species, with two varieties of one.

Rhizobium mutabile from { *Trifolium pratense,* *Trifolium repens,* *Melilotus alba,* *Lathyrus odoratus*

Rhizobium curvum from *Phaseolus pauciflorus*

Rhizobium Frankii var. *majus* from *Phaseolus vulgaris*

Rhizobium Frankii var. *minus* from *Pisum sativum*

Rhizobium nodosum from { *Dalea alopecuroides* *Robinia Pseudacacia* *Cassia Chamaecrista*

Rhizobium dubium from *Amphicarpaea comosa*

The generic name *Rhizobium,* which Frank had proposed, was retained. He made no mention, however, of any of the earlier species or variety designations. He characterized the organisms entirely on the basis of the morphology of the organisms in the nodules. There is, however, some doubt whether his descriptions were in all cases based on the organism responsible for nodule formation. In a later paper, 1894b, he expanded his earlier descriptions of these organisms to include cultural and additional morphological characteristics, and added a new species, *Rhizobium sphaeroides,* from *Pisum sativum.* In several cases he reported the presence of two of his species in the same nodule. For the most part his descriptions of the organisms studied do not agree with our present knowledge. Spores were found in all of the species and in several of them two or three spores

in a cell. It is probable that the tendency of these organisms to exhibit granules upon staining was confused with spore formation.

Gonnermann, in 1894, cultured several organisms from the root nodules of leguminous plants, principally lupines. These he classified into two species on the basis of morphology, and established several varieties of each. Discarding all of the earlier nomenclature, he named his organisms *Bacillus tuberigenus* and *Micrococcus tuberigenus,* designating the varieties by numerals. It is probable that he was not working with the true nodule organism.

Kirchner, in 1895, studied the organism from soybean nodules and concluded that this organism is distinct from the others. The name *Rhizobacterium japonicum* was applied to it.

Mazé, in 1899, used the term *Rhyzobium Pasteurianum* Laurent, although in the papers of Laurent, which the authors have examined, no such term is to be found. He did state in his 1891 paper, however, that in his opinion the group *Rhizobium* was to be identified with the *Pasteuria* of Metchnikov.

Chester, in 1901, placed the nodule bacteria in his group, *Mycobacterium,* but gave no specific names.

Hiltner and Störmer, in 1903a, gave the name *Rhizobium radicicola* to a group comprising the bacteria of the root nodules of *Pisum, Vicia, Lathyrus, Phaseolus, Trifolium, Medicago, Anthyllis, Onobrychis,* and *Robinia. Rhizobium Beijerinckii* was applied to the organisms causing nodule formation on *Lupinus, Ornithopus,* and *Soja,* with *Genista,* and *Sarothamnus* tentatively placed in the same group.

Moore, in 1905, accepted Beijerinck's specific designation *radicicola,* but, because of his belief that the organism has only a single flagellum transferred it to the genus *Pseudomonas.*

Orla-Jensen, in 1909, suggested the generic term *Rhizomonas* for the organisms of the root nodules of the leguminous plants.

Winslow, *et al.,* in 1917, in their studies of bacterial classification and nomenclature placed the nodule bacteria of the Leguminosae in the genus *Rhizobium* and established *Rhizobium leguminosarum* as the type species. The genus *Rhizobium,* in turn, is placed in the family Nitrobacteriaceae. Löhnis and Hansen, in 1921, criticized the stand of Winslow, *et al.* in placing the organisms in close relation to the nitrifying bacteria and far removed from *Bacillus aerogenes, Bacillus coli,* and *Bacillus radiobacter.* They separated the nodule bacteria into two groups on the basis of flagellation and cultural characters. They suggested the possibility of dividing the organisms into two species, *Bacillus radicicola* and *Pseudomonas japonica* or *Bacterium japonicum.* However, they did not believe this wise and followed the plan of including all the root nodule organisms under the term *Bacillus radicicola.*

Shunk, in 1921, published the results of his studies on the flagellation of the root nodule bacteria of 41 kinds of leguminous plants. Two distinct types of organisms were found with respect to flagellation, a monotrichous type and a peritrichous type. Following Migula's classification, he suggested that they belong to two genera, *Pseudomonas* and *Bacillus.* While Shunk himself did not give any species designations, many American writers accepted his classification into the two genera and used the terms *Bacillus radicicola* for the peritrichous forms and *Pseudomonas radicicola* for the monotrichous forms.

Bergey in the first edition of his *Manual of Determinative Bacteriology,* 1923, placed the root nodule bacteria in the genus *Rhizobium* Frank under the tribe, Azotobactereae, and the family, Nitrobacteriaceae. Two species were established, *Rhizobium leguminosarum* Frank and *Rhizobium radicicolum* Beijerinck. The first was said to occur in the nodules of *Lathyrus, Pisum, Vicia,* etc., while the second occurred in the nodules of Trifoliaceae, Hedyereae, Viceae, and Phaseoleae. Many errors in descriptions are apparent. These were corrected in the second edition, 1925, in which *Rhizobium leguminosarum* Frank was listed as the type species, occurring in the nodules of *Lathyrus, Pisum,* and *Vicia. Rhizobium radicicola* Beijerinck was listed as a monotrichous form, from the nodules of *Lupinus, Soja,* and *Ornithopus.* In the third edition, 1930, the scheme of classification proposed by Baldwin and Fred, 1929b, and discussed later in this chapter, was followed, except that *Rhizobium radicicolum* (Beij.) Bergey *et al.* was included and was made the designation for the organisms of *Lupus, Soja,* and *Ornithopus.* This introduces a conflict since the species designation *Rh. japonicum* had been applied by Baldwin and Fred to the nodule organisms of *Soja Max.*

Dangeard, in 1926, proposed the formation of a new tribe, Hyphoideae, in the Bacteriaceae to include the bacteria which form the root nodules of the Leguminosae. This name was chosen because of the resemblance of the "infection threads" to the hyphae of certain fungi, as pointed out by Vuillemin, in 1905, who suggested that these "infection threads" be called "hyphoidees." In this tribe, Dangeard placed the genus *Rhizobium* and established ten species within the genus; *Rh. Trifolii, Rh. polymorphum, Rh. Fabae, Rh. Meliloti, Rh. Loti, Rh. simplex, Rh. torulosum, Rh. Phaseoli, Rh. minimum, Rh. Sojae.* Dangeard's personal studies were largely cytological. In making his classification into species, however, he considered the work of others on serological reactions and cross inoculation. Unfortunately Dangeard failed to observe the laws of priority in forming his species designations, and consequently many of them are invalid.

Buchanan, 1926, presented a resumé of the synonymy of these organisms and concluded:

1. The bacteria of leguminous plants are sufficiently distinctive in morphology, physiology, cultural characters, and habitat, to justify their separation from other bacteria into a distinct genus.

2. The resemblances among all types of bacteria producing the nodules of leguminous plants is so great as to justify the inclusion, for the present at least, of all these organisms within a single genus.

3. Two names, *Phytomyxa* and *Rhizobium,* are available for this genus. It is believed that the name *Rhizobium* is to be preferred.

4. If a single species of nodule bacteria is to be recognized, it should be termed *Rhizobium leguminosarum* Frank.

5. If the peritrichous forms are to be separated from the monotrichous as different species, the former should bear the specific name, *Rhizobium leguminosarum* Frank, and the latter *Rhizobium japonicum* Kirchner.

6. If the various cross-inoculation groups are to be recognized as va-

rieties, they should bear the varietal names proposed by Beijerinck in so far as these are appropriate.

7. If the cross-inoculation groups are raised to the rank of species, it will be necessary to designate as the type of *Rhizobium leguminosarum* Frank the organisms from *Lathyrus,* and of *Rhizobium japonicum* Kirchner the organisms from *Soja.* The remaining varietal names could then be made specific epithets.

Baldwin and Fred, in 1929b, reviewed the synonymy of this group with particular reference to species designations. The desirability of recognizing more than one species was discussed, and it was concluded that there were sufficient data available on the morphological, cultural, and physiological characters to justify the formation of five bacterial species. The ability of the organism to cause the formation of nodules upon the roots of certain species of the Leguminosae and not upon others, was the principal characteristic chosen in deciding upon the species boundaries. To some extent, morphological, physiological, and cultural characters were considered, however. The species so far proposed are as follows:

1. *Rhizobium leguminosarum* Frank

The organism causing the formation of nodules upon the roots of *Lathyrus, Pisum, Vicia,* and *Lens.*

(a) Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, and white. Consistency is slimy and occasionally viscous. Considerable gum is formed.

(b) Fermentation of carbohydrates. Slight acid production from glucose, galactose, mannose, lactose, and maltose.

(c) Morphology. Peritrichous flagellation. Bacteroids from nodules are commonly irregular with many X, Y, star- and club-shaped forms. Vacuolated forms predominate.

The species designation *leguminosarum* was proposed by Frank in 1879, when he erroneously placed the nodule-forming organisms in the genus *Schinzia.* The greater portion of his work, as judged by his figures, was done with *Lathyrus* and *Orobus,* and *Orobus* is now placed in the genus *Lathyrus.* Frank recognized only the one species. If new species are to be established, the old species designation *leguminosarum* must be retained, and it would seem appropriate to apply it to the species including the organism causing the root nodules on *Lathyrus.*

2. *Rhizobium trifolii* Dangeard

The organisms causing the formation of nodules upon the roots of *Trifolium* spp.

(a) Growth on mannitol agar is rapid. The colonies are white, becoming turbid as they grow older. Often the cultures become so mucilaginous that long threads may be drawn when the growth is touched with a needle. Streak cultures at first show a transparent growth along the

line of inoculation. Later this growth becomes mucilaginous and flows down the inclined surface of the agar, accumulating as a slimy mass at the bottom. Produces large amounts of gum.

(b) Fermentation of carbohydrates. Slight acid production from glucose, galactose, mannose, lactose, and maltose; usually slightly greater than *Rhizobium leguminosarum.*

(c) Morphology. Peritrichous flagellation. Bacteroids from nodules are pear-shaped, swollen, and vacuolated. Rarely X and Y-shaped forms.

Beijerinck, in 1888, proposed *trifoliorum* as a varietal name for the organism isolated from *Trifolium.* Dangeard, in 1926, used the species term *trifolii.*

3. *Rhizobium phaseoli* Dangeard

Causes the formation of nodules on *Phaseolus vulgaris, Ph. angustifolia,* and *Ph. multiflorus.*

(a) Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, and white. Consistency slimy and occasionally sticky, but not so marked as in *Rh. trifolii.*

(b) Fermentation of carbohydrates. Very slight acid fermentation of glucose, galactose, mannose, sucrose, and lactose.

(c) Morphology. Peritrichous flagellation. Bacteroids from nodules are usually rods with few branched forms. They are usually smaller than in *Rh. leguminosarum* and *Rh. trifolii* and are often vacuolated.

Beijerinck, in 1888, was able to distinguish the organisms of *Phaseolus* nodules from others and applied the term *Bacillus radicicola—Phaseolus* type. He did not, however, give it a definite varietal name. Schneider, in 1892, was the next to propose a specific designation for this organism. He proposed the term *Rh. Frankii* var. *majus* for the organisms symbiotic with *Phaseolus vulgaris. Rh. Frankii* var. *minus* was proposed for those symbiotic with *Pisum sativum.* Schneider's terminology is invalid from two standpoints. He failed to utilize any of the earlier specific and varietal names. His characterizations of the organisms are so meager and conflicting that it is impossible to tell with certainty with which organisms he worked. Dangeard, in 1926, used the term *Rh. phaseoli.*

4. *Rh. meliloti* Dangeard

The organism causing the formation of root nodules upon *Melilotus, Medicago,* and *Trigonella.*

(a) Growth on mannitol agar is fairly rapid but not as fast as that of *Rh. leguminosarum, Rh. trifolii, and Rh. phaseoli.* Growth is moderate to abundant. The streak is raised, glistening, opaque, and pearly white. Consistency is buttery with considerable gum, but usually not viscous.

(b) Fermentation of carbohydrates. Strong acid production from glucose, galactose, mannose, and sucrose.

(c) Morphology. Peritrichous flagellation. Bacteroids from nodules include club-shaped and branched forms.

Dangeard first proposed this name in 1926, as applied to the organism symbiotic with members of the alfalfa-sweet clover cross-inoculation group. Schneider in 1892 proposed the name *Rh. mutabile* for the organisms symbiotic with *Trifolium pratense, T. repens, Melilotus alba,* and *Lathyrus odoratus.* Schneider's terminology is invalid because of his failure to observe the rules of priority and because of incomplete descriptions.

5. *Rh. japonicum* (Kirchner) comb. nov.

The organism causing the formation of root nodules on *Soja Max.*

(a) Growth on mannitol agar is slow and scant under ordinary conditions. Streak is slightly raised, glistening, opaque, and white. Consistency is buttery with little gum formation. Pentose sugars give better growth than the hexoses.

(b) Fermentation of carbohydrates. Little if any acid formation. After prolonged incubation acid from xylose and arabinose.

(c) Morphology. Monotrichous flagellation. Bacteroids of nodules are long, slender rods with only occasional branched and swollen forms.

This specific designation for the organism of the soybean root nodules was first proposed by Kirchner in 1895.

The five species described above compose the list which was proposed in 1929. Specific designations for the organisms from the nodules of other leguminous plants were not then proposed, because of insufficient study and lack of definite information. Since that time a special study of the lupine nodule bacteria has been made by Eckhardt, Baldwin, and Fred, 1931. From their description and from data presented by Müller and Stapp, 1925, the following species designation is suggested:

6. *Rh. lupini* (Schroeter) comb. nov.

(a) Growth on yeast-water mannitol agar is scant to moderate with alkaline reaction; on litmus milk there is no serum zone, no reduction of litmus, and a slightly alkaline reaction; meager growth on potato and parsnip slants and carrot agar. Growth on beef-peptone gelatin with extremely slow liquefaction; sensitivity to crystal violet varies among the different strains.

(b) Fermentation of carbohydrates. On galactose an alkaline reaction serves to differentiate *Rh. lupini* from all the fast-growing rhizobia (*Rh. phaseoli, Rh. meliloti, Rh. trifolii,* and *Rh. leguminosarum*). An initial alkaline reaction followed more quickly by an acid reaction on rhamnose and xylose separates *Rh. lupini* from the slow-growing *Rh.*

japonicum and the *Rhizobium* from cowpea. In general, *Rh. lupini* produces slight to moderate acidity on pentose sugars and no change or alkaline reaction on hexoses, disaccharides, and trisaccharides.

(c) Morphology. Flagella 1-4, usually 2 or 3, according to Müller and Stapp; it will be remembered from the section on flagellation, Chapter 5, that the lupine bacteria are among those in dispute. Bacteroids of *Rh. lupini* are vacuolate rods, seldom if ever branched. Dangeard, 1926, and Milovidov, 1926, would not consider them bacteroids on this ground.

CHAPTER 9

THE FORMATION OF NODULES, THEIR HISTOLOGY AND CYTOLOGY

"Be sure of it; give me the ocular proof."
—SHAKESPEARE

It is best to begin this chapter with a frank admission that, in spite of an enormous amount of observation and investigation of the root nodules of leguminous plants, we do not yet fully understand the complex structure and function of nodules. Milovidov in 1925 voiced this opinion when he said, ". . . more thorough studies in this direction are indispensable for we are far from having a very clear idea . . . of the entire and exact history of nodule development in the various leguminous plants, of the differences in manner and process of infection, of the stages in the metamorphosis of the bacteria within the nodule." His is perhaps a pessimistic view of the progress to date, for it must be admitted that a great deal of the normal story of nodule formation has been determined. But it will be evident in the pages which follow that many details of nodule histology and cytology, as well as of the physiological relation between the bacteria and their hosts, remain to be settled.

Many of the earlier descriptions of nodules were based upon direct observation of fresh material in free-hand section. Even by 1900a Miss Dawson held that free-hand sections are to be preferred to the regularly imbedded material sectioned by the microtome. For rapid examination and perhaps for rough work on certain phases of morphology, free-hand methods are feasible, but certainly for detailed cytological studies of nodule formation and structure, the best of modern methods should be applied. Each investigator has his own preference in the matter of fixatives and stains and recommends different methods for different purposes. The following procedure is representative and may be used satisfactorily for general work:

Fixation in Flemming's solution (medium)

Dehydration and *hardening* in 15, 30, 50, 70, 80, 95, and 100 per cent alcohol

Clearing in alcohol-chloroform mixtures (⅔ alcohol plus ⅓ chloroform; ⅓ alcohol plus ⅔ chloroform) and pure chloroform

Infiltration in chloroform-paraffin mixture, 45° paraffin, and two changes of 52° paraffin

Imbedding in 52° paraffin

Sectioning by microtome to 3-8 micra thickness

Staining. The method will depend upon the particular structures to be demonstrated.

a. Flemming's triple stain of safranin, crystal violet, and orange G is valuable for general nodule structure, vascular elements, starch grains, and gross outline of the infection threads (contents not differentiated). The orange G is often omitted to prevent too much loss of color from the bacteroids.

b. Heidenhain's iron alum-haematoxylin with counterstain of light green or erythrosin shows the details of the bacteroids and contents of the infection threads. It is also excellent for the general cytology of the plant cells.

c. Cowdry's differential stain of acid fuchsin and methyl green is suitable for the differentiation of mitochondria and bacteria.

The usual process of nodule formation as described in the literature may be summarized as follows:

MODE OF INFECTION

Ward in 1887 proved nodules to be not "natural" structures of the leguminous plants, but the result of infection. He made the first experimental infections by applying crushed nodules of the broad bean and pea to the uninfected roots of the respective plants. He even described something of the mode of infection: the appearance of a bright refractive spot on the tip or side of a root hair, the curvature of the hair at this point, and the growth of an infection filament from the bright spot down the root hair. These facts of infection have been abundantly confirmed by later work, and other details have been added.

Frank, 1890b, stated that the roots of the Leguminosae secrete a peculiar substance which attracts the "spores of the infective fungus." This would imply motility on the part of the rhizobia, if they are to exhibit chemotactic response. It is, of course, true that the bacteria are motile at certain stages in their life history. (See Chapter 5). But is there definite "seeking" of root hairs by the bacteria? Most observers would say there is not, because the ability of the bacteria to spread through soil seems decidedly limited. Nobbe, Schmid, Hiltner, and Hotter in 1892a concluded that the rhizobia do not move readily through soil, even though aided to some extent by water currents. Otis, 1898, speaking of the spread of soybean bacteria in soil, asserted that the bacteria of themselves spread very slowly. Water currents are apparently not effective, and nothing short of mechanical mixing of the soil can bring about rapid spread. Rhodin, 1914, reported similar findings.

The actual rate of movement of the rhizobia has been studied by a number of investigators: Kellerman and Fawcett, 1907; Ball, 1909; Kalantarov, 1914; Frazier and Fred, 1922; and Thornton and Gangulee, 1924 and 1926. Kellerman and Fawcett observed that in moist soil at 25°C. the rhizobia move in a horizontal

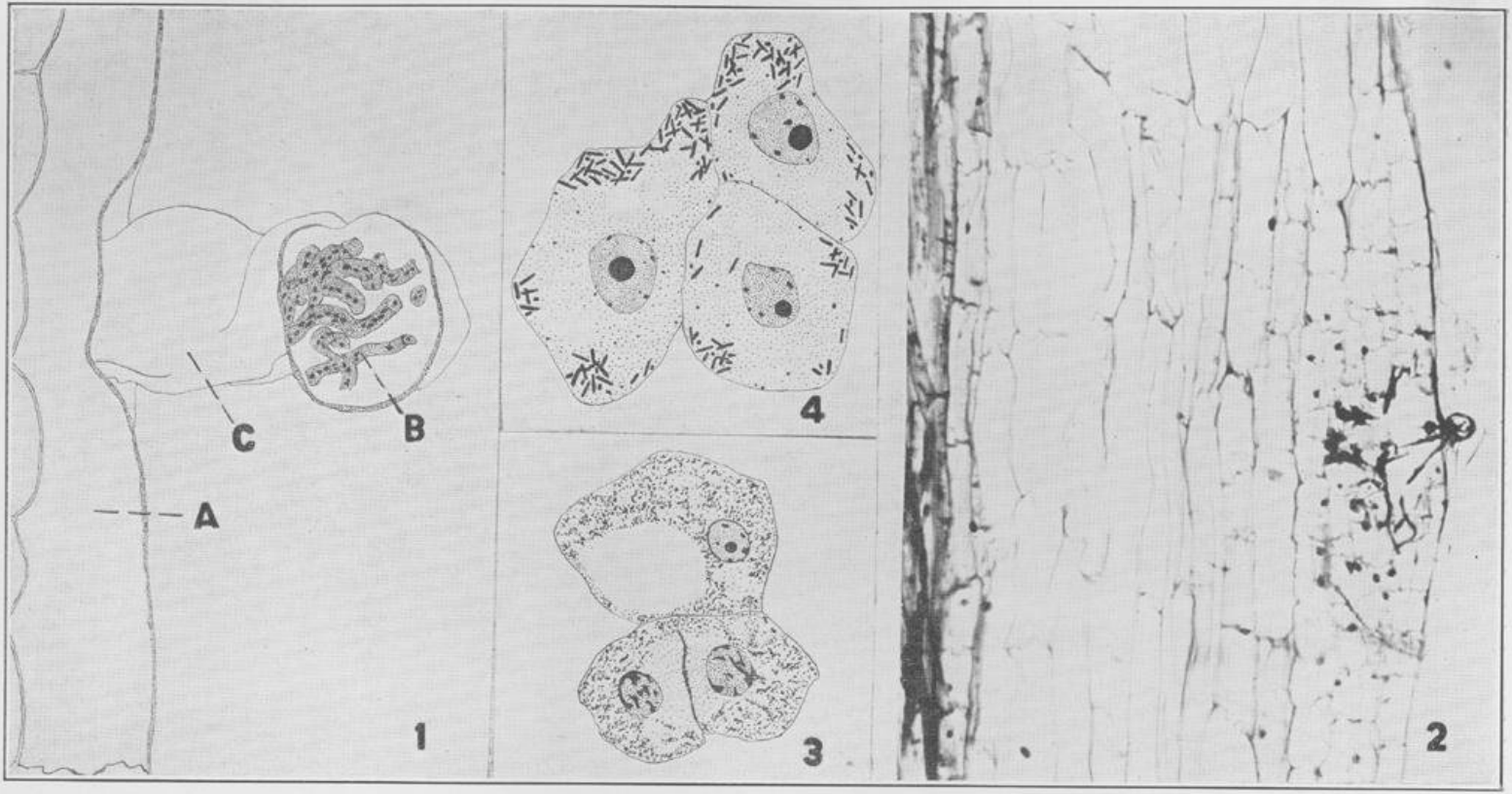
direction at the rate of 2.5 cm. in 48 hours. In barely moist soil, or at a lower temperature, 10°C., the rate is about 2.5 cm. in 72 hours. Ball likewise found the rate of spread in moist soil to be 2.5 cm. in 48 hours, but he considered that the displacement was actually due to the combined effect of vital movement, multiplication of the bacteria, and soil-water currents. Kalantarov gave the rate of spread in sterilized soil as 0.52 cm. in 24 hours, and Thornton and Gangulee placed it at 2.5 cm. in 24 hours. Under field conditions the spread is very slow indeed. Frazier and Fred, 1922, observed no infection of uninoculated control plants of soybean, although they were raised under field conditions and only 3 feet from well nodulated plants. Neither cultivation, dust, nor rain during the entire season sufficed to spread infection in their experiment. In view of the negligible spread of the bacteria by their own vital movement, a number of investigators, Peirce, 1902; Fred, 1911a; Burrill and Hansen, 1917, have considered that chance contact of the bacteria with the numerous root hairs of the plant is sufficient to account for infection.

The exact mechanism of the entrance of the bacteria is still unknown. It is supposed that the cell wall of the root hair is softened or dissolved at the point of the bright spot, and that the bacteria enter there. Giltner in 1915 asserted that the wall is actually dissolved by "the feeble action of an enzyme, cytase." Doubtless his statement referred to Hiltner's report, 1900a, that a filtrate of a culture, passed through a Chamberland filter, exerts a softening effect upon root hairs and causes typical curvature of them. Beijerinck, however, had reported in 1888 that cellulose-dissolving enzymes were absent from his cultures. No recent work has been found on this subject. Viermann, 1929, in a paper on the lupine nodule and its development, held that infection occurs through the amyloid spots at the tips of very young root hairs.

Besides infection through intact root hairs, there may conceivably be infection through epidermal cells of young root tips. This type of infection may occur in agar or liquid cultures in the laboratory where root-hair development is limited, or where inoculation is made by stroking the culture onto roots already one or two inches long. It has been suggested that in the soil infection always takes place through small wounds, as it certainly may experimentally. Bréal, 1888b, made inoculations by deliberately pricking the roots of his seedlings with a needle dipped in a suspension of the bacteria. But wound infection is not probable in nature, for as Peirce has pointed out, the characteristic curvature of the root hair after infection indicates subsequent growth, unequal on opposite sides of the hair. Wounded hairs would not give such marked and regular response. Furthermore, the same type of curvature occurs under laboratory conditions, where wounds can play no part.

THE INFECTION THREAD

Soon after bacteria enter a root hair, an infection thread begins to form and to grow toward the base of the root hair. It is ordinarily a single strand, peculiarly refractile and glassy, but in certain cases, as in Fig. 1 of Plate 22 illustrating infection of the *Phaseolus* bean, the infection thread may branch and become multiple even before leaving the root hair. According to Prazmowski, 1890, the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infection thread reaches the epidermal cell in about 2 days. From thence it penetrates the cortex of the root, growing more or less directly toward the central cylinder in such plants as alfalfa and clover. In the bur clover, which Peirce studied in 1902, the directness of the course is so marked as to suggest attraction by the host cells and "chemotropic growth of the strand or if the bacteria are motile in the cells, chemotactic movement of the bacteria". But in the *Phaseolus* bean, as illustrated in Fig. 2, there is no direct line of penetration, the branches of the infection thread spreading irregularly through the outer layers of cortical parenchyma. In its progress through the cortex, the infection thread may pass through or between host cells (Němec, 1915). Those strands within the cells are spoken of as intracellular infection threads and those between cells as intercellular zoogloea. In the nodules of various plant species there is a tendency toward one or the other type; most nodules have the intracellular type. In passing from cell to cell the intracellular infection threads must traverse cell walls. In doing so they spread out into characteristic funnel-shaped enlargements which have been pictured since the early descriptions by Eriksson, 1873, and Ward, 1887. Prazmowski, 1890, showed exaggerated expansion of the strand at the middle lamella of the cell wall and occasionally the escape of some bacteria into the intercellular spaces. Recently also, Milovidov, 1925, and Thornton, 1930a, have noted in old nodules the invasion of intercellular spaces by bacteria from the infection threads. These bacteria in the coccoid rod form attack the middle lamellae of the plant-cell walls, thereby causing disintegration of the tissues.

The nature of infection threads has long been debated. Early investigators like Eriksson, 1873; Ward, 1887; and Vuillemin, 1888, considered them to be actual fungal hyphae, but they differed in their conception of the rôle of these structures in nodule formation. Ward and others believed them to be the causal organism. Schneider, 1892, observed that infection threads were not always present and concluded that they had nothing to do with the smaller organisms which in his opinion, caused nodule formation. Frank, 1890b, although recognizing *Rhizobium leguminosarum,* a micrococcoid organism from the soil, as the infective agent, considered the filaments to be myxoplasm consisting of a homogeneous mixture of the protoplasm of the host cell and of that of the parasite. The bacteroids he thought to be fragments of the protoplasm of the parasite set free by the dissolution of the host's part of the myxoplasm.

Adherents of the fungal-hypha theory, of course, tried to establish structural and chemical similarity between the parasitic filaments and true fungal hyphae. Eriksson, 1873; Ward, 1887; Vuillemin, 1888; Koch, 1890; and Laurent, 1891, reported the presence of a cellulose membrane. Moeller, 1892b, and Schneider, 1893c, stated that a membrane is present but is deposited by the host cells to wall off the infection. Prazmowski, 1890; and Frank, 1890b, saw no membrane. Kny, 1879a; Prillieux, 1879 and 1890; Lundström, 1888; and Zukal, 1897, seeing no membrane and no definite boundaries, considered the threads plasmodia and the organism therefore as belonging to the Myxomycetes, Mycodomaces, or Myxobacteriae. By these investigators the bacteria in the cells were interpreted as the spores of the plasmodia, "buds" set free from the hypha-like strands or zoospores formed in special sporanges of the non-septate hyphae.

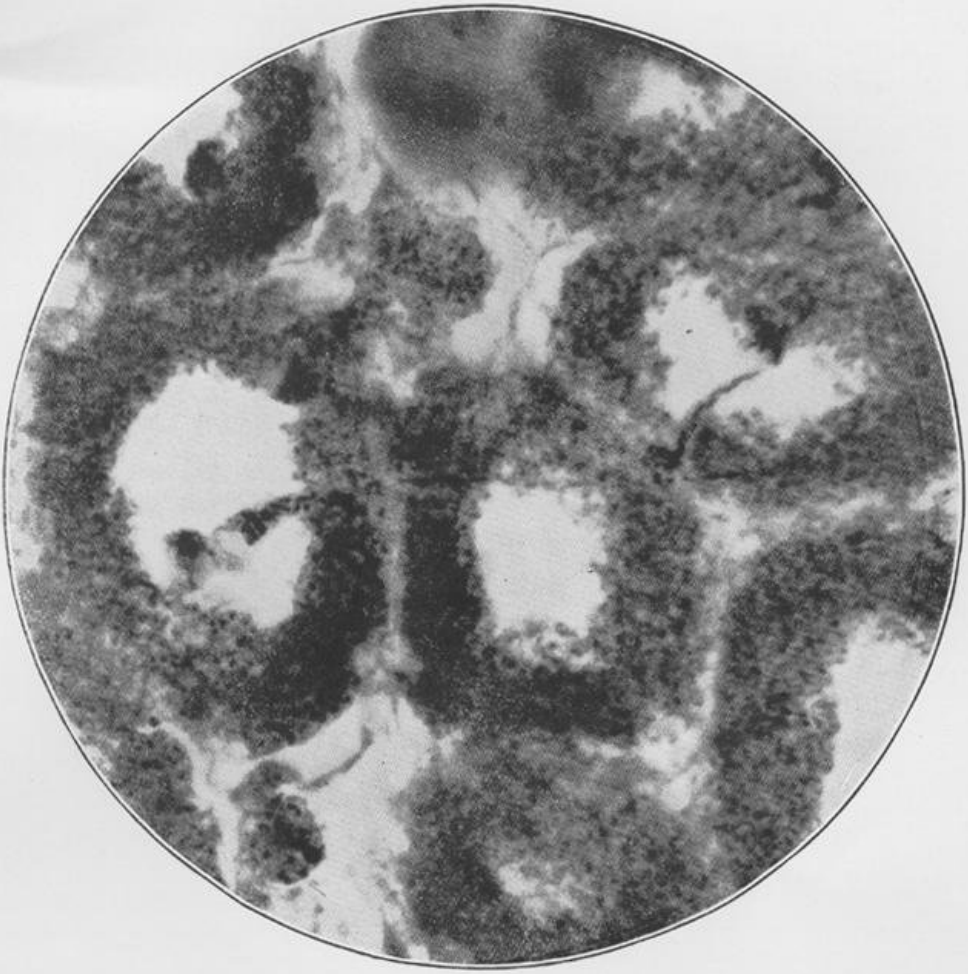
After Beijerinck's isolation of the bacteria, in 1888, and Prazmowski's re-

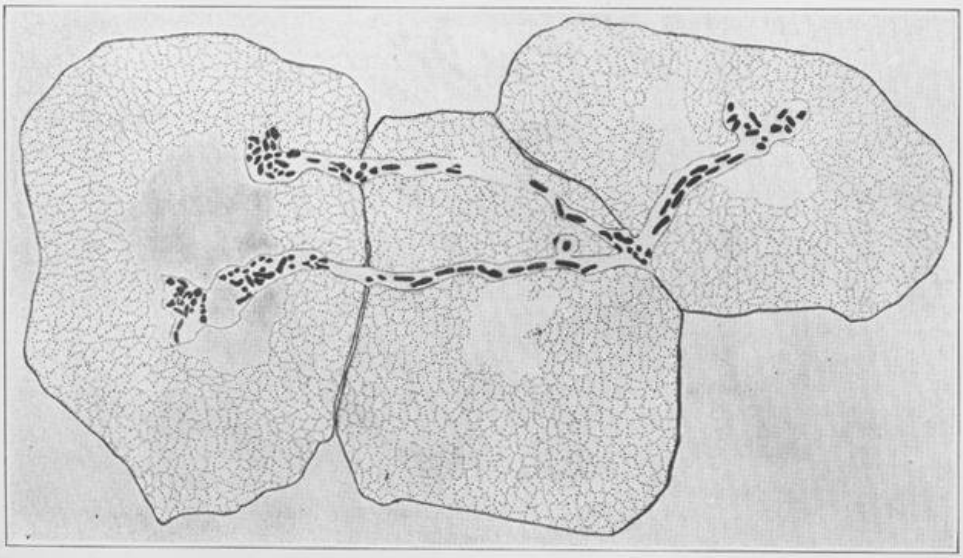
port, in 1890, of the story from the time of infection to the death and dissolution of the nodule, it was recognized that the filaments are not the infecting agents, but that they contain bacteria. There are then two conceptions of the form of the infection threads: they may be either zoogloeal strands imbedding the bacteria or tube-like sheaths containing them. Dawson, 1900a; Peirce, 1902; Fred, 1911a; and others have favored the zoogloeal strand theory, and it is now generally held. But recently Burrill and Hansen, 1917, have described it as "not a zoogloeal strand made up of small bacilli but a solid hypha-like structure bearing remarkable resemblance at times to a tube." This conception is strengthened by a recent report showing the sheath to consist of cellulose, hemicellulosic, and pectic material. Its composition, as well as mode of formation, thus proves it to be a plant product (McCoy, 1932).

THE FORMATION OF THE NODULE

As to the point of origin of the nodule there is little agreement, perhaps because of the differences in the penetration of the infection thread as reported in different plants. Yet the location of the primary nodule tissue is important in deciding whether or not the nodule is a modified lateral root, as so often stated in popular writings. Botanists agree that lateral roots arise in the pericycle. Van Tieghem and Douliot, 1888; Beijerinck, 1894; Paratore, 1899; Peirce, 1902; Němec, 1915; Whiting, 1915; and Terby, 1925, considered that nodules also originate by division of pericyclic cells, and that they usually develop opposite protoxylem points, as do lateral roots. On the other hand, Eriksson, 1873; Prillieux, 1879; Frank, 1879; Tschirch, 1887; Ward, 1887 and 1889; Prazmowski, 1888 and 1889; Atkinson, 1893; Schneider, 1893c; Burrill and Hansen, 1917; Spratt, 1919; and Brenchley and Thornton, 1925, were of the opinion that division and differentiation of cortical cells give rise to the nodule tissues. Most of these investigators specified that only the inner layers of the cortex or cells adjoining the endodermis are stimulated to division. In this respect also the *Phaseolus* bean must be cited as an exception; in it the primary cluster of infection threads penetrates only the outer layers of the cortex (See Fig. 2, Plate 22), and induces nodule formation by stimulation of the adjacent cortical cells. In any case, the evidence is against the nodule arising as a result of infection of a young lateral root. As Burrill and Hansen pointed out, the nodule differs from a lateral root in several respects. It has no central cylinder, no root cap, and no epidermis. Furthermore, it does not digest its way out from the cortex of the main root but remains covered with a considerable layer of cortical parenchyma.

The nodule possesses great complexity of tissues and a very high degree of specialization, facts which indicate a long-standing association between the bacteria and their hosts. At first the entire young nodule is meristematic in the sense that all of its cells are undifferentiated and capable of active cell division. But the infection thread soon branches and invades cells in all directions. Upon entering a cell, the branch of the infection thread may remain peripheral or may cross the middle of the cell. Usually it grows toward the nucleus. The infection thread soon liberates bacteria into the cytoplasm of the host cell. These bacteria, at first lying free and single in the host's cytoplasm (See Fig. 4, Plate 22), rapidly mul-





tiply and often fill the entire cell. The bacteria in the infection thread (Plate 23, Fig. 1 and 2) and those first set free in the cells are coccoid bodies or very short rods and may be stained uniformly. Out in the plant cytoplasm they soon become banded, then swollen, vacuolate, and often branched. This is the "bacteroid stage", so called by Brunchorst in 1885a. The tissue containing bacteroids is the "bacteroid tissue" or "bacteroid area." It is also called the "medulla" of the nodule by Dangeard, 1926. In an old nodule it constitutes the largest portion of the nodule tissue. A fully-developed bacteroid tissue would contain 100 to 1000 million bacteria, according to an estimate by Ewart and Thomson, 1912. Greig-Smith, 1907, asserted that a large proportion, perhaps 99.8 per cent, of the bacteria in the nodule are dead. Nevertheless, there may still be 1¼ million live bacteria in a nodule ⅛″ in diameter. The total number of cells in such a nodule is then 625 million, a figure well within the estimate of Ewart and Thomson.

The remaining meristem surrounds the bacteroid area in a round nodule and forms a cap over the growing end in one of the elongated type. Normally the cells of this region retain their power of division because of the bulwark of uninfected and recently-infected cells between them and the bacteroid tissue. Because the meristem does not extend across the basal region between the nodule and the central cylinder of the root which bears it, no secondary thickening is possible there. A constriction then appears at the base as the nodule grows in length and width (Peirce, 1902). The lupine nodule is an exception, there being infection for a considerable distance around the pericycle and consequently a general thickening of the root (Whiting, 1915). Outside the meristem is the region of the nodule cortex, a thin layer of flattened cells. Outside that is the root cortex, which is stretched and broken by the enlarging mass of the nodule tissue within. But the nodule tissue does not emerge from the root cortex as does a young lateral root. Dangeard, 1926, described two layers of the root cortex. The inner is capable of cell division and growth, and thus of surrounding the nodular mass, even though the latter may grow to be several millimeters in length and width. The outer layer of cortical cells, however, is dissociated and broken away.

As the young nodule develops, vascular bundles are differentiated in the nodule cortex and are connected with the vascular system of the host root. It is supposed that the vascular system serves for the transport of nutrients to the nodule cells and their bacteria and of nitrogenous substances (form unknown) from the bacteria to the host plant. The system is adequate for a time, but when the nodule grows large and further vascular connection is not possible through the constricted base of the nodule, degeneration and death of the nodule cells result (Peirce 1902). Vascular strands were seen by Treviranus, 1853; Woronin, 1866; and Eriksson, 1873, and were especially described by Vuillemin, 1888. Recently Brenchley and Thornton, 1925, have correlated the degree of development of the vascular system in the nodules of *Vicia Faba* with the functioning of the nodules on plants grown in the presence and absence of boron. In the presence of boron they found full normal development and functioning, and in its absence nodules with abortive or weak vascular systems and corresponding loss of function. It will be remembered that Warington, 1923, 1926; and Brenchley and Warington, 1927, showed that boron in small amount is essential for the normal growth of *Vicia Faba* and certain other plants. In the absence of boron both root and stem tips

show abnormality of the meristem with withering and blackening, poor development of vascular tissue with later discoloration and blocking of tracheids, abnormal formation or destruction of the cambium, and blackening and atrophy of the cortex. In the nodule cortex, as in many other parenchymatous tissues of plants, there may be calcium oxalate crystals (Wendel, 1918, and Spratt, 1919). Sometimes the nodule cortex may show remains of the primary infection thread (Němec, 1915); that in the bean appears as in Fig. 3, Plate 28.

The general structure of a nodule as discussed in the foregoing paragraphs is illustrated in Plate 24, a camera lucida drawing of a median section of a mature clover nodule.

TYPES OF NODULES

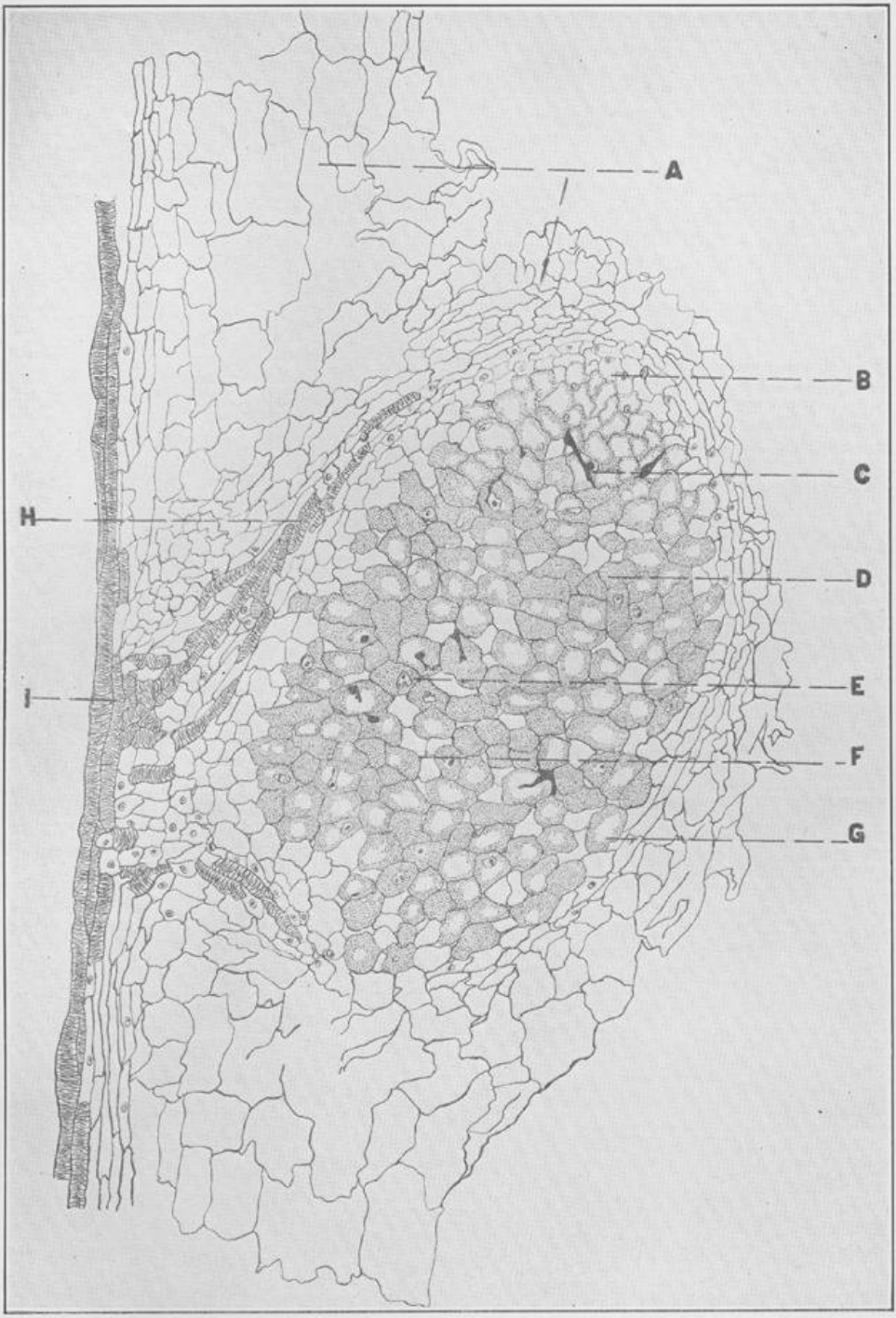
The tissues of nodules as described above are those present in the typical nodule, and the arrangement of tissues is that of the simple round or elongated form. Other types of nodules occur and have been variously classified. Perhaps the most extensive survey of nodule histology is that of Dangeard, 1926, and of his pupil, Lechtova-Trnka, 1931. The former dealt mainly with herbaceous Papilionoideae and the latter mainly with woody Papilionoideae, Caesalpinioideae, and Mimosoideae. Differences in details of structure are many and complex, and it will be possible to refer to the various types only in very general terms. According to gross structure, nodules are said to be simple or compound; round, elongated, or branched; single or clustered.

The size and location of nodules differ considerably in different kinds of plants. Perhaps the earliest recognition of these differences is seen in the illustration of Eriksson, 1873, reproduced in Plate 25. Tschirch, 1887, distinguished two types of nodules, that of the lupine and that of all other Leguminosae. The former involves a thickening of the central cylinder of the root, and the latter only an outgrowth of tissue between endodermis and epidermis. As an example of the second type, Tschirch cited the nodules of the locust, *Robinia*.

Miss Spratt in 1919 proposed a more intricate classification of nodules, in which internal structure is considered. The following types were recognized by her:

I. *Genista type*: spherical with spherical meristem outside the bacteroid tissue, which later becomes localized and thus acquires a very uneven surface and shape. Vascular supply forms one broad zone across the base of the nodule and subsequently branches, forming a varying number of strands. Bacteroid tissue in distinct areas with sterile tissue between. Large development of protective covering; large areas repeatedly split off and renewed as the nodule grows. Infection threads rare. Nuclei with nucleoles remain a long time. Found on woody plants with copious development of periderm, *i.e.*, trees and shrubs. Calcium oxalate crystals (styloids) present in the parenchymatous tissue of plant and nodule. Subgroupings of I:

1. *Lupinus, Ornithopus, Cytisus,* and *Desmodium* with primary vascular strand tetrarch, its four divisions correlated with four growing points.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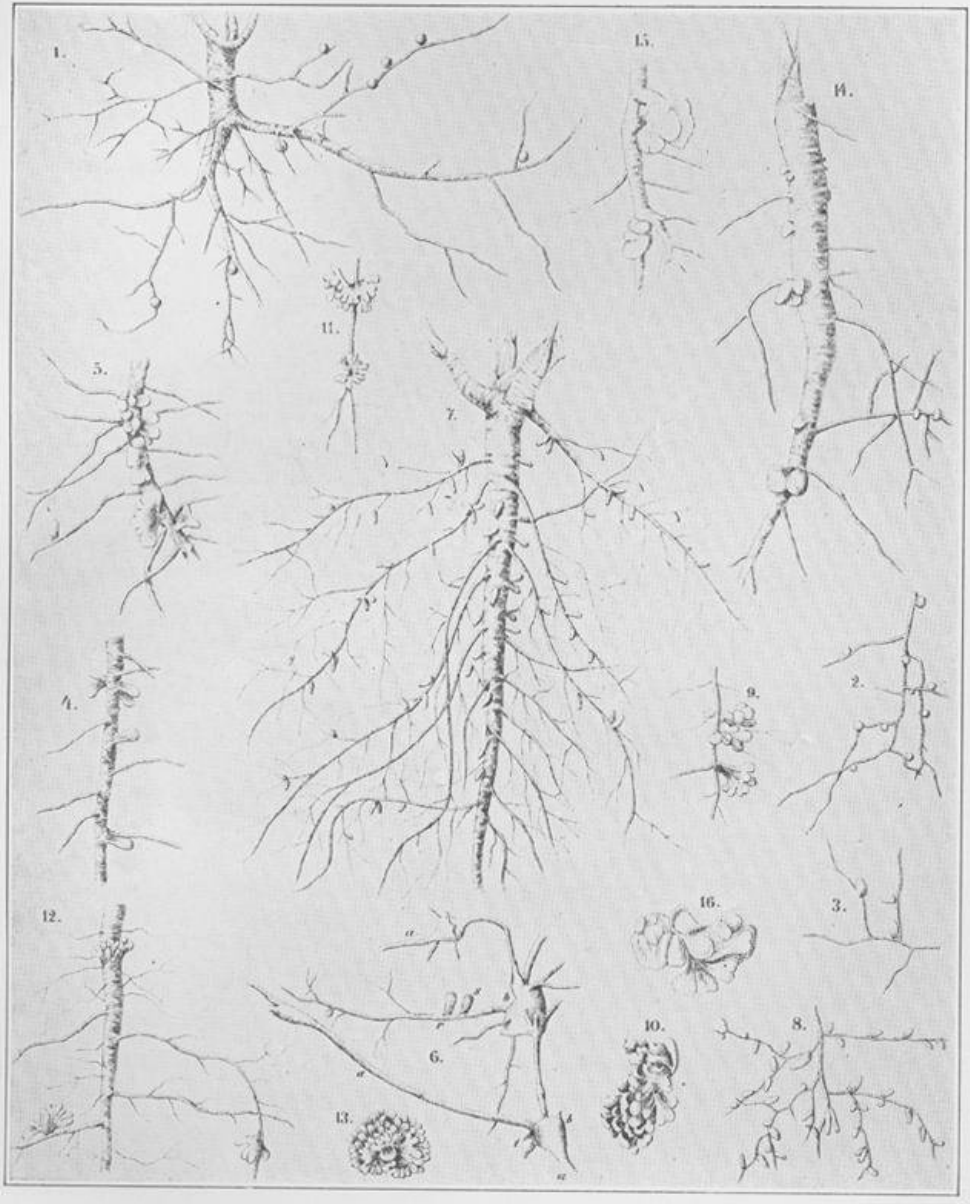
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2. *Genista, Ulex, Spartium,* and *Amorpha* with primary vascular strands diarch, each dividing in two and crossing some distance before dividing again. Bacteroid zones separated by narrow bands of parenchyma. Usually one apical meristem to each bacteroid zone.

II. [1]*Phaseolus and Trifoleae type*: similar to I, but with bacteroid zone always in undivided central zone. Growing point frequently localized apically; therefore, elongation and branching. One vascular strand. More infection threads in young nodules of *Phaseolus* and *Coronilla;* in all ages of nodules of *Trifolium.* Nuclei well-defined in young bacteroid tissue, later distorted and destroyed. Large central vacuoles. Styloids in outer tissue of *Phaseolus* nodules. Sub-grouping of II:

1. *Phaseolus, Coronilla,* and others with vascular strands divided at base below bacteroid tissue into four strands which function some time before branching.

2. *Ononis, Anthyllis,* and others with vascular strand divided at the base into two branches.

3. *Trifolium, Lotus,* and others with single strand which passes obliquely to one side of the nodule and remains undivided for a time. Later it branches, but at some distance from the base.

III. *Viceae type*: elongated with well-defined apical meristem and a basal intercalary zone which produces a small amount of tissue. Frequently branched and in clusters. One continuous bacteroid tissue; apical portions traversed by innumerable infection threads. In young nodules threads through whole tissue; later only in new areas. Nuclei at first large and prominent; later they lose spherical outline and become elongated, irregular, and separated into granular bodies; still later they are further dispersed. Two vascular strands on opposite sides each with separate attachment to the root stele.

Group III includes *Vicia, Pisum, Lathyrus, Galega, Stizolobium,* and *Coluta.*

IV. *Mimosoideae type*: Nodules of I, II, and III are annual; of IV are perennial. Nodules of *Robinia, Acacia,* and *Sophora* are included. Much protective covering; dark with tannin in *Robinia* and *Acacia,* not in *Sophora.* Two vascular strands with separate attachments to the root stele. In *Acacia* meristem all around the bacteroid area; activity renewed from time to time. Nodules bean-shaped. In *Sophora* and *Robinia* growing point is apical and the nodule elongated and transversely dented between periods of

[1]*Authors' footnote.* The nodules of *Phaseolus* and *Trifolium* to the casual observer would seem very different; the bean nodule is typically round and the clover nodule elongated, finger-shaped, and occasionally branched. The grouping as given by Miss Spratt is necessary for her classification, because they have in common an undivided bacteroid tissue and a single vascular strand from the root stele. In the sub-grouping Miss Spratt recognizes the difference in the branching of the vascular system but fails to point out the significant difference between the clover and bean types; namely, that in the clover the meristem is localized apically and gives rise to elongation, whereas in the bean it surrounds the bacteroid area and provides for equal growth in all dimensions.

growth. Constrictions consist of cortical cells, which in *Sophora* are crossed by anastomosing vascular strands and infection threads for the migration of the bacteria into new bacteroid areas. Cells, elongated in the long axis of the nodule, become multi-nucleate; later nuclei disappear. Many empty nodules.

Milovidov in two recent papers, 1926 and 1928d, on the cytology of the lupine nodule distinguished three types of nodules with respect to the mode of infection of the bacteroid tissue. To quote from his paper:

a. *Normal type in the majority of Leguminosae.* Infection is produced by aid of infection threads intruding progressively into the cells produced by the meristem.

b. *Type of Serradella.* There is intercellular zoogloea which plays the principal rôle in infection. Intracellular infection threads play only a limited rôle and normally soon disappear.

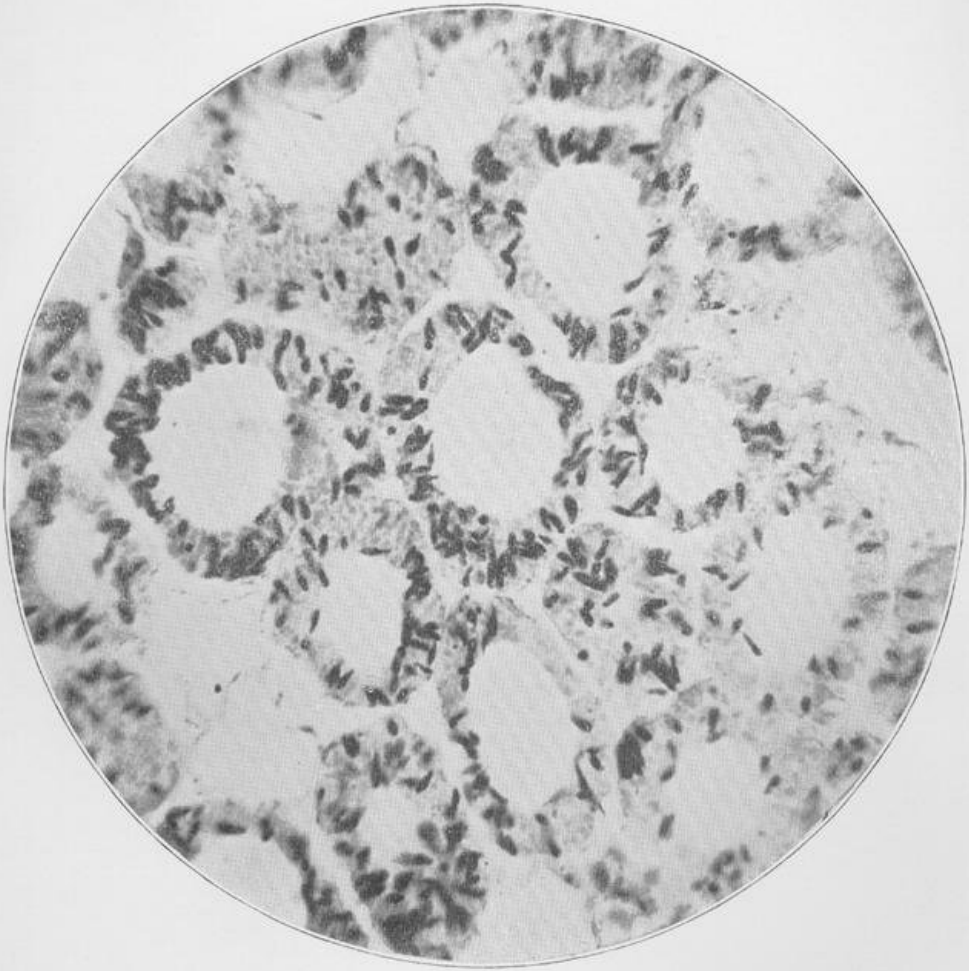
c. *Type of Lupinus.* The formation of bacteroid tissue results from the active division of the infected cells.

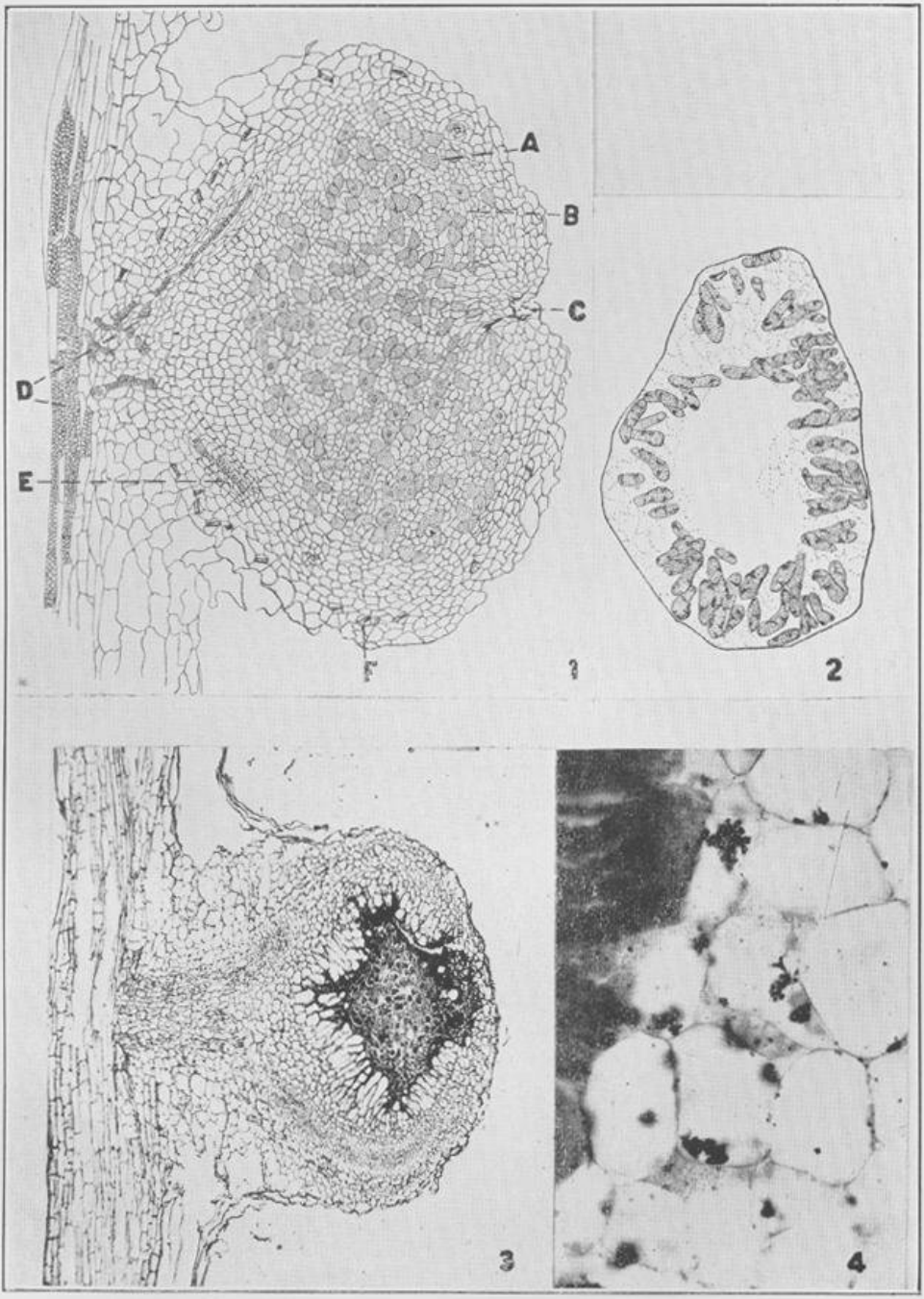
The lupine type was especially studied by Milovidov. He found many mitotic figures, even in infected cells of the bacteroid area, in which the bacteria appeared to have congregated in two groups at the poles of the spindle. Subsequent division into daughter cells separated the groups of bacteria and thus extended the infection. Káš, 1930, confirmed Milovidov's classification of nodules according to mode of infection within the bacteroid tissue. From his own observations he placed the soybean with *b,* Type in *Serradella,* and suggested that further study of the cowpea should reveal a similar relation because of its reported kinship to the soybean as shown by cross inoculation (Leonard, 1923a).

CHANGES IN THE HOST CELLS AS A RESULT OF INFECTION

Certain striking changes produced in the host cells as a result of bacterial infection have been observed by many investigators and have been variously interpreted as evidence of the parasitic or symbiotic relationship between the bacteria and the plant.

Hypertrophy. A very obvious effect of the growth of the bacteria within a cell of the nodule is the hypertrophy of the protoplast (See Plate 24). Peirce, 1902, after an experiment in which he confined nodules in plaster casts, concluded that the internal pressure of the bacterial mass is responsible for the swelling of the cells. Most observers, however, record progressive swelling from the region of newly-infected cells to the region of old bacteroid cells. Surely the beginning of hypertrophy is apparent before the cells are filled with bacteria and is rather to be interpreted as a result of increased cellular activity after infection. Perhaps there is stimulation by toxic products of bacterial metabolism, as Molliard, 1912, suggested from his study of the effects of bacterial filtrates upon plant roots. The hypertrophy of the cells involves both cytoplasm and nucleus. The extent of it

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may be appreciated from the following figures for nuclear hypertrophy quoted from Miss Wendel, 1918.

	Diameter of nuclei in micra	
	Normal	Infected
Pisum arvense	14	40
Acacia lophantha	10	33.8
Lathyrus tuberosus	—	30
Acacia armata	—	25-30

Miss Terby, 1925, observed a tendency toward reversible hypertrophy in cells recently infected. That is, she saw in sections a region just within the meristem in which the cells were slightly smaller than the uninfected meristematic cells. This condition she interpreted as due to a partial recovery by active resistance against the infection. No other observer has reported reversible hypertrophy. Rather has the hypertrophy been considered progressive up to the complete degeneration of the cell.

Vacuolation. Perhaps the next most common change in the infected cell is its vacuolation. It was noted by Brunchorst, 1885a, by Beijerinck, 1888, and by most investigators since then. As the cell becomes hypertrophied, large vacuoles appear in the cytoplasm. Generally these vacuoles fuse into a large central vacuole, which crowds the nucleus and the dense cytoplasm with its bacteria to the periphery. Often the dense cytoplasm is confined to a narrow layer in which the bacteria are packed side by side. There is a tendency in such cells for the bacteria to be arranged radially between the vacuolar membrane and the cell wall (See Plates 26 and 27, Fig. 2).

Of course vacuolation is not caused by the infection. Vacuoles appear during the maturation of most plant cells, and in many cases the mature cell consists of a large central vacuole and a scarcely distinguishable layer of dense cytoplasm along the walls. The bacteroidal cell, then, corresponds to a mature cell, just as the meristematic cell of the nodule corresponds to a meristematic cell of any growing region.

Changes in the nucleus. When an infection thread penetrates a cell, it often crosses directly to the nucleus and extends beside or around it (Beijerinck, 1888). The nucleus soon shows evidence of that hypertrophy which involves the whole cell. Later it becomes irregular in shape, collapsed, grooved, and even amoeboid (Viermann, 1929), and is often displaced to the side of the cell (Plate 24 and Plate 28, Fig. 2). The amoeboid appearance of many of the nuclei led some investigators (Schneider, 1893c; Paratore 1901b; Štefan, 1906; and Spratt, 1919) to report amitosis in the nodule cells. Miss Terby, 1925, granted that the appearances do suggest amitosis, but she pointed out that in cases of actual division of the nucleus, the bi-nucleate condition might as well be interpreted as fragmentation of the nucleus, a stage in its complete degeneration. The fragments are often unequal in size and are not subsequently set apart by cell division. There are also occasional multi-nucleate cells whose condition is obviously evidence of degeneration, and in still older cells the nuclei are entirely disintegrated.

Disappearance of the nucleolus. Miss Terby reported changes in the nucleolus as one of the earliest effects of bacterial invasion. Nucleolar hypertrophy

begins but is soon followed by decrease in the volume and finally by disappearance of the nucleolus. This is not true of all nodules, however, as will be noted below.

Production of chromocenters (Terby). Miss Terby, 1925 and 1927, correlated the disappearance of the nucleolus in the white-clover nodule with the appearance of certain small, globular bodies in the interior of the nuclei of infected cells. These bodies, stained by nuclear stains and suggesting areas of concentrated chromatin, were noted in nodule cells by Vuillemin, 1888; by Paratore, 1899, 1901b; and by Wendel, 1918. Miss Wendel suggested the similarity of these chromatin bodies to *prochromosomes,* at least in the case of *Lathyrus tuberosus.* Miss Terby, however, reported that the chromatic bodies in the clover nodule are present only in infected cells, are not equal in number to the chromosome number of the host plant species, and appear at the time of the disappearance of the nucleolus as if formed from its substance. For these reasons she considered them not prochromosomes but *chromocenters.*

However, a recent study of the nodules of the *Phaseolus* bean (McCoy, 1929) does not admit of the same conclusion as were drawn by Miss Terby. In the first place, the nuclei of the root tips of the bean, which were fixed as control tissue for comparison of normal and nodular tissues, show unmistakable chromocenters. Wager in 1904 also found these chromatic bodies in the normal nuclei of *Phaseolus* root tips. They are also present in uninfected cells of the main root and in the cortical tissues of the nodule. It is certain, then, that they are not the result of infection. It is true that the chromocenters are larger and more conspicuous in the infected cells, but that condition is undoubtedly an effect of hypertrophy. Neither does Miss Terby's second point, that the chromocenters are composed of nucleolar material abnormally dispersed in consequence of infection, seem to be borne out by the condition in the bean. There are two reasons for considering the chromocenters of the bean not to be of nucleolar origin. In the clover, Miss Terby was able to correlate the disappearance of the nucleolus with the appearance of the chromocenters. This is not the case in the bean, for its nucleoli are the most persistent of the nuclear structures. In the second place, the fact that the Němec fixative does not preserve the chromocenters but does fix the nucleoli very well is evidence that they are of different constitution. The phenomena of fixation, while perhaps not conclusive, would suggest that the chromocenters are a part of the nuclear reticulum, made conspicuous in the bacteroid cells by the general hypertrophy of the nuclei.

Arrest of cell division. Cells of the meristematic region of the nodule are capable of normal mitotic division as are also cells of the adjoining recently infected region. As infection progresses and causes more and more abnormality in the cell structures, the occurrence of division figures becomes more and more rare. Ultimately the power of division is lost, and all further changes in a given cell are the result of growth and degeneration of existing structures. This progressive effect of infection led Miss Terby to divide the nodules of *Trifolium,* which she studied, into the following areas: region of intact cells, region of infected cells still dividing, and region of infected cells incapable of division. The regions were still further divided to emphasize the progressively inhibiting effect of infection upon mitosis; there were traced a series of stages from the normal mitoses of the uninfected meristematic region to the older infected region where mitoses are im-

possible. The conclusion drawn is that the vacuolate condition of the cell, produced by the extreme hypertrophy, interferes mechanically with spindle formation. This and the degenerate condition of the nucleus account for the loss of the power of division. There is also the possibility that in the older infected cells toxic products of bacterial metabolism help to inhibit nuclear and cell division.

STARCH AND MITOCHONDRIA IN NODULES

Several investigators—Frank, 1890b; Schneider, 1892 and 1893c; Dawson, 1900b; Peirce, 1902; Štefan, 1906; Wendel, 1918; and Dangeard, 1926—have recorded the presence of starch grains in root nodules. Brunchorst, 1885a, who was apparently the first to observe them, referred to two general types of starch deposit. In *Lupinus* he noted peripheral layers of starch in bacteroid cells. In the nodules of *Vicia Faba* and *Phaseolus* sp., on the other hand, he found starch not in the bacteroid cells but in special starch-bearing cells interspersed with the bacteroid cells. In the latter case there is also starch in the region of the nodule cortex. Štefan, 1906, also indicated two types of starch deposit in nodules. These are illustrated by *Melilotus* and *Trifolium* nodules. In the former the bacteroid area contains many small starch-bearing cells between the bacteroid cells; the bacteroid cells themselves do not contain starch. In the latter the starch accumulates in the periphery of the bacteroid cells as well as in the nodule cortex. Miss Wendel, 1918, in commenting upon Štefan's division of storage types, said that there is probably not so sharp a difference. She noted peripheral layers of starch in the bacteroid cells of *Melilotus officinalis, Pisum sativum, Galega officinalis, Medicago intertexta, Lathyrus maritimus,* and *L. tuberosus, Vicia Faba and V. sativa,* and expressed the opinion that in such cases some starch must have been diverted from the starch cells.

Dangeard, 1926, speaking of the pea nodule, reported starch not present in the cortex or central cylinder of the root, absent in young nodules, absent or rare in the growing tips of older nodules, but more abundant in the main bacteroid area. In infected cells it is present as a parietal layer of grains showing more or less flattening and irregularity of form. The occasional uninfected cells of the bacteroid area are often packed with starch grains.

Frank, 1890b, suggested that there may be some relation between the starch and the activity of the bacteria, for it is well known that as the nodule begins to degenerate, the starch begins to disappear. From this it has been inferred that the starch serves as a source of energy for nitrogen fixation. Rippel and Poschenrieder, 1928, attempted to study the effect upon nitrogen fixation when starch formation is limited by darkening the plants or by cutting off their tops. They found a definite decrease in nitrogen fixation under such conditions. From their results it appears that the bacteria require far more energy than is furnished by the storage starch of the nodule; they must be fed by a continuous supply of carbohydrate from other parts of the plant. This finding might have been predicted from the observation that starch accumulates during most of the life of the nodule erinck in 1888 reported the bacteria unable to attack starch. What then is the and only after the onset of degeneration begins to disappear. Furthermore, Beijrelation of the bacteria to the starch in the nodule? That is a problem which needs

further investigation, involving as it does the whole relation of the bacteria to their food supply and of their energy requirement for nitrogen fixation.

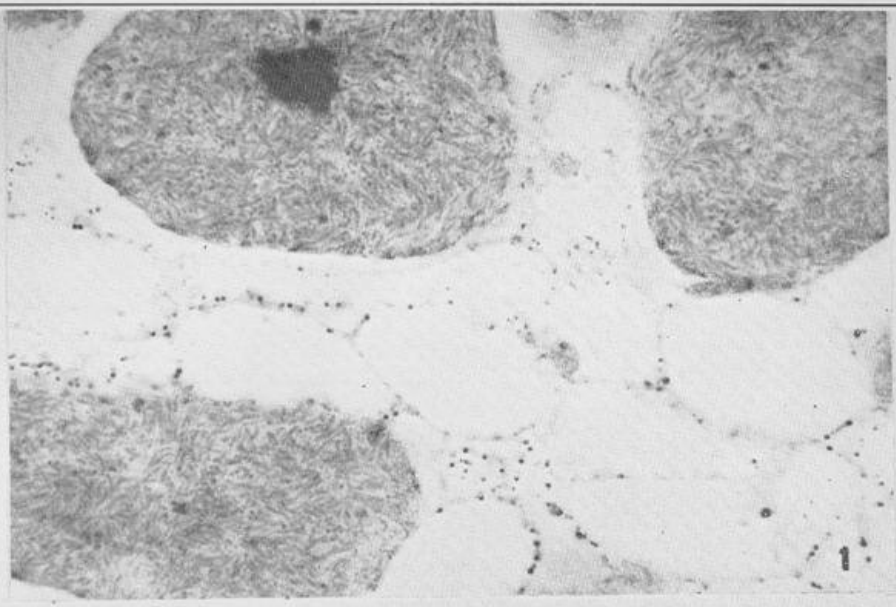
A recent contribution to this phase of the nodule problem (McCoy, 1929) suggests that the mitochondria of the nodule are in some way concerned with the behavior of the starch. In the young infection spot in the *Phaseolus* bean root the mitochondria are conspicuous rod-shaped bodies, more numerous in the infected cells than in the more distant cortical cells of the root proper. As the bacteroid area develops, they become very abundant in the nodule and rare in the adjoining root cells and are changed in form from rods to spheres (Plate 28, Fig. 1 and 2). Some of these mitochondria apparently function as proplastids and are responsible for the abundant deposit of starch in the bean nodule. This starch remains inaccessible to the bacteria, at least until well into the stages of degeneration (in which the mitochondria also seem to be involved; see Fig. 4, Plate 27). In view of the excessive deposit of starch in the bean nodule, it is suggested that the bacteria actually suffer malnutrition which is reflected in their failure to infect many cells of the bacteroid area and, in certain cases, in their actual attack upon the host's cytoplasm (see Fig. 3, Plate 27). The latter is the more striking when one considers that such nodules contain quantities of starch which the bacteria are apparently powerless to attack.

The mitochondria in nodules have been considered by several investigators, but from an entirely different point of view. In his series of studies on the nature of mitochondria, Wallin, 1922a, and b, cited the nodule bacteria as examples of bacteria symbiotic in the cells of plants. He further declared that the mitochondria in the cells are identical with the "juvenile" form of the bacteria, and on this basis he pictured bacteria in the root, stem, and leaf cells as well as in the nodule. He went on to develop his theory that all mitochondria are bacteria which have become obligate symbionts in the living cell as a result of ages of association. Cowdry, 1923; Duesberg, 1923; and Milovidov, 1928a, b, and c, answered Wallin's contention by devising differential staining methods by which mitochondria and bacteria can be identified and differentiated in the same cell. Milovidov, 1928e, has also shown by the ingenious scheme of centrifuging living tissue, followed by fixing and staining, that the mitochondria and bacteria behave differently. The bacteria are thrown down by centrifugation, whereas the mitochondria, being of apparently the density of the cytoplasm, remain in place. There is now no doubt that the mitochondria are a class of bodies normal to the cytoplasm of the host cells, and entirely distinct from the bacterial infection which causes the formation of the nodules.

Incidentally it should be said that Wallin recognized other forms of the bacteria which he distinguished from the mitochondria. He divided the white-clover nodule which he studied into the following three general areas, differentiated by their three types of "bacteria-like" organisms.

a. Younger apical part containing small bacilli in the infection threads and free in the cells. "Juvenile" forms of the organism equivalent to mitochondria.

b. Central part containing bacteroid or "mature" forms.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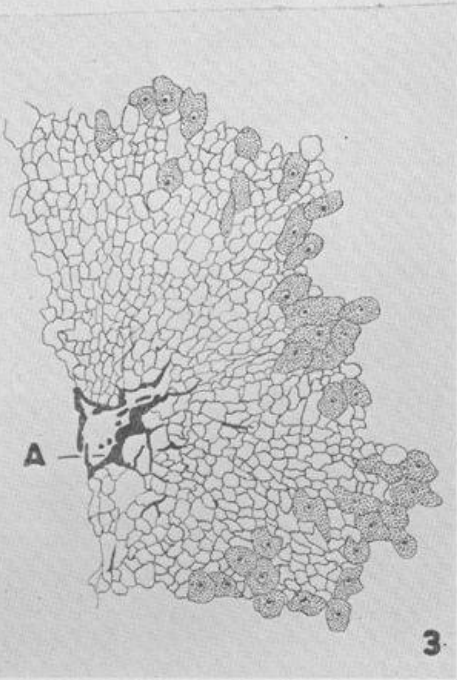
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3

c. Part next to the body of the plant containing old and therefore "senile" forms. Senile forms of the clover bacteria are described as round or globular bodies, probably corresponding to the "pre-swarmer" stage of Bewley and Hutchinson, 1920, and to the "spherical" forms of Löhnis, 1921.

By way of summary it should be emphasized that the development of root nodules on the Leguminosae represents a high degree of specialization and adaptation on the part of both symbionts. The nodules are essentially masses of parenchymatous tissue in which the bacteria live. They are connected with the vascular system of the root proper by a complete system of vascular bundles, which serve for transport of materials to and from the nodule. The nodule itself arises from meristematic tissue formed in response to the stimulation of infection. Its continued growth is provided for in the persistence of meristematic regions at the growing tip or periphery of the infected area. It is distinctly not a modified lateral root, for it has no central cylinder, root cap, nor epidermis. Furthermore it does not digest its way out from the cortex of the main root but remains covered with a considerable layer of cortical parenchyma. Anatomically then, it differs from non-leguminous nodules, many of which are clearly modified roots.

CHAPTER 10

RELATIONSHIP BETWEEN LEGUMINOUS PLANTS AND BACTERIA

"Prove all things; hold fast that which is good."
—I THESSALONIANS 5:21

PART I. THE EFFECT OF THE LIVING PLANT ON THE MICROÖRGANISMS IN THE SOIL

The rhizobia and related organisms. Aside from the general effects of plant residues on the microörganisms in the soil, we have to consider the influence of the living plants upon the microörganisms in the immediate vicinity of its roots. This subject has recently been well reviewed by Starkey, 1929a, b, and c, and Gräf, 1930. Both have reported that practically all investigators have found bacteria decidedly more numerous close to the roots of plants. Lyon and Wilson, 1921, and Cranner, 1922, explained this stimulation of the microflora as due to organic matter given off by the roots. Miss Gräf, 1930, confirmed this view by demonstrating that there are distinctly more microörganisms directly on the root surface than in the surrounding soil. It is not certain whether the supposed organic matter arises from actual excretion by the roots or from mere sloughing off of the outer layers of cells, as the roots grow.

There is some evidence of specificity in the action of different plants. This view has recently been discussed by Perotti, 1921 and 1926, who contended that each plant creates an environment in the soil which is particularly adapted to the life of certain kinds or groups of microörganisms. Perotti tried introducing into the soil extracts of plants belonging to representative plant families and was thus able to show encouragement of the growth of different microörganisms. Hiltner, 1904c, held a similar view. He described the region immediately surrounding the roots of a plant as a "rhizosphere" and stated that in this region nitrifying bacteria are not active, inasmuch as the soluble nitrogenous compounds are constantly taken up by the numerous microörganisms of other types. The free-living nitrogen-fixing bacteria, however, may be especially active.

There is some indication that the rhizosphere of the leguminous plant affords unusually favorable conditions for bacterial development. Beijerinck, 1908, found *Azotobacter* most numerous in the soil under leguminous plants. Caron, 1895; Stoklasa and Ernest, 1905; Löhnis, 1926b; Creuzberg, 1928; Starkey, 1929a; and Gräf, 1930, all reported larger numbers of bacteria in general in the immediate vicinity of leguminous plants. Representative examples of their data are given herewith, Table 13.

TABLE 13

Bacteria in millions per gram of soil

	Löhnis 1926b	Creuzberg 1928
No crop	39	11.5
Corn	54	----
Wheat	54	12.2
Rye	--	11.9
Oats	--	10.5
Vetch	73	15.6
Pea	98	15.9
Cowpea	69	----
Lupine	--	17.8

	Starkey 1929a		Gräf 1930	
	Near roots	Away from roots	Near roots	Away from roots
Rye	28.6	13.2	87.8	18.4
Corn	41.0	24.3	----	----
Wheat	----	----	19.7	10.0
Alfalfa	93.8	17.8	64.9	4.1
Clover	----	----	162.0	8.6

Hoffmann, 1914, found that extracts of soils which have been in various crops behave differently in respect to the support of various microörganisms. In most instances the growth in extracts of clover soils is better than in that from oat soil, but inferior to that from corn soil. Starkey's later studies, 1929b, also indicated that leguminous plants are not necessarily more stimulating to the soil microflora than are the non-leguminous plants. The effect varies with age, being generally greater during early stages of growth. Biennial and perennial plants are effective over a longer period than are annuals.

The rhizobial portion of the soil population as it obtains in the rhizosphere and in the adjoining soil has never been adequately studied, partly because of the difficulty of making differential counts. On the basis of plate counts with several media, Joshi, 1920, reported that soil bearing a leguminous crop contains more rhizobia than does the same soil bearing a cereal crop. An uncropped soil shows even fewer rhizobia than the cereal-bearing soil. Wilson, 1926b, made determinations of the number of rhizobia by the simple expedient of testing out the highest dilution of a given soil sample capable of inducing nodule formation on a specific host plant. Although his tests were not made primarily to determine the effect of specific leguminous plants on the numbers of rhizobia, some interesting comparisons are available. One plot which carried clover in 1926, the year of the test, contained over 1,000,000 cells of *Rhizobium trifolii* per gram. Another plot, which had not raised peas or vetches since 1911, still contained over 20,000 of *Rh. leguminosarum* per gram. The latter figure, however, is large for a soil which has been harboring rhizobia for a number of years. In one or two cases a whole gram of soil was required to demonstrate any *Rh. leguminosarum*, and in the data for alfalfa soils, the figures for the number of *Rh. meliloti* ranged from none in 5 gm. to 100,000 per gram. In a recent paper, Wilson, 1931b, has reported further studies on the detection of certain species of rhizobia in soil. Forty-four samples representing 4 soil types were tested quantitatively for *Rh. trifolii* and *Rh.*

leguminosarum, and it was found in 42 cases out of 44 that *Rh. trifolii* predominated. Soil reaction, moisture, or seasonal variation seem not to account for this capacity of the soils to maintain the population of one organism at a higher level than the other. It is suggested that the mineral constituents of the soil, sulphates particularly, are in part responsible.

There is indirect evidence that plant growth has a beneficial effect upon the development of rhizobia in soil. Temple, 1916, stated that a sterilized soil with the addition of a small amount of leguminous matter furnishes the best medium for the growth of rhizobia. Wilson and Lyon, 1926, in effect recreated the rhizosphere and the adjoining soil in a laboratory experiment. They arranged two series of sterilized soil in test tubes. One series was planted to corn and timothy, the other left with "no crop." Both were inoculated with a pure culture of rhizobia and at intervals of about three months plate counts were made. In every case the planted-soil samples surpassed the unplanted in support of large numbers of rhizobia.

Bacteria of the *B. radiobacter* type seem to be peculiarly susceptible to stimulation by the roots of higher plants. Löhnis, 1926b; Creuzberg, 1928; Smith, 1928; Starkey, 1929a, b, and c; and Gräf, 1930, have noted that these organisms are distinctly stimulated in the immediate vicinity of plant roots, particularly in the case of leguminous plants. Smith, 1928, reported some variation among the Leguminosae in respect to their stimulatory effect. Among those tested, Smith listed field peas, vetch, and soybeans in descending order.

Just what effect the increased number of microörganisms in the vicinity of plant roots may have on the general biological processes in the soil is not at all understood. There have been several suggestions that a part, at least, of the beneficial action of the Leguminosae in soil fertility is due to stimulation of general soil microflora. Lyon, 1918, stated, "I do not intend to question the well-known fact that the nitrogen content of a soil may sometimes be augmented by the growth of legumes even when the above-ground portions of the plants are removed, but I think it is fairly questionable whether the beneficial effect of legumes on soil productivity is due entirely to this increase in the nitrogen content or whether it is in part accomplished through some other influence which the plant exerts on the bacterial activities within the soil." Löhnis, 1926b, stated, "Therefore, it is very probable that wherever an increase becomes noticeable in the soil nitrogen under and after legumes, this is much less due to the nitrogen fixation by the bacteria in the root nodules, than to the action of non-symbiotic soil organisms assimilating elementary nitrogen." And in the same paper, " . . . the beneficial after-effect exerted by legumes harvested for hay is to a considerable extent due to favorable changes in the microflora of the soil, which are still marked, and are even increasing a few weeks after the surface growth of the legumes has been removed." "It is probable that in field tests the crop increases ascribed to green-manuring are in fact more frequently due to the special after-effect of the growing legumes than to their manuring effect after having been plowed under where they have grown."

The importance of this phase of the problem is at once apparent and would seem to call for additional study.

PART II. THE RELATIONSHIP BETWEEN THE NODULE BACTERIA AND THE HOST PLANT

The entrance of the rhizobia into the plant and the effect of the number of organisms on nodule formation. The mode of infection and the subsequent development of nodule tissues have been presented in detail in Chapter 9. They will be referred to here only as they may enter into our interpretation of the relationship of the plant and bacteria. It will be recalled that the root hair is the usual portal of entry for the bacteria, although occasionally they may find their way through abrasions or wounds in the epidermis. The time required for penetration by the bacteria is difficult of actual determination; indications are, however, that it is relatively short, if conditions are favorable. Wilson, 1917, found that an exposure of 5 hours was sufficient for generalized infection of the root system, although sporadic nodules might appear on plants given much shorter exposure. Longer exposure, even to 14 days, resulted in little or no increase in the average number of nodules per plant. Similar findings were reported by Giöbel, 1926, and S'Jacob, 1927. Peirce, 1902, showed that in the case of bur clover the characteristic curvature of root hairs develops within 24 hours of infection and Prazmowski, 1890, reported that about 2 days elapse as the infection thread grows down the channel of the root hair.

The number of rhizobia necessary to induce nodulation and the influence of heavy inoculations in increasing the number of nodules have been studied by many workers. As early as 1903, Steglich observed that artificial inoculation often increases size and number of nodules and plant growth on soils which already contain a limited number of appropriate nodule-forming organisms. Thornton, 1929b, from a series of field experiments with alfalfa reached the same conclusion and attributed the beneficial effect of artificial inoculation in part, at least, to the early association of the rhizobia with the host and the consequent strengthening of the seedling. Wilson, 1926a, and Wilson and Leland, 1929, reported that artificial inoculation increases the crop yield even in soils containing as many as 100,000 of the appropriate rhizobia to the gram.

Many investigators (Fruwirth, 1891; Süchting, 1904; Kellerman and Robinson, 1906; Temple, 1916; Fellers, 1918b, for example) found that heavy inocula give better results than light inocula. Noyes and Cromer, 1918, found that a commercial culture used at the recommended rate gave only 20 per cent nodulation of the plants and at double this rate 75 per cent nodulation. Hawkins, 1923, reported that an application of 20 times the normal rate of culture gave three times the crop yield with alfalfa. Alway and Nesom, 1927, increased the inocula to 30 times the recommended rate and thereby materially increased the percentage of plants bearing nodules. Harper and Murphy, 1928, by doubling the rate of application of culture were able to increase both the percentage of plants bearing nodules and the number of nodules per plant.

Opposed to these findings are those of Noble and Hiltner, 1899b and 1901, and Hiltner, 1900a and 1902. In their 1899 paper Nobbe and Hiltner maintained that 25 times the usual rate of inoculation gives no increase in nodulation over the normal. In 1900a Hiltner reported variation of the inoculum from 100 times to 1/100 of the normal application without appreciable effect upon the number,

size, and activity of the resulting nodules. In the opinion of Nobbe and Hiltner the "virulence" of the culture is much more important than the number of organisms. Nightingale expressed a similar view in 1922. Fellers, 1919, found little correlation between the number of bacteria applied to the seed and the number of nodules developed on the plant. The numbers of bacteria per seed, however, were large in all his experiments. Perkins, 1925a, used comparatively small numbers of bacteria per seed and raised his soybean plants in glass tumblers of sterilized sand fortified with a mineral-salts solution suitable for plant nutrition. Under such conditions he claimed that the bacteria were not permitted to multiply, owing to lack of carbohydrate. He was thus able to show parallel increase in number of nodules with number of bacteria per seed up to a limit, which in his experiments appeared to be at approximately 50 rhizobia per seed. With greater initial inocula, up to 10,000 per seed, he found no marked increase in nodulation. Thornton, 1929b, questioned the experiments of Perkins on the grounds that his assumption that the initial inoculum was the effective number surrounding the seedling is not necessarily correct. In a confined space, such as that of the small volume of sand used in the Perkins experiment, organic nutrients from excretion and decay of dead rootlets would soon support growth of the rhizobia to the saturation point. Thornton therefore repeated the work with alfalfa under field conditions; he also found some correlation between numbers of bacteria and of resulting nodules, at least within the range of 2,500 to 20,000 organisms per seed, initial inoculum. A graph of the results, presented in his paper, indicates that the higher rates of inoculation, though they do increase nodulation, do not do so in direct proportion to the number of organisms applied. There are then other limiting factors, chief of which is probably the so-called *immunity* of the plant. In the first place, there is a remarkable limitation of the number of root hairs infected, even in the face of overwhelming numbers of rhizobia surrounding the roots. Thornton has counted the infected hairs of alfalfa seedlings in agar culture with excessive inocula and has found only about 4 per cent of the hairs infected. Peirce, 1902, estimated the infection of root hairs of bur clover as 1 in 1000 on plants grown in the open field. Other aspects of the plant's resistance to infection will be presented in detail later in the chapter.

A summation of the evidence so far presented indicates that relatively few bacteria are able to give maximum infection under ideal conditions, but that under field conditions a large inoculum is preferable to a small one. The number of nodules developed, however, is not necessarily indicative of the benefit resulting to the host plant.

The immediate reaction of the host to infection by rhizobia. The first visible effect of infection by the rhizobia is a deformation of the root hair, a characteristic curvature of the tip, described as early as 1887 by Ward. The cause of this curling reaction is not well understood. It has been suggested that unequal growth of the root-hair wall around the injury at the point of infection is responsible for the bending and curling. Very soon after infection, the bacteria may be seen in the root hair in the form of a well-defined strand or "infection thread." The nature of the thread has been discussed in Chapter 9 in as much detail as we yet know. Commonly the infection thread penetrates to the deeper layers of the root cortex, branches, and enters host cells in all directions. The invaded cells, to-

gether with those immediately adjoining, are stimulated to division and by their growth and differentiation eventually form the nodule tissues. The power of the rhizobia to stimulate growth of higher-plant cells is indicated by the results of Molliard, 1912, who found that sterile filtrates of rhizobial cultures cause hyperplasia of the pericycle and hypertrophy of the cortical parenchyma of the pea. Another case in point, although involving a non-leguminous plant, is the report of Němec, 1929, to the effect that the rhizobia streaked upon the cut surface of kohl-rabi induce a rapid overgrowth of callus tissue. This is apparently accomplished without the entrance of the bacteria into the cells.

In the nodule, however, the rhizobia soon emerge from the infection thread and come to lie free in the cytoplasm of the infected plant cells. According to a recent paper by Thornton, 1930b, they may escape either directly from zoogloeal masses on the young naked infection thread (*i.e.* before the sheath forms about the thread) or later by the bursting of blister-like swellings which appear irregularly along the sheathed threads.

The symbiotic relation of the rhizobia to the leguminous plant. The intimate relation of the rhizobia and their hosts, the Leguminosae, is usually considered one of symbiosis. The precise meaning thus implied hinges upon our definition of symbiosis. Strictly speaking, the cells, in which the bacteria are, eventually suffer from their presence. For a time, however, infection does not apparently interfere with the functions of a cell, since mitoses may occur in recently invaded cells. In fact, in the lupine (Milovidov, 1928d) and the bean (McCoy, 1929), in which infection threads are nearly or entirely lacking, the main mechanism for the spread of infection is the active cell division. Mitotic division has been observed in a cell actually containing an extension of the infection thread (Thornton, 1930b) and also in cells containing numerous free bacteria (Terby, 1925). Whether or not these mitoses are normal is another question. Fred, 1911a, suggested that they are not, in view of the irregularities observed in the separation of the chromosomes in mitosis in the lupine nodule. Eventually, however, the cell suffers hypertrophy, nuclear degeneration, and loss of the power of division. In the sense that the bacteria are nourished by the cell and that they "bite the hand which feeds them," the relation may be called parasitic. Certainly in the early stages of nodule development the plant contributes much and receives no known benefit. Later, though the actual cells of the nodule may be destroyed, the plant as a whole profits by the association.

It is generally conceded that the principal benefit to the leguminous plant accruing from the symbiosis is the utilization of atmospheric nitrogen, a fact which is emphasized again and again in this book. There are, however, other aspects of the influence of the rhizobia upon their host. Frank, 1890b, emphasized a greater growth energy in all parts of the plant, a richer formation of chlorophyll, a greater assimilation of carbon dioxide, as well as the regular increase in nitrogen content and in total dry weight of tissue. In a broad sense, however, all of these effects are secondary and indirectly the result of enhanced nitrogen metabolism of the plant.

The degree of benefit to the host plant is variable and unstable and dependent upon many factors. Beijerinck as early as 1888 appreciated that the relation between plant and bacteria is one of a subtle state of balance. The outcome is

uncertain and subject to disturbance by any factors which vary the nutrition and vigor of the host or the effective state of the rhizobia. In general, any factor which tends to promote vigorous growth of the plant, with the exception of a plentiful supply of combined nitrogen, will enhance the benefit derived by the host plant. Such factors, as they affect nodule formation, will be set forth in Chapter 11.

It is interesting to trace the gradual realization that not all nodulation of a leguminous plant implies benefit to the plant. We thus arrive at our present conception of strain variation among the rhizobia. Nobbe, Hiltner, and their associates were among the first to point out the variable relation between plant and bacteria. Their views are well summarized in the 1904b paper of Hiltner, in which he defined six conditions which may obtain:

1. The bacteria are not able to gain entrance into the roots. (At this time Hiltner recognized two species of rhizobia, but the cross-inoculation groups were not yet established).
2. The bacteria gain entrance into the root but are immediately absorbed because they are too weak to withstand the plant. Small root swellings later entirely vanish.
3. The bacteria gain entrance into the roots and produce nodules; however, the bacteria are completely or to a great extent absorbed by the cells, so that the nodules remain inactive.
4. The bacteria produce active nodules which stimulate nitrogen fixation.
5. The "virulence" of the bacteria is so great that the plant is injured.
6. The bacteria are pure parasites upon the plant, especially when the plant is unfavorably nourished or weakened through other influences. The bacteria do not change into bacteroids. Nitrogen fixation does not occur, and the nourishment of the bacteria results at the expense of the plant.

Süchting, 1904, presented another view. He believed that the bacteria produce a "toxin" which stimulates the plant to produce an antibody, and that the relationship between these two substances determines whether the bacteria are able to invade the plant and produce functional nodules. Süchting also drew a distinction between the "virulence" or nitrogen-fixing ability of the organisms and the "vegetative energy" or ability of the organisms to invade the plant and to proliferate therein. According to his theory, these two characters are distinct and may operate independently.

In support of the hypothesis of antibody formation by plants, Cappelletti, 1923a and b, 1924, 1926a and b, and 1928, reported that the nodules of leguminous plants possess agglutinins which are specific for the particular root-nodule organism. He found agglutinins in the nodules of *Lathyrus odoratus, Pisum sativum,* and *Vicia Faba,* but none in *Phaseolus vulgaris.* The agglutinins appear in the nodules at about the time of the development of flower buds, reach a maximum with the unfolding of the flower, and disappear with the formation of seed. This apparently correlates with the development of bacteroids in the nodule. The

agglutinins found by Cappelletti are present only in the nodules, not in roots, stems, or leaves. So far as is known, the results of Cappelletti have not been duplicated by other workers. Kořínek, 1924, found no true agglutinins in an investigation of agglutinins in plants infected with *B. tumefaciens, B. prodigiosus,* or *B. radicicola.* Sardina, 1926, failed to detect agglutinins in plants infected with plant pathogens; *Vicia Faba* was among the plants studied.

Any immunity which may be set up in the plant does not inhibit the formation of additional nodules. It has been noted by many workers that under suitable conditions nodules may form as long as the plant makes active growth (Prucha, 1915; Giltner, 1915; and Whiting, 1915). This is particularly true of perennial plants on which the nodules decay at the close of each growing season and form again the following year on the new young roots. The excessive nodule formation induced by certain ineffective strains of rhizobia (Helz, Baldwin, and Fred, 1927) is a further example of lack of immunity on the part of the plant.

Frank, 1890b, stated that the needs of the plant, and not the time of year or age of plant, control nodule formation. Much the same idea was expressed by Schindler, 1885; Tschirch, 1887; and Maassen and Müller, 1906, and was reaffirmed by Müller and Stapp, 1925, who stated that the state of nourishment and general health of the plant are more important than the efficiency of the organism in determining the number and size of the nodules. On the other hand, Hiltner, 1902, felt that a certain degree of immunity is set up by the entrance of the rhizobia into the plant, and that only bacteria of a higher "virulence" than those already present can effect secondary invasion.

Israilsky, 1929, reported that the infection of the plant by one strain of the organism results in an immunity which inhibits infection by other strains. His work is based upon serological classification. Bialosuknia and Klott, 1923; Ohkawara and Yoshida, 1925 (see Jimbo, 1930); and Jimbo, 1930, however, have reported the isolation of two or more serological races from a single plant. No evidence is given in these reports as to the virulence or effectiveness of the strains isolated. Miss Löhnis, 1930b, reported that a secondary inoculation of clover with an effective strain of rhizobia excites further nodule formation on plants already carrying nodules of an ineffective strain. The reverse is not true. Dunham and Baldwin, 1931, were able to infect alfalfa, clover, peas, and soybeans simultaneously with effective and ineffective strains of rhizobia. In studies on secondary infection it was found much more difficult to secure nodulation by the second strain, although it was accomplished in several instances. No correlation could be established between the infective power of a strain and its ability to aid plant growth. It proved much more difficult to infect with an effective strain plants already bearing nodules formed by an ineffective strain than nodule-free plants of the same age and nutrition. Evidently the first lot of nodules irrevocably changes the plant, and since the change is in the direction of resistance, we may call it a degree of immunity.

There is one other line of evidence in support of the proposition that the plant exerts some determining influence in the face of infection. Schindler, 1885, and Tschirch, 1887, noted that nodules do not appear before the opening of the first true leaves. Müller and Stapp, 1925, and Thornton, 1929d, recently made a similar observation. The time in days, as given by Otis, 1898; Temple, 1916; and Dug-

gar, 1929, agrees approximately with this idea. Although the high nitrogen content may account in part for the temporary immunity of the seedling, Thornton, 1929d, showed that seedlings of the first leaf stage possess an extractable material which both encourages growth of the rhizobia and stimulates nodule production on younger seedlings.

Because of the experimental difficulties involved, little work has been done on the harmful effects of the rhizobia. Wunschik's, 1925, work on the effects of repeated passage of the rhizobia through the host plant convinced him that the "vegetative energy" of the rhizobia is so enhanced by the process that the benefit derived by the plant is lessened. By a comparison of the activities of effective and ineffective strains of the rhizobia under varying conditions, Allen and Baldwin, 1931a, and Dunham and Baldwin, 1931, have concluded that there are at least two phases to the action of the bacteria within the host plant; that is, contribution to the process of nitrogen fixation and effect upon the growth of the plant. This effect may be either stimulatory or inhibitory to growth, and from the evidence in hand the latter seems more probable.

It is interesting to note that several of the earlier writers observed a less perfect accord between the bean plant (*Phaseolus*) and its associated species of rhizobia. Frank, 1890b, and Schneider, 1892, claimed that the bean bacteria are purely parasitic. Dawson, 1900b, and Dangeard, 1926, have also observed that the association seems to be different from that of other leguminous plants and their rhizobia. A recent cytological study (McCoy, 1929) suggests that the cause of the failure of the bean bacteria to aid their host is a reflection of their malnutrition resulting from the abnormally large deposit of starch in the bean nodule. A deficiency of available carbohydrate then reacts to hold in check the synthetic activities of the bacteria and may account for the functional deficiency of the nodules.

In view of this supposed parasitic existence of the bean bacteria, it is of interest that in 1911 the Michigan Agricultural Experiment Station, 1914, discontinued the issue of cultures for inoculation of bean crops because of an accumulation of unfavorable reports in previous years. However, the culture was again distributed the following year. Recently there have been two reports of definite utilization of atmospheric nitrogen and enhanced plant growth on the part of nodulated bean plants (Wilson and Leland, 1929; and Sears and Clark, 1930). It seems possible that the effective or ineffective state of the particular bacteria used may account for the success or failure of the nodulation. It is conceivable that a large proportion of the strains of *Rh. phaseoli* exist in the ineffective state, whereas the reverse is true of most rhizobia.

It is at least possible that rhizobia and leguminous plants may enter into a partnership without actual nodule formation.[1] Such a case has been reported by Friesner, 1926, and Fehér and Bokor, 1926, who reported that the roots of *Gleditsia triacanthos* bear peculiar cylindrical swellings caused by an organism which is probably a species of *Rhizobium*. The plant appears to benefit from the association, as other Leguminosae benefit from the rhizobia in their true nodules. Earp-Thomas, 1907; Hiltner, 1907; and Joshi, 1920, have reported

[1]The observations of Leonard and Reed, 1930, on *Cassia* and of Gutschy, 1931, on a soybean, growing with a non-nodule-forming strain of rhizobia, are suggestive of this.

that Leguminosae may benefit from the mere presence of rhizobia, regardless of whether nodules are formed. Other workers, however, have failed to confirm this observation.

Cross-inoculation groups. The specificity of the relationship existing between the leguminous plant and its rhizobia is worthy of note. Members of the genus *Rhizobium* are apparently able to infect only members of the family Leguminosae, and conversely, practically all species of the Leguminosae are infected by some species of *Rhizobium.*[2] Not all species of Leguminosae are infected by the same type of *Rhizobium,* however. Recognition of this specificity has lead to a classification of leguminous plants into groups, within which the rhizobia are interchangeable. In many cases a single plant group includes several different species or even genera of the Leguminosae; in other cases a single plant species is the only known host to a particular *Rhizobium.*

A detailed list of the plant-bacteria or so-called cross-inoculation groups has been given in Chapter 8. There is as yet no satisfactory explanation for the specificity which exists. A correlation between the acidity of the cell sap and the limiting acidity for growth of the rhizobia concerned has been suggested. Baldwin, Fred, and Hastings, 1927, have established a certain correlation between the protein constitution of the seeds and the cross-inoculation grouping. Neither of these explanations establishes an exact correlation, and it seems probable that some other factor or factors in the physiological complex of the host determines infection or non-infection.

The work of Richmond, 1926c, is interesting in this connection. By grafting the tops of navy bean plants, *Phaseolus vulgaris,* on the roots of the lima bean, *Ph. limensis,* and *vice versa,* he demonstrated that the conditions in the root are the factors determining infection. On grafted plants with lima bean roots, only rhizobia of the lima bean are able to induce nodule formation. And conversely, on plants with navy bean roots, the rhizobia of the navy bean alone may enter. Seeds produced on such grafted plants were apparently altered in such a way that the plants of the second generation could be infected by either the rhizobia of the navy bean or of the lima bean. The data supporting this latter statement are meager. Recently Hansen and Tanner, 1931, have repeated Richmond's grafting experiment and have arrived at negative conclusions as far as nodulation is concerned. They have also done serological work on the seed proteins of normal and grafted plants and have found no indication of changed nature of the seed protein complex.

Strain variation. Even before the specificity of the plant-bacteria relationship was fully recognized, it was noted that nodules on the roots of leguminous plants did not always result in fixation of atmospheric nitrogen and consequently increased plant growth. Frank, 1892b, described two types of nodules on the pea, distinguished as "amylodextrin and albuminoid nodules." The former were characterized by their reddish-brown color with iodine, a reaction due chiefly to absorption of iodine by numerous granulae of amylodextrin in the bacteroids and probably also by accumulated starchy reserve in the plant cells. Beijerinck, 1888, divided nodules into two broad groups—one in which the plant gains the

[2]The few known exceptions are listed in Chapter 3.

ascendency and is benefited, and the other in which the bacteria are stronger, destroying the plant tissue and acting as parasites.

Further search of the early literature reveals a growing realization that not all strains of any species of *Rhizobium* are alike in ability to benefit the host plant; see, for example, the papers of Lawes and Gilbert, 1890 and 1891; Nobbe, Schmid, Hiltner, and Hotter, 1892a; Nobbe and Hiltner, 1893; and Dehérain and Demoussy, 1900a and b. During this early period some care was taken to apply this knowledge in the preparation of cultures. As the value of inoculation

TABLE 14

The effect of various strains of Rhizobium meliloti on the yield and nitrogen content of alfalfa

(After Stevens, 1925a)

Strains	Dry weight*		Nitrogen		Total nitrogen*
	Roots	Tops	Roots	Tops	
	gm.	gm.	per cent	per cent	mg.
111	6.719	10.621	1.68	2.59	387.9
100	13.165	12.942	1.79	2.65	578.6
106	9.704	11.070	1.66	2.63	454.1
107	6.926	9.813	1.84	2.60	382.5
104	2.942	5.712	2.16	2.61	212.5
102	2.813	6.078	2.22	2.60	220.4
101	3.931	6.596	1.84	2.41	230.5
105	3.685	5.181	2.01	2.24	180.2

*30 plants

TABLE 15

*The effect of various strains of Rhizobium japonicum on the yield and nitrogen content of soybeans**

(After Wright, 1925b)

Strains	Wisconsin Black		Ito San		Manchu	
	Dry weight	Nitrogen fixed	Dry weight	Nitrogen fixed	Dry weight	Nitrogen fixed
	gm.	gm.	gm.	gm.	gm.	gm.
1	815	12.84	894	14.07	853	15.91
2	965	21.51	961	16.86	886	11.41
3	873	16.08	871	13.46	834	13.33
4	788	15.07	888	15.40	861	15.31
5	598	7.05	659	4.48	765	7.04
6	734	9.54	731	5.07	622	4.87

*Three-year average of field results. 50 plants in each case.

became better known and the practice more extensively followed, this fact seems to have been disregarded and did not again come into prominence until the work of Stevens, 1925a, with *Rh. meliloti* and of Wright, 1925a and b, with *Rh. japonicum*. By carefully controlled and extensive studies, these investigators demonstrated conclusively that different strains of rhizobia vary in ability to aid plant growth. The accompanying Tables 14 and 15 from Stevens, 1925a, and Wright, 1925b, illustrate the differences existing between strains of a species.

These studies were followed by other comprehensive studies with strains of

Rh. leguminosarum by Helz, Baldwin, and Fred, 1927; of *Rh. trifolii* by Baldwin and Fred, 1929a; and *Rh. lupini* by Eckhardt, Baldwin, and Fred, 1931. Many other reports of the same import are appearing in various parts of the world. In the United States, Vandecaveye, 1927b and 1928; in Scotland, Cunningham, 1928; and in England, Thornton, 1929a, have found variations among strains of *Rh. meliloti.* Bialosuknia and Klott, 1923, in Poland; Leonard, 1930, in Louisiana; and Miss Löhnis, 1930b, in Holland have reported the same for *Rh. leguminosarum* and Harper and Murphy, 1928, for *Rh. japonicum.* In their studies of irregular cross-inoculations, Sears and Carroll, 1927, obtained evidence of strain variation, as did also Sears, Gifford, and Myers, 1930, with the rhizobia of soybeans and cowpeas. A study of the non-reciprocal cross between garden bean and Dalea revealed to Sears and Clark, 1930, variations in the ability of the rhizobia from Dalea to benefit the bean plants. And so with the reports of Miss Löhnis, 1930b, for the clover and pea; of Allen and Baldwin 1931a, for clover, soybean, and pea; and of Dunham and Baldwin, 1931, for alfalfa, pea, clover, and soybean, the record of strain variation of the rhizobia is continually growing. There is no doubt of the fact of variation among strains of the rhizobia. There is, however, uncertainty as to the essential cause of ineffectiveness and still more question concerning conditions under which the rhizobia are ineffective.

The relation of laboratory cultivation to physiological efficiency. Contemporary studies with the pathogenic bacteria of animals indicated to the early workers with the rhizobia that cultivation on laboratory media might change their characteristics, with respect to either infective power or effectiveness in aiding plant growth. As early as 1893 Nobbe and Hiltner reported that organisms grown on a gelatin medium for several weeks were not effective in aiding plant growth, although nodules were formed. A similar view was expressed by Kühn in 1896 and Lauck, 1899. Frank, 1899, further declared that rhizobia, on being grown in a gelatin medium, actually lost their ability to form nodules.

Largely because of the failure of some of the early commercial cultures (see Chapter 13), it was generally considered that cultivation on artificial media was undesirable. Remy, 1902, stated that the efficiency of the rhizobia begins to decrease as soon as they are placed upon artificial media, and he recommended that only crushed nodules be used for inoculation. In 1907 a patent was granted to Earp-Thomas, who had invented a procedure for carrying the nodule bacteria in nodules until they were needed for the production of commercial cultures. Ferguson, 1906, and Makrinoff, 1913, accomplished essentially the same thing by using nodules directly as the source of inoculum in the preparation of commercial cultures. Hiltner and Störmer, 1903a, however, found no advantage in the use of crushed nodules as compared with pure cultures on agar for purposes of inoculation.

The use of medium high in nitrogen for culture of the rhizobia was considered undesirable by several of the early workers, Hiltner and Störmer, 1903a; Hiltner, 1904b; Süchting, 1904; Moore, 1905; and Simon, 1908-09, and 1911. On the other hand, Lewis and Nicholson, 1905, and Prucha, 1915, have reported that the nitrogen content of the medium is of no importance in determining the infective ability of the organism. Prucha, 1915, gave it as his opinion that "every living nodule-producing organism in a vigorous condition, will, if given a chance,

cause nodule development no matter on what kind of media it has been propagated." Giöbel, 1926, with sand cultures and S'Jacob, 1927, with solution cultures found that the infective power of the rhizobia is not affected by at least a relatively short exposure to a medium with a high content of nitrogen. The result of long-continued cultivation on rich nitrogenous media has not been studied since the recent advances in the knowledge of strain variation. It is probable that such a study would prove profitable as it is well known that conditions of culture are not without effect upon bacteria in general. It is known that the dye sensitivity of the rhizobia may be modified to some extent by culture on increasing concentrations of the dye. (See Burke and Burkey, 1925). Simon, 1908-09, stated that by cultivation in sterilized soil it is possible to rejuvenate a culture which has become degenerate through cultivation on artificial media. Just what factors in the soil environment are thus stimulatory to the rhizobia Simon did not mention. A similar statement was made by Śnieszko, 1929.

Contrary to the admitted possibility of variation resulting from cultural conditions, the fact remains that culture of the rhizobia on artificial media is not so degrading as formerly supposed. Hiltner and Störmer, 1903a, reported that a culture carried on agar for 3 years was not distinguishable from another carried in the same way for only 1 year. Garman and Didlake, 1914, likewise found the infective power of the organisms constant after cultivation on laboratory media. The recent studies on strain variation have shown to Wright, 1925b; Stevens, 1925a; Helz, Baldwin, and Fred, 1927; Baldwin, and Fred, 1929a; Allen and Baldwin 1931a, and Dunham and Baldwin, 1931, that maintenance of rhizobia on laboratory media of low nitrogen content, at least, effects little or no change in infective power or effectiveness of the strains. The attempts of Burrill and Hansen, 1917, and of Burke and Hohl, 1930, to change the infective ability of the organisms by culture on media containing extracts of foreign host plants were entirely unsuccessful.

Cultural characters as indicators of the effectiveness of strains of the rhizobia. The reports of Stevens, 1925a, with *Rh. meliloti* and of Wright, 1925a, with *Rh. japonicum* indicated a possible association of certain cultural differences with effectiveness of the several strains. Later work in this laboratory with other species of *Rhizobium* and with a larger number of strains of the two species mentioned has failed to show definite correlation between any known cultural or physiological character of the rhizobia and their state of effectiveness (Helz, Baldwin, and Fred, 1927; and Baldwin and Fred, 1929a). Tittsler, 1928, found direct correlation between the cataphoretic velocity and "inoculating ability" of fifteen strains of the rhizobia from alfalfa. No details are given as to the method of measuring the "inoculating ability" of these strains. Similar studies by Zucker, 1929, upon a large number of strains whose effectiveness had been established by the previous studies of Stevens, 1925a; Wright, 1925b; Helz, Baldwin, and Fred, 1927; and Baldwin and Fred, 1929a, failed to give results definite enough to be of value in differentiating the strains.

Plant passage and the effectiveness of the rhizobia. In the literature of the early period when it was thought that any of the rhizobia could be adapted to any host plant, there are to be found several hints that passage of the organism through its host plant may change its effectiveness. Lawes and Gilbert, 1890

and 1891; Frank, 1890b; and Nobbe and Hiltner, 1900, for example, made statements that may be so interpreted. Apparently the first attempt to study this problem with a reasonably adequate technique is that of Remy, 1902. He compared the growth of lupines inoculated with crushed nodules and with pure cultures of rhizobia. The plants grown from seed inoculated with crushed nodules (i. e., directly from a previous plant passage) showed a more vigorous growth and greater fixation of nitrogen. While his results are suggestive, they are not in themselves conclusive. Süchting, 1904, carried out a more extensive investigation, from which the data gave much the same indications.

Wunschik, 1925, attempted a more elaborate study of this question and concluded that plant passage does bring about a change in the effectiveness of the rhizobia. There is increased effectiveness up to the fourth passage and decreased effectiveness thereafter. According to his theory, plant passage induces an adaptation of the rhizobia to the plant; the most favorable nitrogen utilization occurs when a balance has been reached between the vegetative energy of the plant and that of the bacteria. Further plant passage increases the vegetative

TABLE 16

Changes in the physiological efficiency of Rhizobium trifolii (strains 200, 201, and 202) induced by plant passage, as measured by the growth and nitrogen content of nodulated plants

Culture	Strain 201		Strain 200	Strain 202		
	Alsike clover	White clover	Red clover	Alsike clover	White clover	Red clover
	Dry Weights					
	gm.	gm.	gm.	gm.	gm.	gm.
Control	1.01	0.97	1.15	1.01	0.97	1.15
Agar culture	4.32	4.17	3.64	0.97	1.08	1.07
1st plant passage	1.27	3.75	3.58	0.95	1.05	1.10
2nd plant passage	1.51	3.77	3.60	3.87	1.31	2.31
3rd plant passage	2.01	2.10	2.97	3.89	1.56	2.57
4th plant passage	1.98	2.12	3.01	3.41	3.21	2.55
5th plant passage	2.11	1.57	2.26	3.46	3.18	3.12
6th plant passage	2.10	1.61	1.98	3.11	3.41	3.91
	Nitrogen					
	per cent	per cent	per cent	per cent	per cent	per cent
Control	0.91	1.11	0.94	0.91	1.11	0.94
Agar culture	3.92	3.98	3.47	1.01	0.98	1.17
1st plant passage	1.18	3.15	3.51	1.09	0.97	1.12
2nd plant passage	1.33	3.21	3.40	3.41	1.17	1.77
3rd plant passage	1.78	1.66	2.99	3.39	1.40	1.90
4th plant passage	1.81	1.50	3.01	3.26	2.06	1.97
5th plant passage	2.00	1.09	2.34	3.20	2.97	2.77
6th plant passage	2.29	1.01	2.01	2.90	3.21	3.18

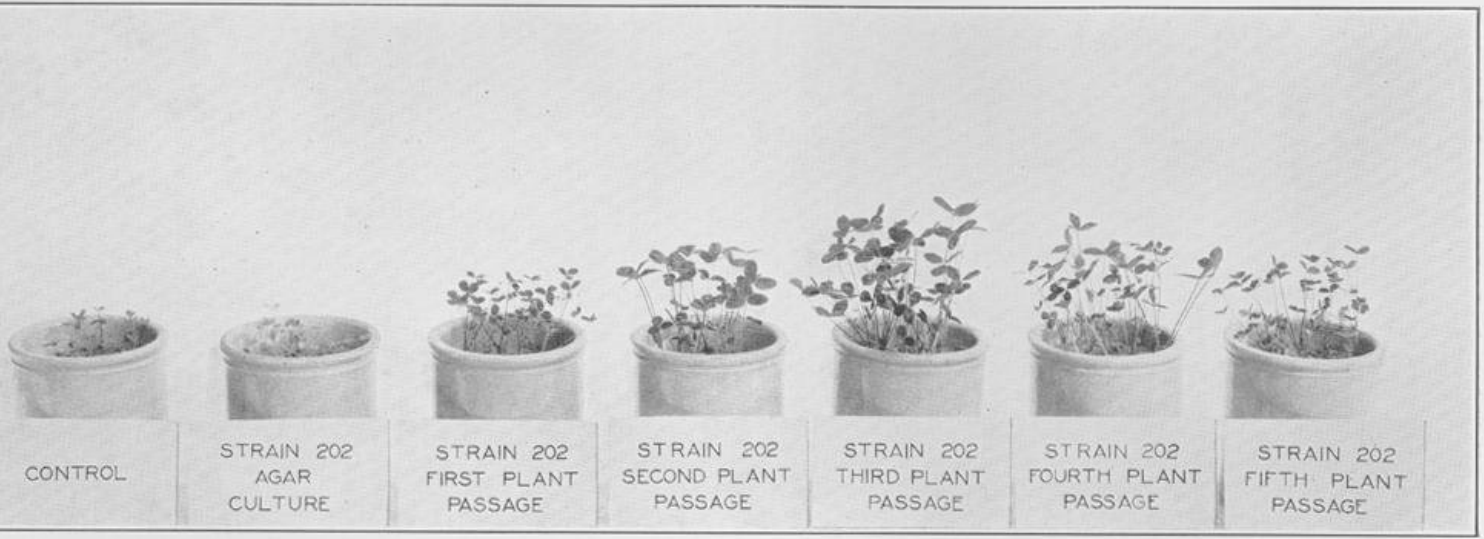
The experiments with alsike and white clover are identical, except for the host plant species. Planted 5-10-29, and harvested 7-6-29. The plant passage cultures are suspensions of crushed nodules from red clover plants 60 days of age.

The red clover experiment is the same except for the host plant species, strains of rhizobia used, and date of harvest, which was 8-5-29.

energy of the rhizobia to a point at which they are less efficient in aiding the plant. His work is vitiated to some extent by his failure to use adequate controls and by the fact that his original inoculum was a commercial culture, in which there may have been strains of varying degrees of effectiveness. A popular presentation of his results was published by Ehrenberg, 1925. Stapp, 1929, working with field peas and serradella, failed to confirm Wunschik's results. Although he did obtain certain differences in dry weight and nitrogen percentage among his test plants, he did not consider them significant. Allen and Baldwin, 1931a, studied the effect of repeated plant passage on both effective and ineffective strains *of Rh. trifolii, Rh. leguminosarum,* and *Rh. japonicum.* Their findings indicate that effective strains of the organism may become ineffective by repeated passage through the host plant, and *vice versa.* In some experiments the change of condition is effected by a single plant passage. In other cases the change is much more gradual and requires several passages to give complete reversal. In long-continued experiments it appears that the changes are cyclic; *i.e.,* an effective strain becomes ineffective and upon further passage returns to the effective condition. Essentially similar results were obtained with all three of the species of rhizobia investigated. The above Table 16 from Allen and Baldwin's publication gives the data of three typical plant passage experiments with *Rh. trifolii.* In these experiments the different series of plants were grown in sterilized sand low in nitrogen, and treated exactly alike except for the original inoculum. In the case of strains 200 and 201, which were originally effective strains, repeated passage through the host plant served to reduce the effectiveness. The reverse occurred with strain 202, which was originally ineffective. In the alsike clover experiment with strain 201, there appeared a sudden drop in efficiency coincident with the first plant passage. The same inoculum, when tested on white clover, exhibited no sudden change but rather a gradual loss of effectiveness through 5 plant passages. Plate 29 illustrates similar results secured in another experiment.

Effectiveness of a given strain of rhizobia seems to be a specific entity in the physiologic complex of the organisms operating within a given host plant, since change in effectiveness is not reflected in any known change in cultural, physiological, or serological properties of the organisms. Strains recovered from the host plants after several plant passages are in no way distinguishable from their parent cultures. The factors operating in the plant to produce change in effectiveness have not been determined. The importance of the change is beyond question. It is self-evident that ineffectiveness may be of considerable practical importance under field conditions.

Effectiveness as influenced by host-plant specificity. If effectiveness were a property of the bacteria alone, the problem would be complicated enough, but in reality effectiveness of the bacteria is an expression of their benefit to another organism, the host plant. The interaction of the bacteria and plant is necessarily complex, and it is to be expected that a replacement of the host by another species (though it be of the same inoculation group) will make a difference in the net result of the association. It is possible that a strain may be effective as tested by one association and yet be ineffective under other circumstances. Several reports dealing with this subject have appeared. Nobbe, Hiltner, and Schmid, 1895, noted


CONTROL
STRAIN 202 AGAR CULTURE
STRAIN 202 FIRST PLANT PASSAGE
STRAIN 202 SECOND PLANT PASSAGE
STRAIN 202 THIRD PLANT PASSAGE
STRAIN 202 FOURTH PLANT PASSAGE
STRAIN 202 FIFTH PLANT PASSAGE


CONTROL
13
20


CONTROL
20
13

that a strain of *Rh. leguminosarum,* though it formed nodules on both *Pisum sativum* and *Vicia villosa,* was not equally beneficial to the two hosts. Buhlert 1902a and b, confirmed the report with respect to the efficacy of nodules on the pea produced by inoculation with a culture from the broad bean, and Mann, 1918, stated that rhizobia from peas form nodules on vetch but give no fixation of nitrogen. Bialosuknia, 1923, and Bialosuknia and Klott, 1923, again reported the ineffectiveness of the rhizobia of the pea when brought in association with the broad bean, and further stated that the condition is not changed by two passages through the broad bean. Helz, Baldwin, and Fred, 1927, found that certain strains of *Rh. leguminosarum* are effective on *Pisum sativum* and *Lathyrus odoratus* but ineffective on *Vicia Faba,* whereas other strains produce the reverse effect. Other strains are effective on all hosts tested (that is, of the Pea Group), and still others are ineffective on all hosts. The following table and Plates 30 and 31 give the results of this experiment.

Host-plant specificity influences the effectiveness of certain strains of Rhizobium leguminosarum.

Strain	*Pisum sativum*	*Vicia Faba**	*Lathyrus odoratus*	*Vicia villosa*	*Lens esculenta*
	Dry weight per plant				
	gm.	gm.	gm.	gm.	gm.
Control	.314	.049	.429	.163	.455
3	.915		1.363	.375	.735
13	.777	.077	.488	.395	.626
15	.400		.310	.150	.450
20	.955	.066	1.268	.343	.663

*The *Vicia Faba* seed were planted in February and the others in April.

Miss Löhnis, 1930b, and Allen and Baldwin, 1931a, found that a strain of *Rh. trifolii* may be effective with one species of clover but ineffective with another. Hiltner, 1902, reported a similar experience with *Lupinus luteus* and *Lupinus angustifolius* and inferred that best plant growth is secured when the rhizobia of the partnership are derived from a previous plant of the same species. Eckhardt, Baldwin, and Fred, 1931, also found that strains of *Rh. lupini* are not equally effective on the various species of *Lupinus.*

Morse, 1915, and Voorhees, 1915, have carried the conception of plant-bacteria specificity one step further in their report of variation in effectiveness of *Rh. japonicum* as manifested in different varieties of the soybean. Leonard, 1916; Fred and Bryan, 1922b; and Perkins, 1925b; failed to confirm the results of Morse and Voorhees.. As a possible explanation of the difference of opinion, Fred and Bryan pointed out that the growing period of various soybeans differs. Consequently the time of formation and of disappearance of the nodules may not be uniform. Different stages of development at the time of harvest may then account in part for the apparent differences in effectiveness of nodulation. Recently, however, Erdman and Wilkins, 1928, have again reported varietal specificity. They secured best nodulation of the Peking variety of soybeans by inoculating with soil from around the roots of Peking-variety plants or with a pure culture directly from this variety. In another report from the same station,

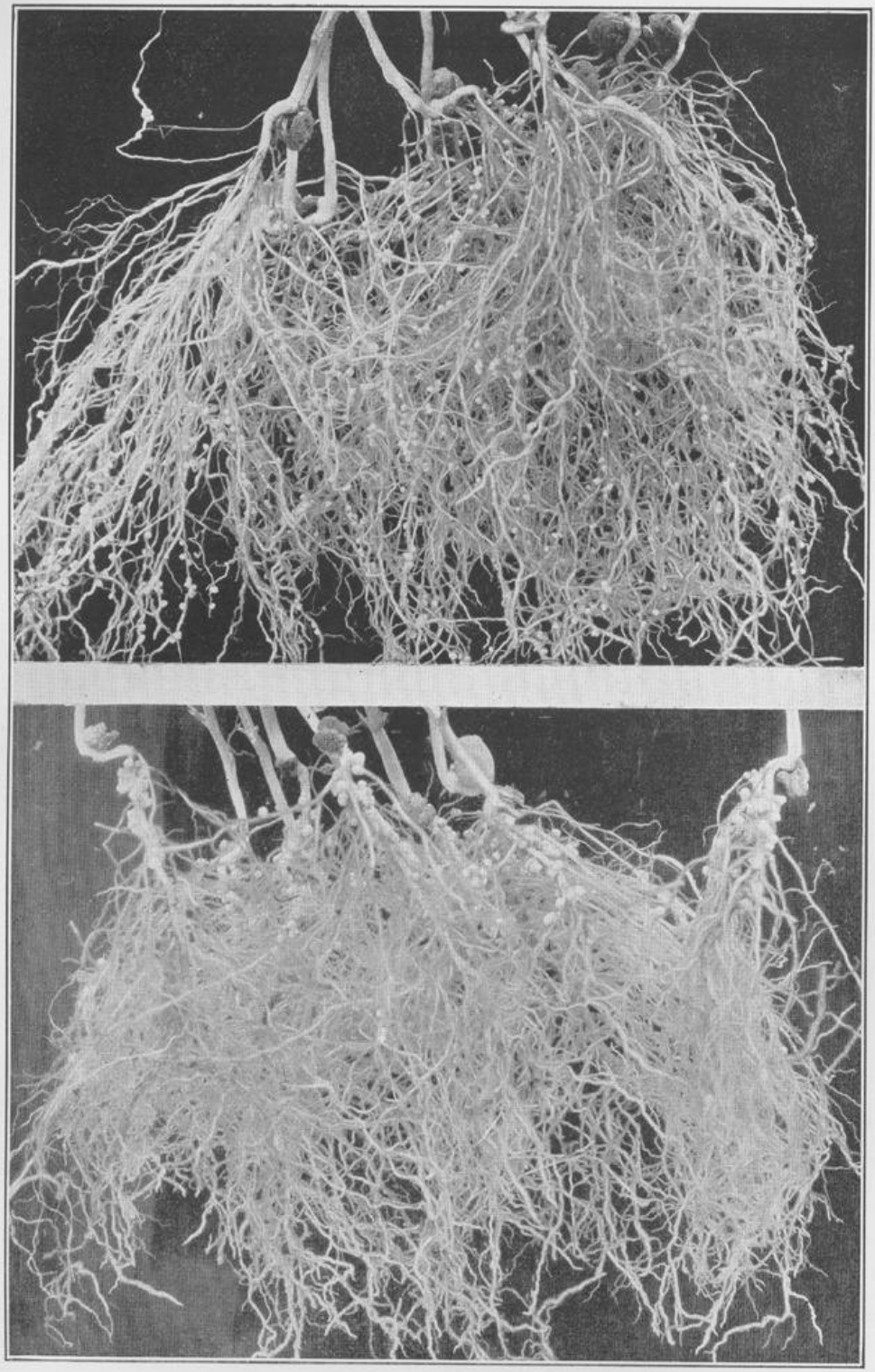
Pohlman and Walker, 1929, indicated that the apparent differences in the infection of varieties of soybean by various strains of the bacteria are due to other factors than the adaptation of the rhizobia to a particular variety of the host.

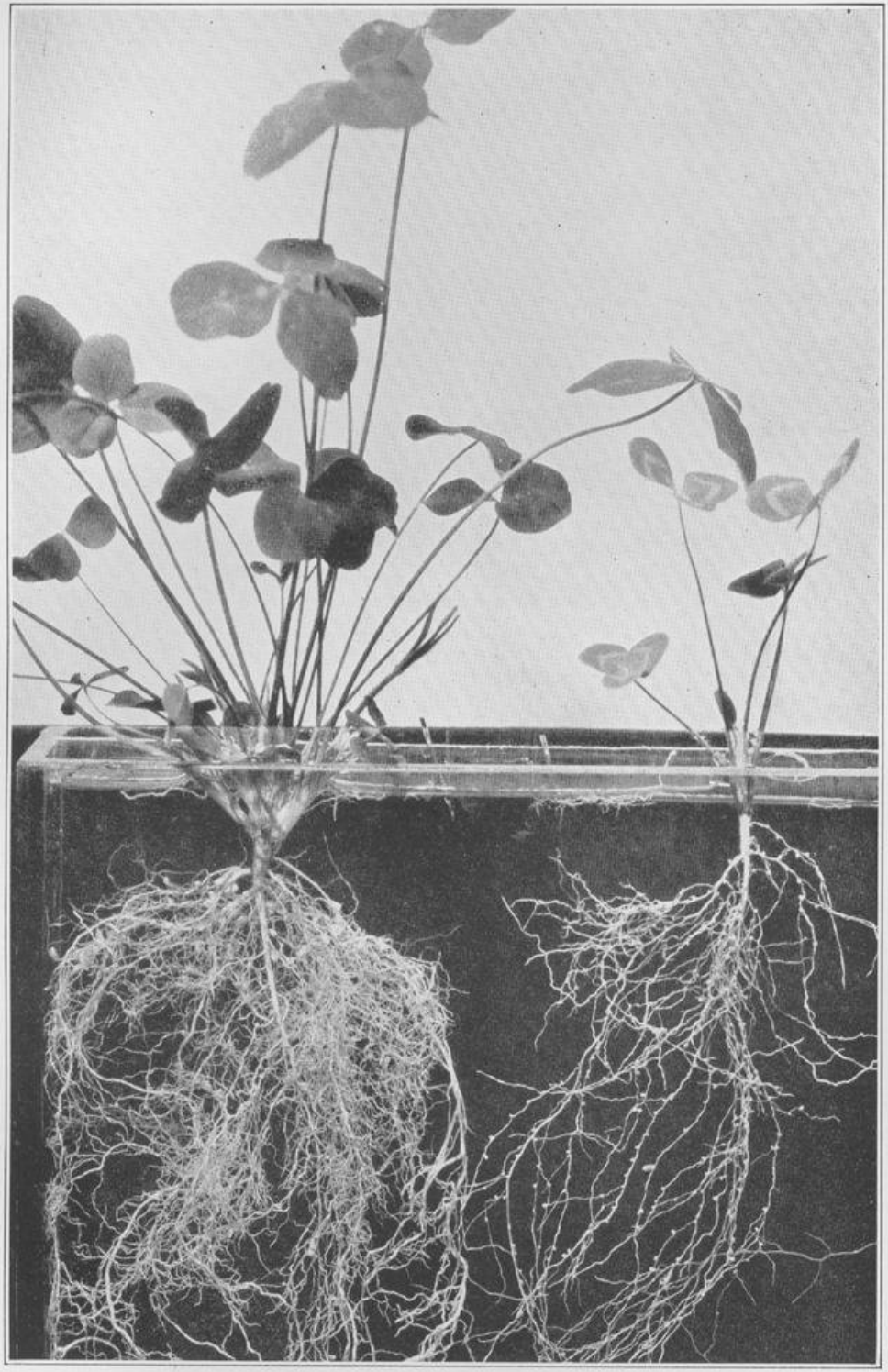
The apparent correlation between the type of nodulation and the benefit to the host plant. The size and shape of nodules are to a large extent characteristic of the plant species on which they occur, although modified to a certain extent by soil conditions, Leonard, 1927. In all cases the young nodules are spherical or somewhat ovoid. On the bean, soybean, and cowpea, the nodules remain spherical as they grow in size, but on the clovers and alfalfa, apical growth makes for elongation with increasing age. In some cases of apical growth, *e.g.* on the pea and vetch, branching occurs and the nodules become spherical or fan-shaped convoluted bodies.

Even before the true nature and function of nodules were known, there were attempts to correlate the size and abundance of these bodies with the vigor and growth of the host plant. Schindler, 1885; and Atwater and Woods, 1889 and 1890, suggested that nitrogen fixation by the leguminous plant was in some way dependent upon the number and size of the nodules on the roots of that plant. This idea gained a strong hold in the minds of many workers, who frequently recorded the number and size of nodules developed in the field or in an experiment as tangible evidence of the success of inoculation. The following statement from Dangeard, 1926, is typical of the view held by many, "Generally the vigor of plant growth is a function of the number of nodules existing on its roots." Few actual data to support such a statement, however, are to be found in the literature.

Giöbel, 1926, in his study of the effect of nitrate on nodulation and nitrogen fixation in the soybean reported that the fixation of nitrogen is roughly proportional to the mass of nodular tissue developed. Erdman, 1926, presented a study of the weight and content of nitrogen in small, medium, and large nodules of several varieties of the soybean. The percentage of nitrogen in the three classes of nodules is approximately the same. Erdman, therefore, concluded that the mass of the nodules is more important than their number in determining benefit to the host plant. On this basis he proposed a classification of nodulation in which one large nodule should be equivalent to ten small or two medium-sized nodules. Erdman and Wilkins, 1928, used this classification as the criterion of success in their study of various methods of inoculation. In three experiments dry weights of the plants and percentages of nitrogen were recorded; a positive correlation was found between these data, as expressing the effectiveness of the nodulation and the nodule mass. But since large, medium, and small nodules were thrown together in each case, it is impossible to determine whether the plants with few large nodules were better or poorer than those with many small nodules.

Boswell, 1929, classified nodule development on the peas as good, medium, poor, and none, apparently on the number and size of the nodules, although his basis of classification is not definitely stated. Using such a basis of classification, he observed, "Under uniform environmental conditions, plant weights and productivity are associated directly with degree of nodule formation." Thornton, 1929a, from results of a pot-culture experiment reported that the top weights of young alfalfa plants increase in nearly direct proportion to the number of nodules





upon their roots. Other workers, however, have differentiated between the two factors, number and size, in the relation of the nodules to plant benefit. Frank, 1890b, stated, "Die Zahl der Knöllchen ist aber eben kein Werth für die Beurtheilung der Produktion derselben, sondern sie giebt nur die Häufigkeit der Infektionen an." Beijerinck, 1918, made a similar statement, "The number of tubercles is of no consequence; it evidently suffices if only a few come to development."

Lawes and Gilbert, 1891, described two types of nodulation of the pea. In one case the nodules are small and scattered over the root system; in the other, "agglomerations of nodules somewhat as a raspberry or mulberry" are formed. Plants of the first type in their experiments possessed an estimated leaf-surface of 267 square inches, whereas those of the second developed 481 square inches. The papers of Nobbe, Hiltner, and their associates, particularly those of Hiltner, 1900a, 1902, and 1904b, and of Hiltner and Störmer, 1903a, are important for their early emphasis of the significance of the type of nodulation. Hiltner asserted that the position of the nodules is a function of the nutrition of the plant, the character of the soil, the distribution of the rhizobia, and their "virulence." He suggested that the first nodules formed on a plant contain more virulent bacteria than do those later formed on the lower roots, and he strongly emphasized that strains of rhizobia for the preparation of commercial cultures be selected from isolations from the first-formed nodules. This point was shown to be false by Remy, 1902, and others. Hiltner and Störmer, 1903a, describing a so-called parasitic strain of rhizobia on the pea, observed that it induced the formation of small nodules scattered over the entire root system. This phenomenon has been confirmed by several recent investigators. The work of Dehérain and Demoussy, 1900a, is interesting because it appears to be an exception to the general experience that a few large nodules are most beneficial. They found that lupine plants bearing large nodules contained less nitrogen than those bearing small nodules. Hiltner, 1902, likewise reported that large nodules on the lateral roots of the yellow lupine are absolutely worthless from the standpoint of the plant.

Recent work on strain variation by Stevens, 1925a; Wright, 1925b; Helz, Baldwin, and Fred, 1927; Cunningham, 1928; Thornton, 1929a; Baldwin and Fred, 1929a; Löhnis, 1930b; and Leonard, 1930, has shown that the more effective strains produce few, but large, nodules usually located on the upper parts of the root system, whereas ineffective strains form numerous small nodules widely distributed over the root system. Plate 32 is a photograph of the type of nodulation produced by effective and ineffective strains of *Rh. leguminosarum*. Plate 33 is a similar illustration for strains of *Rh. trifolii*.

The importance of soil type and plant nutrition in determining nodule size and placing was pointed out by Maassen and Müller, 1906. They stated that "ist der Ernährungs- und Gesundheitszustand der Pflanzen für die Anzahl der Knöllchen und für ihre Grösse massgebender, als die Wirksamkeitshöhe (der Virulenzgrad) der Bakterien." This view was reaffirmed by Müller and Stapp in 1925. By raising pea plants in the shade, they induced the formation of numerous small nodules widely scattered over the root systems, whereas upon plants grown in normal light the same culture gave rise to much larger nodules. Billings, 1906, in a field experiment with alfalfa made

some observations showing the fallacy of any arbitrary statement that a few large nodules necessarily mean efficient partnership with the rhizobia (however true the converse may be). According to Billings' observations spots of "poor soil" in the field were associated with yellow plants bearing clusters and aggregates of nodules; the rest of the field containing "good soil" yielded good green plants with smaller, more numerous, and single nodules. No analysis of the cause of this condition was made. The rhizobia of all parts of the field were presumably alike and the difference observed was due to local conditions of the soil or within the plants. S'Jacob, 1927, carefully studied the effect of various inorganic ions on the growth of the leguminous plant and discovered that the nodule size and placing is to some extent governed by the nutrition of the plant. The calcium and phosphate ions are particularly influential in this respect. A high concentration of phosphorus permits the formation of fewer nodules than does the normal concentration. However, the nodules on plants in a high phosphorus substrate grow to a larger size. Boswell, 1929, evidently agreed that the condition of the plant is of importance in nodule development, for he wrote, "If the nitrogen-fixing organisms are to benefit the plant greatly, good plants must be grown that can benefit the masses of organisms in the root-nodules."

Jones and Tisdale, 1921, studied the effect of temperature on nodule formation and reached the conclusion that the maximum weight of nodules on the soybean occurs upon plants grown at 24° C. Diminution in weight of nodules as the temperature is increased or decreased is much greater than the diminution in weight of either tops or roots. Thus temperature is an important factor in any experiment in which it is attempted to correlate nodule mass and benefit derived by the plant as reflected in dry weight of tissue.

In short it may be stated that *under normal and comparable conditions* the development of large nodules, few in number and concentrated on the tap root and first lateral roots, is the desirable type of nodulation and is indicative of positive benefit to the host plant. On the other hand, large numbers of nodules, particularly of small nodules scattered over the entire root system, may be of the parasitic type, from which the plant derives little or no benefit. However, the number of nodules present at the actual time of harvesting may be misleading; particularly in the case of field-grown plants the nodules may largely disappear as the plant reaches maturity or earlier under certain conditions of drying of the soil (see the latter part of this chapter).

Anaerobic conditions and parasitism by the rhizobia. Even before the true nature of nodules was recognized, it was noted by Rautenberg and Kühn, 1864; Schindler, 1885; and Buckhout, 1890, that leguminous plants growing in water culture bear few nodules. Hiltner, 1900a, pointed out that upon *Robinia* plants whose roots are entirely submerged, there are formed a large number of small nodules scattered over the root systems. On plants raised partly out of the water, the nodules developed on the upper part were few in number and grew to a larger size; these plants made better growth. Nobbe and Hiltner, 1899c, reported that nodulated leguminous plants grown in water culture fix far less nitrogen than similar plants grown in sand. Moore, 1905, held that lack of air to the nodule operates to keep the bacteria in the rod form and prevents their conversion to the bacteroid form, which is active in nitrogen assimilation and benefit to

the plant. Recent publications by Jones, 1926; Fehér and Bokor, 1926; and Thornton, 1930a, have again claimed that deficiency of air interferes with the functioning of nodules and tends to make the organisms "become parasitic."

Parasitic strains of rhizobia and soil sickness. The fact that under certain conditions, strains of rhizobia tend to become ineffective when subjected to continued plant passage (Allen and Baldwin, 1931a), suggests that such a condition might arise in fields where a particular leguminous crop has been cultivated for several years. This possibility was pointed out as early as 1908-09, by Simon, who demonstrated that soil sickness for clover and peas may be produced by repeated growth of the respective crops. Simon attributed soil sickness to increased virulence of the rhizobia, although he apparently made no allowance for change in general soil fertility, plant disease, etc. Budinov, 1907, made isolations of rhizobia from good and "clover-sick" soils in an attempt to find differences in the number or character of the organisms harbored in each. His results gave no positive evidence which implicates the rhizobia in clover sickness.

Recently Leonard, 1930, has described a case of the failure of peas, which was apparently due to parasitic rhizobia. In a field to which no inoculation had been added and in which no crops of the pea cross-inoculation group had been raised for at least 6 years, there were a few isolated spots in which the plants made good growth. In the major part of the field, however, the plants were yellow, stunted, and sickly. The good plants bore large clusters of convoluted nodules, and the poor plants many small nodules widely scattered over the root systems. Isolation of organisms from these plants yielded parasitic or ineffective strains from the stunted plants and effective strains from the healthy plants.

Undoubtedly many cases of soil sickness for leguminous crops are due to factors other than ineffectiveness of rhizobia; plant disease, acidity of the soil, accumulation of toxic wastes, and other possibilities have been suggested. The subject needs more thorough study in all its aspects, but there does seem to be ample evidence that the presence of ineffective strains of rhizobia may at times be the immediate factor. The occurrence of the ineffective strains in nature is a phase of the subject almost untouched at the present time. Baldwin and Fred, 1930, made an initial survey of Wisconsin soils for ineffective strains of *Rh. leguminosarum.* According to their findings, the ineffective rhizobia may be widely distributed and appear to be harbored in the wild Leguminosae.

The mechanism of nitrogen fixation. The fact of utilization of atmospheric nitrogen by the leguminous plant when in association with the appropriate rhizobia was definitely established by Hellriegel in 1886a and was soon confirmed by a host of other investigators. Yet the exact mechanism of this fixation and the part played by each of the symbionts is not at all clear. To the authors it appears that perhaps the effort to divorce the rôles of plant and bacteria in the process of nitrogen fixation is fundamentally wrong. The plant-bacterial association is so intimate that one might conceive of the association as constituting a new form of life, possessing new and unusual properties. May it not be that in partnership plant and bacteria can accomplish what neither could do alone? Perhaps the one carries out initial stages which the other seizes upon and assimilates, thus accomplishing the net effect which we call nitrogen fixation. The hypothesis most generally accepted is that the rhizobia fix the nitrogen in the root nodule and

that some compound of nitrogen is then utilized by the higher plant. Beijerinck's early observation, 1888, that not all nodules contain bacteroids, and his belief that only those containing bacteroids are the nodules effective in fixing nitrogen, were followed by more extensive studies by Nobbe and Hiltner, 1893. They were convinced that there is no benefit to the plant nor fixation of nitrogen until the bacteria are changed to the bacteroid state. Their view was probably due in part to the earlier and widely accepted belief that the bacteroids were "protein-storage bodies" which nourished the plant with nitrogen (Brunchorst, 1885a; Beijerinck, 1888; Prazmowski, 1890; and Frank, 1891). Hiltner and his associates reaffirmed this view, and for many years it was prevalent in the literature.

Recently the investigations of Pfeiffer, 1928; and Miss Löhnis, 1930b, have shown that the coincidence of bacteroid formation and nitrogen fixation is not invariable. Miss Löhnis did find that her effective strain of *Rh. leguminosarum* produces the bacteroid form in the nodules, while her ineffective strain does not, but with *Rh. trifolii* she found bacteroidal forms produced by both effective and ineffective strains. She concluded, "It seems very probably, therefore, that in pea the occurrence of bacteroids is indispensable for the supply of nitrogen to the host plant. The study of the clover nodules, however, showed that from the occurrence of bacteroids in the nodule, nothing can be inferred as to the existence of any assimilation of nitrogen in the host-plant." Miss Löhnis also studied the distribution of starch in the nodules and with peas noted an abundance of starch granules in the nodules produced by the ineffective strains. No such relationship was apparent with clover. The bean nodule also contains a large accumulation of starch, a fact which may be associated with the reputed parasitic nature of the rhizobia of the bean (McCoy, 1929).

Mazé, 1898, and Greig-Smith, 1907, advanced the theory that nitrogen fixation is associated with the formation of gum or slime by the rhizobia. Mazé held that the slime, itself a product of sugar decomposition, is able to combine with nitrogen and thus becomes the nitrogenous compound taken up by the plant. Consequently those conditions, either in the plant or in artificial culture, which encourage the formation of slime are conducive to nitrogen fixation.

Schloesing and Laurent, 1892a and b, and Alpe and Menozzi, 1892, first demonstrated that the gain in nitrogen by a nodulated leguminous plant is actually accompanied by an intake of atmospheric nitrogen. In their experiments entire plants were confined in an air-tight vessel, and determinations of the gaseous nitrogen within the vessel were made before and after a sufficient period of plant growth. Frank, 1889 and 1890b, believed that assimilation of nitrogen occurs through the leaves, and that the presence of the bacteria merely enhances a process preëxisting in less degree in the normal leguminous plant. Stoklasa, 1895, also believed that fixation of nitrogen occurs in the leaves. Gasometric experiments by Kossowitsch, 1892, and by Whiting, 1915, have demonstrated that the nitrogen is actually absorbed through the roots of the nodulated plant, presumably through the nodular tissues, since nodule-free plants are inert. Still there is not complete agreement as to the seat of nitrogen assimilation. In one of his last published papers Beijerinck, 1918, showed that nodules removed from the plant do not fix atmospheric nitrogen. From this he argued that they do not do so on the plant. The conditions of his experiment, however, were far different from those

obtaining in nodules attached to the plant, and it is very doubtful whether his conclusion concerning the functioning of nodules on the plant is valid. Probably the work of Burk, 1927b, is the most careful yet done to refute the early idea of Frank and others that the fixation of nitrogen is a function of the plant alone. He showed that plants of *Pisum sativum,* grown without rhizobia, either with or without added nitrogen in the substrate, show "a small unqualifiable loss of nitrogen," after the seed nitrogen and any initial nitrogen of the substrate are exhausted.

The time at which nitrogen fixation occurs in relation to the age of the plant has been studied by many workers. In general they are agreed that the process of nitrogen fixation begins as soon as, or shortly after, the formation of the nodules and continues as long as the nodules remain firm and healthy, and the plant is actively growing. Müller and Stapp, 1925, noted that young pea plants, originally nodule-free, show a definite effect from inoculation within 10 days after the culture is applied. The plants by that time are plainly greener and show signs of more adequate nitrogen nutrition. Several workers have observed what is termed the "hunger period" immediately following the absorption of all the seed nitrogen and before the fixation of atmospheric nitrogen starts (Bréal, 1899b; Wunschik, 1925; and Pfeiffer, 1928). Although this period may occur under some conditions, it does not appear to be necessary and inevitable. From a study of the rate of nitrogen fixation as related to the age of cowpea seedlings, Whiting and Schoonover, 1920b, pointed out that a small fixation of nitrogen is sometimes evident before the appearance of the first true leaf. Surely at such an early stage there is still reserve nitrogen in the cotyledons. Other factors are more intimately concerned with the rate of fixation, according to the findings of Whiting and Schoonover. Chief among these are the rate of growth of the seedling and particularly the rate of carbohydrate synthesis as related to sunlight and leaf surface. The coordination between carbohydrate synthesis and nitrogen fixation has been pointed out by several workers (Schindler, 1885; Müller and Stapp, 1925; Leonard, 1926; Rippel and Poschenrieder, 1928; and Thornton, 1930a). In view of this interdependence of nitrogen fixation and carbohydrate synthesis, it follows that the rate of fixation increases with increase of effective leaf surface. *i.e.,* as the plant approaches maturity (Brown and Stallings, 1921; and Giöbel, 1926.)

A further line of evidence that the seat of nitrogen fixation is the nodular tissue is to be found in the data for nitrogen content of the nodules *versus* that of other parts of the plant. (See Chapter 12 for complete data and references.) As the plant develops from the seedling stage to maturity, the percentage of nitrogen gradually falls, as indicated in the following analyses of soybean nodules (Erdman, 1929).

Percentage of nitrogen in the nodules of four varieties of soybean at various dates

Dates	Manchu	Dunfield	Midwest	Peking
July 28	5.52	5.24	4.46	4.80
August 20	5.79	5.42	5.44	4.98
September 2	5.25	4.50	4.37	4.71
September 28	4.20	4.17	4.95	3.88
October 10	2.37	4.02	3.70	3.57
October 13	----	3.59	3.75	3.55
October 26	----	----	----	3.18

In many species of leguminous plants, particularly in the case of annuals, the nodules are emptied as the plant approaches maturity. This is particularly noticeable with beans, upon whose roots the empty hulls of the nodules may be found at the time of seed formation (Schindler, 1885). Stoklasa, 1895, determined the nitrogen in the nodules of lupines, as the plants developed through blossoming and setting of seeds. He found that the percentage of nitrogen progressively decreases as seed formation proceeds; in fact, by the time the seed is ripe, the percentage of nitrogen in the nodule has fallen to approximately that of the remainder of the root.

	Blossoms formed	Seed forming	Seed ripe
	per cent	per cent	per cent
Nitrogen in nodules	5.22	2.61	1.73
Nitrogen in roots	1.64	1.84	1.42

Such findings would seem to indicate that the nodule is the source of the nitrogen from which the plant draws to meet its needs for protein storage in the seeds. The decrease of nitrogen in the nodule at this stage is apparent only because the nodule itself is on the down grade of its activity, its contents softening and being absorbed or decaying. It is thus necessary to know the age and condition of nodules in order to correctly interpret analyses of their nitrogen content. There may, however, be another factor influencing the level of nitrogen to be found in nodule tissue. Virtanen, 1927 and 1928b, obtained the following data showing the influence of pH on the nitrogen content of nodules.

Percentage of nitrogen in nodules

	Peas	Red clover	Alsike clover	White clover
pH 6.0	4.23	4.31	8.05	5.63
pH 5.5	3.71	3.61	7.14	5.36
pH 5.0	3.29	3.05	5.95	5.03

The dry weights and nitrogen content of the plants in Virtanen's experiment also reflected the unfavorable effect of the lower pH.

The forms of nitrogen in the nodule have been studied by several workers, (Troschke, 1884; Hutchinson and Miller, 1911; Sani, 1910; Klein, 1913; Herke, 1912; Whiting, 1915; Whiting and Schoonover, 1920b; Strowd, 1921; Stallings, 1926; Parisi and Masetti-Zannini, 1926; and Virtanen, 1928b). Sani, 1910, demonstrated the presence of free amino acids, such as glycine and 1-asparagin, in the nodules of *Vicia Faba*. Strowd, 1921, reported the following distribution of the nitrogen in soybean nodules:

	per cent
Water soluble	28.6
Soluble in 10 per cent NaCl sol.	34.1
Soluble in 70 per cent alcohol	7.0
Soluble in 0.3 per cent NaOH sol.	36.0

In another experiment he found that the hydrolyzed nitrogenous material gave 16.3 per cent of the nitrogen in the amino form, 19.3 per cent as amide nitrogen, and 62.6 per cent as basic nitrogen.

The investigations of Parisi and Masetti-Zannini, 1926, are probably the most complete yet published on the subject of the nitrogen content of nodules. They found considerable amounts of free amino acids in the nodules of *Vicia Faba* and lesser amounts in those of *Lupinus albus*. The solubilities of the nitrogenous compounds of the nodules are reported as follows:

	Vicia Faba		*Lupinus albus*	
	Percentage of		Percentage of	
	Dry substance	Total N	Dry substance	Total N
Soluble in alcohol	0.23	3.35	----	----
Soluble in water	1.80	26.23	1.32	31.96
Soluble in 10 per cent NaCl sol.	----	----	Trace	----
Soluble in 0.05 per cent KOH sol.	2.00	29.15	2.83	68.52
Insoluble	2.94	42.85	0.44	10.65
Total	6.97	101.58	4.59	111.13

Upon hydrolysis of the proteins of the *Vicia Faba* nodule they obtained:

	per cent
Ammonia nitrogen	18.80
Humic nitrogen	12.53
Diamino nitrogen	16.91
Mono amino nitrogen	50.87

The significance of the amino-acid content of nodules is shown by the results of Virtanen, 1928b, 1929b; and Virtanen and Hausen, 1931a and b, who showed that red clover is able to utilize amino acids better than inorganic nitrogen, whereas the reverse is true of white clover.

As might be expected from the high content of nucleic acids in bacteria, the phosphorus content of nodules is high. Troschke, 1884, found 16.19 per cent P_2O_5 in the ash of lupine nodules and only 8.84 per cent in the ash of the roots of the same plants. Virtanen, 1928a and b, found the phosphorus content of the nodules of red, white, and alsike clover higher than that of either the roots or tops. On the contrary, in the pea the percentage of phosphorus in the root tissue is slightly higher than in the nodules.

It is a common statement that the nitrogen content of the leguminous plant is increased as a result of effective nodulation. Weber, 1920, has inquired more specifically into the nitrogen content of seeds developed by lupine plants with and without nodules. He also compared nodule-free plants supplied with ammonia and nitrate nitrogen in lieu of nodules.

The influence of inoculation and fertilization on the nitrogen and alkaloid content of the seed of Lupinus angustifolius

Plants	Total nitrogen	Alkaloid content
Nodule-free	100	100
Nodule-free plus $(NH_4)_2SO_4$	229	558
Nodule-free plus $NaNO_3$	236	678
Nodule-bearing	554	1648

Apparently the types of nitrogen compounds supplied through the nodules are better suited to the storage of nitrogenous reserves in the seed than are the inorganic forms of nitrogen usually considered requisite to a complete plant-nutrient solution. Behlen, 1924, and Giöbel, 1926, found that application of fertilizer did not change the nitrogen content of well-nodulated plants. Iwanoff, 1927, in a study of the seeds of leguminous plants gathered from widely separated geographical regions, found a remarkable constancy in their protein content.

The chemistry of the actual process of fixation of nitrogen is not yet understood. From the chemical standpoint there are three possible processes by which the nitrogen may be fixed—oxidation, reduction, or direct union of nitrogen with some organic compound. Tests for the presence of simple compounds of nitrogen in nodules have proved negative, when adequate precautions have been taken to exclude such compounds from the culture medium. For example, Whiting, 1915, and Whiting and Schoonover, 1920b, failed to detect ammonia, nitrite, or nitrate, and Strowd, 1921, found no cyanide. However, when the plants are grown in soil or nutrient solution containing ammonia, nitrites, or nitrates, these compounds may be found (Stoklasa, 1895; Loew and Aso, 1908; Hutchinson and Miller, 1911; Klein, 1913; Strowd, 1920; and Stallings, 1926). The various theories for the process of biological nitrogen fixation have been discussed and critically examined by Blom, 1931. Consideration of a variety of established physiological facts, *i.e.*, influence of nitrates, ammonium compounds, and oxygen on fixation, lead Blom to the conclusion that fixation occurs through a reduction process. He suggested that the fixation proceeds by means of the hydration of nitrogen with iron as a catalyst, and that the first product formed is hydroxylamine and not ammonia. His scheme can be written—

$$N{\equiv}N \text{ (Atmospheric)} \rightleftarrows N{\equiv}N \text{ (Solution)},$$

$$2 \text{ (Organic Fe++)} + N{\equiv}N \rightleftarrows (\text{Organic Fe++})_2\ N{\equiv}N,$$

$$(\text{Organic Fe++})_2\ N{\equiv}N + 2H_2O \rightleftarrows (\text{Organic Fe++})_2\ HONH{-}HNOH,$$

$$\text{Organic Fe++})_2\ HONH{-}HNOH + 2H^+ \rightleftarrows 2 \text{ (Organic Fe+++)} + 2\ HONH_2$$

$$(\text{Organic Fe+++}) + H \rightleftarrows (\text{Organic Fe++}) + H+.$$

In support of this idea Blom cited numerous observations which are consistent with the theory. Probably the most plausible are those which discuss the effect of nitrate on the fixation process; however, all of the evidence is of an indirect nature.

A review of the literature dealing with the energetics of nitrogen-fixation by rhizobia has been made by Wilson and Peterson, 1931, in a general paper concerned with energetics of heterotrophic organisms. They pointed out that any discussion of the energetics of nitrogen fixation by the rhizobia is even more uncertain than that of the free-living forms *Azotobacter* and *Clostridium*. Since it has not been conclusively demonstrated that the rhizobia can fix any nitrogen in the absence of the host plant (indeed, the best evidence at present is to the contrary), it is useless to draw conclusions from the results of the several investigators who have found small positive gains. A review of these investigations will be found in the recent papers of Hopkins, 1929; Allison, 1929; Miss Löhnis, 1930a; and in Chapter 6 of this monograph. Nevertheless, because of the possible relation of all forms of biological fixation of nitrogen, it is well to consider briefly some of the more important reports on the free-living nitrogen-fixing organisms.

Kostytschew, Ryskaltschuk, and Schwezowa, 1926; and Kostytschew and Schwezowa, 1926, claimed that *Azotobacter* fixes atmospheric nitrogen by reduction; that is, that ammonia is the first product formed. Their claim rests primarily on the detection of ammonia in cultures. Still larger quantities of amino acids are present in the cultures, but these Kostytschew believed to represent the second step in the fixation process. The absence of nitrate, nitrite, or urea nitrogen and also the known ability of *Azotobacter* to effect reduction, as of nitrate nitrogen, are also offered by Kostytschew as indirect evidence that the first step in fixation is the production of ammonia. Truffaut and Bezssonoff, 1927, reasoning from the results of Kostytschew with *Azotobacter,* arrived at similar conclusions for *Clostridium pasteurianum.*

Meyerhof and Burk, 1928; Burk, 1930a and b; and Burk and Lineweaver, 1930 and 1931, have made a thorough study of the efficiency of the growth of *Azotobacter* in medium containing free and fixed nitrogen. Burk found that the efficiency varies with the partial pressure of the oxygen present and that the rate of fixation is approximately directly proportioned to the pressure of nitrogen. As a result of these studies he concluded that the first step in the fixation by *Azotobacter* concerns a compound that is in equilibrium with the nitrogen of the air. This key compound is formed instantaneously from free nitrogen as rapidly as it is used in growth, and the concentration present at all times is proportioned to the pressure of nitrogen. Since its formation is represented by an equilibrium reaction with high reversibility, the free energy involved is zero. Subsequent changes of this key compound in the *Azotobacter* cell may require free energy, since the necessary reaction must be irreversible.

Nitrogen fixation by rhizobia in association with the leguminous plant is still more complicated by the complex metabolism of the latter. Suitable technique has not yet been developed for differentiating the energy used by the microörganism from that used by the plant. Nevertheless, it is interesting to consider some of the attempts at penetrating the secret processes of the nodulated plant, speculative though these attempts may be. If we reason by analogy that the fixation is similar to that of *Azotobacter* and the efficiency is of the same order, the figures reached are highly improbable. Thus Christiansen-Weniger, 1923, has shown that for typical field experiments the energy utilized for fixation

would require a carbohydrate destruction of 2 or 3 times the carbohydrate of the total yield of the plants themselves. He therefore argued that the process of fixation by the rhizobia must be much more efficient than that of *Azotobacter*. The obvious suggestion is that the fixation must occur by an exothermic process, in support of which Christiansen-Weniger offered the following evidence. He found that the total dry weight of lupines, beans, and alfalfa grown in the presence of $NaNO_3$ showed only a slight increase over that of nodulated plants grown in the entire absence of fixed nitrogen. From the differences obtained, he calculated the energy utilized for fixation of nitrogen on the basis of 1 gm. of dry plant tissue as equivalent to 4.0 Kg-Cal. A typical set of results follows:

Plant	Total dry weight		Difference	N fixed	Energy for fixation of 1 gm. N
	Without N	With N			
	gm.	gm.	gm.	gm.	Kg-Cal
Bean	50.746	56.572	5.826	1.021	22.800
Alfalfa	60.659	68.487	7.828	1.184	28.884

For the fixation of 1 gm. of N by the commercial methods, the requirement is about twice this amount. On the basis of these results Christiansen-Weniger concluded that the energy involved in the fixation, since it appears to be so small in amount, is probably used for the vital activity of the bacteria, and that the actual fixation of nitrogen is accomplished by an exothermic reaction, which yields additional energy to the bacteria for their cell processes. He stated that the most likely mechanism is the direct reduction of nitrogen.

Rippel and Poschenrieder, 1928, took issue with Christiansen-Weniger, pointing out two sources of error in his work: (1) the nitrogen for the control (nodule-free) plants was supplied as nitrate, which in the plant would require energy for reduction; (2) the final difference in dry weight between the control and nodulated plants is not necessarily a measure of the total carbon that was involved in the fixation. It has been observed by Kostytschew, 1922, that the power of CO_2 assimilation of leguminous plants for a given leaf area is two to three times that of other plants, and that if part of the leaves are prevented from assimilating CO_2, there is a compensatory increase on the part of the other leaves. Hence Rippel and Poschenrieder claimed that the nodules could have utilized a great deal more carbohydrate than indicated by the Christiansen-Weniger results and still have little influence on the final dry weight of the plant. As added evidence that carbohydrates are being actively burned in the nodule, Reinau, 1927, pointed out that the "earth respiration" of leguminous plants is much higher than that of non-leguminous plants such as mustard or rye. Rippel and Poschenrieder, therefore, discarded the idea that the nitrogen is fixed by an exothermic process. They reasoned by analogy from the Heats of Formation of NH_3, and HNO_2, and HNO_3 and N : C ratio of autotrophic *Nitrosomonas* and *Nitrobacter,* that if *Rhizobium* were autotrophic, the N fixed: C assimilated would be of the order of 200. Thus for the formation of 1 gm. dry weight of bacteria (50 per cent carbon), 100 gm. of N would be fixed; this Rippel and Poschenrieder

dismissed as a preposterous figure. They also raised the question as to the origin of the hydrogen necessary for reduction, if such is to be the mechanism of fixation. It is possible that the hydrogen arises from water, but this would require energy. To secure the necessary 3 H for the reduction of one atom of N, 103.5 Calories would be required to liberate hydrogen from water. (Rippel and Poschenrieder considered heats of reaction rather than free energy in all their calculations.) Since only 21 Calories are liberated in the formation of NH_3, there is a net loss of 84.5 Calories. To secure this energy, 22.5 gm. of glucose would have to be oxidized. Hence 620 mg. of N might be fixed per gram of sugar used; in reality, if a share of the energy is diverted to supply the needs of the vital processes of the bacteria, the quantity of nitrogen fixed per gram of carbohydrate used would be somewhat lessened. Even so the figure is too high, in the opinion of Rippel and Poschenrieder, and it is their conclusion that the hydrogen may arise rather from a fermentation process, similar to that by which hydrogen is derived by intramolecular exchange in alcoholic fermentation. In that case 4 gm. of fermentation hydrogen arise from every 180 gm. of glucose fermented. If this hydrogen were used entirely for the reduction of nitrogen to ammonia, a fixation of about 100 mg. of N per gram of sugar destroyed would result; such an amount is still ten times that fixed by the average young culture of *Azotobacter*. There is, of course, the alternative possibility that the rhizobia fix nitrogen by an oxidative rather than a reductive process. However, in such a case the nitrate formed would require to be reduced to ammonia before its nitrogen could be built into cell substance, and this would involve more energy than that gained by the oxidation.

Allam, 1931, has repeated Christiansen-Weniger's experiment with modifications that should give more reliable results. Firstly, several series of control plants (soybean) were *periodically supplied* with different levels of nitrogen, as NH_4NO_3, in order that the growth of the inoculated plants would be compared with suitable controls, *i.e.*, controls which had grown at the same *rate* as those inoculated. To determine which of the control series corresponded in growth rate with the inoculated series measurements were made of total ash, chlorophyll and leaf surface. The nitrogen content and dry weight of both controls and inoculated plants were determined and from these data the theoretical dry weight of the inoculated plants was calculated on the basis of Mitscherlich's formula. This theoretical weight, (based on nitrogen content of plants) was compared with the weight actually obtained and from these results, the energy required for fixation was determined. In the first experiment conducted in the summer, 104 Cal. were required per gram of N fixed while a second experiment in the fall required only 50.4 Cal.; these differences Allam ascribed to climatic factors. It is to be noted that these are 2 to 4 times as large as the values found by Christiansen-Weniger.

Burk, 1927a, made a theoretical study of the various mechanisms which might account for the fixation of nitrogen by the rhizobia. He stressed the point that the actual formation of the primary compound is the question involved, and that any subsequent reactions, in which this compound is concerned, are a part of the nitrogen metabolism of the bacteria and of the plant. Any energy changes involved in these later reactions should be distinguished from those of the fix-

ation. Only from the point of view of the practical or economic significance of nitrogen fixation must the "board-bill" of the microörganisms be considered a part of the toll exacted for increasing the protein content of leguminous plants. Burk further suggested that the hydrogen of the air might be drawn upon for the reduction reactions. The data available indicate that the atmosphere contains about 0.01 per cent of hydrogen. This is in itself a small amount; yet it is of the same order as the CO_2 content of the air. Burk also pointed out that if the nitrogen were fixed as nitrate, the latter might be reduced in the leaves to NH_3 by radiant energy and at no expense to the plant.

In view of the diverse opinions it must be concluded that the energy relations of the symbiotic fixation are as yet in the realm of unsolved problems.

The passage of nitrogen from bacteria to the plant. If it is granted that the bacteria of the nodule are the agents of nitrogen fixation, the mechanism by which these nitrogenous compounds are passed on to the plant becomes important. Four general theories have been proposed to account for the liberation of the nitrogenous compound, at first supposed to be locked up in the bodies of the bacteria. These are briefly: (1) plant enzymes may attack the bacteria and change their complex nitrogenous substance into a form which may be assimilated by the plant; (2) soluble nitrogenous compounds may be excreted by the bacteria; (3) death and autolysis of the bacterial cells may liberate the nitrogenous compounds in forms available for plant nutrition; (4) a bacteriophage capable of lysing the rhizobia may account for the production of soluble nitrogenous materials.

A number of different enzymes have been detected in the nodular tissue; for example, *urease* by Benjamin, 1915; Werner, 1923; Beijerinck, 1923; and Hutchinson, 1924; *proteolytic enzymes* by Fermi and Buscalioni, 1899, and the Tennessee Agricultural Experiment Station, 1919; *tyrosinase* by Stapp, 1923. In addition Hiltner, 1900a, by filtration of a nodule extract, was able to obtain a substance causing root hairs to swell and curl. None of these investigations, however, attempts to explain the mechanism by which the nitrogenous compounds are made available to the plant. A number of the early investigators (Brunchorst, 1885a; Beijerinck, 1888; Prazmowski, 1890; and Frank, 1891) asserted that the bodies of the bacteria in the bacteroid form are "absorbed" by the plant, and Dangeard, 1926, stated more specifically that the bacteria are dissolved by the parasitized cells, largely through the activity of the cell nuclei, and that the value of the nodule to the plant is dependent upon this phagocytic action.

Very soon after the formation of the nodules and long before there is apparent dissolution of the bacterial cells, the plant shows indications of benefit from nodulation. This fact supports the theory advanced by Nobbe and Hiltner, 1893; Golding, 1905-6 and 1910; Ritter, 1911; and Giöbel, 1926, that the bacteria excrete soluble nitrogenous products which are passed on to the plant. Nobbe and Hiltner, 1900, and Hiltner, 1904b, believed that the absorption of nitrogenous compounds by the plant occurs only after the bacteria are transformed into bacteroids. Golding, 1905-6, sought to support the excretion theory by showing increased nitrogen fixation on the part of a culture of rhizobia growing in a filtering apparatus. Other investigators, however, have not been able to duplicate Golding's results. Mulvania, 1916, attacked the problem in still another way. He used a culture of rhizobia separated only by a dialyzing membrane from the

roots of an appropriate leguminous plant. He observed no benefit to the plant and concluded that nitrogenous compounds were not being elaborated by the bacteria and passed out through the membrane.

Virtanen, 1928b, having shown that the plant may be nourished by amino acids, proceeded to investigate the production of amino acids from bacterial cells. It is possible that the amino acids originate by the action of enzymes of the bacteria themselves, or possibly of enzymes excreted by the plant. To distinguish between these two alternatives, Virtanen experimented as follows. He took equal quantities of a mass of rhizobia from the surface of a pea extract gelatin medium and of clean, crushed root nodules. These two samples were diluted with equal volumes of sterile water; toluol was added to kill the bacteria, and the two suspensions were vigorously shaken. The total nitrogen, amino nitrogen, and ammonia were determined in each sample at the start of the experiment and at regular intervals thereafter. It was found that the rate of hydrolysis of the proteins was approximately equal in the two samples. The end products were apparently amino acids, as practically no ammonia was formed. This together with his experiments on the utilization of amino acids by the leguminous plant led Virtanen to conclude that bacterial enzymes attack the bacterial cells as they die, and decompose their complex proteins to amino acids, which are then assimilated by the host.

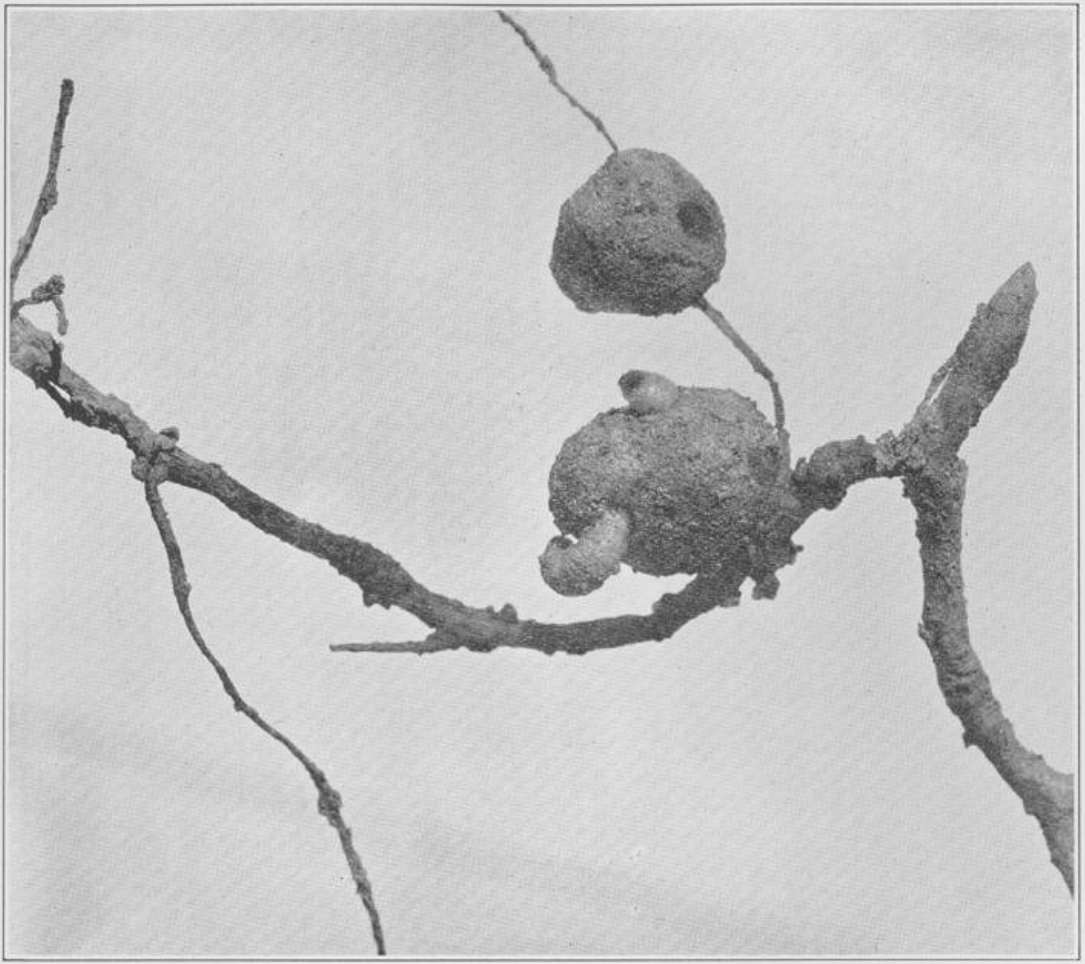
The demonstration by Gerretsen, Gryns, Sack, and Söhngen, 1923, of a bacteriophage which is capable of lysing the rhizobia, led them to propose that solution of the complex nitrogenous compounds of the bacteria within the nodule may likewise be accomplished by bacteriophage. A more complete study of this was presented by Grijns in 1926. Grijns, 1927a, showed that lysis of a concentrated culture of *B. danicus* by its specific phage results in a marked increase in amino nitrogen. It is possible that a similar action by phage takes place in the nodule, but there is in reality little probability of it. Grijns, 1927b, showed that bacteriophage does not influence nodule formation or growth of red clover plants. The findings of Israilsky, 1926; Hitchner, 1928 and 1930; and Laird, 1932, indicate that the action of the bacteriophage on rhizobia is dependent upon many factors, which make it doubtful whether phage is the principal agent in the solution of bacteria within the nodule. The early results of Gerretsen, Gryns, Sack, and Söhngen, 1923, suggested that the bacteriophage might be specific for the various species of rhizobia. Hitchner's results, however, have shown a much narrower specificity; in fact, only one strain of *Rh. trifolii* of the several tested is sensitive to the phage isolated by him. Laird, on the contrary, obtained evidence of more general action of the phage within the *Rhizobium* species. He has shown that the apparent resistance of many strains of rhizobia is due to the presence of resistant individuals. By proper plating procedures it is possible to obtain separate strains, relatively sensitive and relatively resistant to the action of phage. Laird further stated that his phage from *Trifolium* may lyse certain strains of *Rh. meliloti* and *Rh. japonicum*. In the latter part of Laird's work, an attempt was made to correlate phage sensitivity with effectiveness of the rhizobia, in the sense previously defined as ability to aid the host plant. The results with stock cultures fail to show any positive correlation. There does, however, seem to be a tendency

for sensitive strains to produce nodules which are longer, fewer in number, and of the general type of effective strains.

The life of the nodule. As mentioned earlier in this chapter, the root nodules of most leguminous plants are emptied as the plant approaches maturity. This is particularly noticeable in the larger-seeded annuals. In the case of *Phaseolus vulgaris* upon which a second crop of blossoms and seed may be formed if the first crop of seed pods is removed before maturity, the first setting of nodules may be followed by a second; these develop and in turn are emptied as the second crop of seed matures. With the smaller-seeded leguminous plants, in which the demand for nitrogen is not so concentrated in point of time, this process is less conspicuous. The nodules of biennial and perennial herbaceous plants usually disappear during the winter rest period and are re-formed when the plant again resumes active growth (Giltner, 1915). The nodules of certain leguminous trees and shrubs may live over from year to year and in such cases new growth is laid down each year (Spratt, 1919; and Jimbo, 1927). In other words, the formation of new nodules or the development of new lobes upon existing nodules closely parallels the development of new rootlets.

The above statements refer to the normal life of the nodule, conditions for plant growth and initial formation of nodules being favorable. A number of external conditions, however, may shorten the normal period. Giltner, 1915, has observed that even temporary waterlogging of the soil may cause destruction of nodules, and conversely, that excessive drying as in summer drought or lack of watering in the greenhouse may likewise cause destruction of nodules. The shedding of nodules in dry soil has been noted also by Leonard, 1927, and Wilson, 1931a. It is not clear whether this shedding of nodules is due to the mechanical removal of nodules by shrinkage of the soil or to an actual sloughing of the nodules by the plant.

After the early period when the causative organism of the leguminous nodule was mistakenly considered a fungus, there have been occasional reports of true fungi found growing in nodules and in some cases evidently consuming the nodule substance. Thus Vuillemin, 1905, had recognized as a *Pythium* the fungus he previously called *Cladochytrium tuberuclorum.* Lechtova-Trnka, 1931, also found a *Pythium* sp., very similar to *P. tuberculorum* Vuillemin. She also described a new fungus, *Ascorhiza Leguminosarum,* g. nov. sp. nov. The *Pythium* is able to penetrate throughout the nodule, whereas the *Ascorhiza* remains for the most part in the cortex. The frequency with which these two forms were encountered indicates that they are rather common nodule parasites. Insect depredations may also interrupt the normal cycle of a nodule. McConnell, 1915a, reported a case of destruction of nodules by larvae of the bean-leaf beetle, *Cerotoma trifurcata* Forst, and again 1915b, the attack upon the nodules of certain wild Leguminosae by the larvae of *Endiagogus rosenschoeldi* Fahrs. Leonard and Turner, 1918, made a study of the extent of injury by *Cerotoma* larvae and found that fixation of nitrogen by nodulated cowpeas might be seriously interfered with by the infestation of the beetle. Plate 34 shows a *Cerotoma* larva feeding upon a cowpea nodule. Leonard, 1923b, reported that the mealy bug, *Pseudococcus maratinus* Ehrh., attacks the nodules of soybean, navy bean, Japan clover, and chick pea. No evidence is available concerning the amount of damage resulting from such



depredations. Parisi and Masetti-Zannini, 1926, reported that one sample of lupine nodules, which was gathered for their studies on the nitrogen content of nodules, had to be discarded because a large percentage of them contained larvae of some coleopterous insect. The entire bacteroidal tissue had been devoured, and only the cortical shell remained. The occurrence of such insect infestations needs further study in order that their importance may be evaluated.

CHAPTER 11

FACTORS THAT INFLUENCE NODULE PRODUCTION

"Let us first understand the facts and then we may seek the cause."
—ARISTOTLE.

The many and variable factors which affect nodule formation have called forth a very large literature. So great an interest in the problems of nodule formation is to be expected from the standpoint of agricultural importance as well as of scientific interest. The classical investigations of Hellriegel and Wilfarth, 1888, opened the way to an early appreciation of the importance of soil conditions in relation to nodule production. There followed a long series of investigations which have only served to show how elusive and baffling are the problems involved. On certain points the results have been concordant and conclusive, but there are many important questions which still need solution. Perhaps the difficulty lies in lack of appreciation of the structure and behavior of the nodule itself. The physical and chemical factors that influence the bacteria and the plant separately are fairly well known. Their similar influence upon the nodule, however, does not necessarily follow. It must be remembered that the nodule during its formative period is essentially meristematic, in contrast with adjacent more mature cells of the plant root. It is composed of thin-walled tissue easily affected by such factors as moisture change, oxygen deficiency, etc.

THE EFFECT OF FACTORS LARGELY PHYSICAL

Perhaps the most important factors affecting the development and functioning of nodules, as indeed of all forms of life, are air, water, and light. Temperature and soil reaction are also influential and to a large extent determine the behavior of the nodule in relation to nitrogen assimilation. In order to understand the action and interaction of these conditions, it is necessary to remember the general relationship which exists between the leguminous plant and its rhizobia. However beneficial the activities of the bacteria may eventually be in the nitrogen metabolism of the plant, the fact remains that the bacteria are dependent upon the plant for their carbonaceous foodstuff and probably in the early stages of infection also for their nitrogenous food.

Air and moisture. The two factors, air and moisture, will be discussed together, inasmuch as many of the earlier investigations deal with plants in water culture, that is, with a limited supply of air in conjunction with excess of moisture. As early as 1864 the German investigators, Rautenberg and Kühn, reported that in nitrogen-free water the roots of *Vicia Faba* bear hundreds of small nodules about

the size of millet seed. Kny, 1877 and 1878, on the contrary, reported the entire absence of nodules on *Pisum sativum* and *Phaseolus multiflorus* grown in water culture. Buckhout, 1889 and 1890, also failed to obtain nodules on peas in water culture. One would not suppose that lack of the rhizobia could be the cause of his failure, since the addition of rhizobia-bearing soil to the water was without effect. Prazmowski, 1890, asserted that nodule formation in water culture is a variable and more or less accidental phenomenon. Nobbe, 1896b; Nobbe and Hiltner, 1899c; and Golding, 1903, were able to secure nodulation of plants in water culture experiments but stated that the nodules so formed are of little or no benefit to the plant. Even the pumping of air or nitrogen gas through the liquid cultures was of no avail in stimulating activity of such nodules as were present. Nobbe and Hiltner reported that the submerged nodules of the common locust and the pea are of abnormal structure, being largely deficient in bacteroidal tissue. Golding's experiment, 1903, is noteworthy as representing an early attempt to discover whether the nodules are the organs of nitrogen assimilation. He raised plants with roots submerged in nitrogen-free nutrient solution, part of the culture jars with the surface of the liquid covered with a layer of oil. Plant growth in the oil-covered jars was very abnormal, and there was no assimilation of nitrogen. Plants in the open jars grew slightly better and fixed a limited amount of nitrogen. Plants with nodules exposed to the air or with air forced through the culture solution were no better with respect to nitrogen fixation than were plants with roots entirely submerged. Remy, 1907, is also to be numbered among the early investigators who noted defective nodules on plants in water culture.

According to Thornton, 1929e, nodules formed under water may be deficient in vascular strands[1] and thus unable to fix nitrogen through interference with the transport of nutrients to the bacteria or of nitrogenous materials to the plant. In a later paper Thornton 1930a, suggested that lack of air supply to a nodule may limit the number of bacteria in the nodule and thereby hinder nitrogen fixation. In agar cultures Thornton found the most efficient nodules developing at the surface or at some point where shrinkage or cracking of the agar permits free access of air. Plants with nodules deeply embedded in agar grow very poorly and gain no nitrogen. The same plants may be induced to new growth if the nodules become exposed to air by cracking of the agar. A recent paper by Wilson, Hopkins, and Fred, 1931, presents somewhat different results. By a large number of experiments, they have shown it entirely possible that nodulated leguminous plants in agar culture can fix nitrogen, regardless of the position of their nodules within the agar. Under the conditions of the experiments and with the particular strains of rhizobia used, the quantities of nitrogen fixed ranged from 2 to over 10 mg. per 10 plants. These amounts are not intrinsically large but represent a gain for the plants of 2 to 10 times the amount of the original seed-nitrogen. Careful study of the factors involved seems to indicate that, providing aeration is sufficient to meet the needs of the plant at its current level of metabolism, aeration directly of the nodules is not so important as the combination of other factors which govern plant growth; as for example, temperature, humidity, light, and gas exchange in relation to transpiration and photosynthesis.

[1]This statement is not clear but apparently refers to the paper of Brenchley and Thornton, 1925, in which it was reported that lack of boron in a culture solution may be responsible for defective formation of the vascular tissue.

Aside from the effect of aeration on the actual fixation of nitrogen, there is the decided benefit of aeration to the growth of the plant and of the nodules themselves. Kellerman and Robinson, 1906, found that the conditions of aeration in a light sandy soil are conducive to a marked increase in the number of nodules and also in size of the plants. The following year Reynolds, 1907, reported that aeration results in increased growth and quality of peas. The observation of Frank, 1890b, that the number of nodules is very much decreased in the deeper layers of soil may perhaps be associated with the factor of aeration. On the other hand, it must not be forgotten that the deeper layers contain the later roots, grown after the first setting of nodules and upon which there is little tendency to form new nodules, providing those formed higher up on the system are adequate. That nodules may be formed deep in the soil and upon the fine lateral roots is evident from the type of nodulation produced by certain ineffective strains (Baldwin and Fred, 1929a). These, however, are developed under conditions of fair aeration in soil or sand of moderate moisture-content. In water-logged soil the story may be entirely different. An experiment with soybeans in Virginia will illustrate how excessive water may affect even mature plants. The crop developed normally until, as the plants neared maturity, excessive rain produced water-logging of the soil. As a result new nodules were formed in clusters and masses upon young rootlets near the surface of the soil. After the water subsided, these nodules were left exposed to air and light and gradually changed to a greenish-brown color. A similar case was reported by Jones, 1926, involving the formation of nodules on adventitious roots of mung bean plants grown on land that was flooded. He suggested that the nodules so formed are parasitic rather than symbiotic to the host plant.

Granted that excess of moisture is undesirable for the formation and functioning of nodules, what is the effect of low moisture, and what is the optimum? Gain, 1893, was perhaps the first to carry out such experiments. Using clover, peas, horse beans, and lupines in soils of different moisture content, he made counts of the nodules and found that both the total number and the size of nodules in moist soil greatly exceed those in dry soil. Much the same thing was reported by von Seelhorst, Freckmann, and Bünger, 1904; Prucha, 1915; Wilson, 1917; Perkins, 1924a and b; and Gangulee, 1926a. To cite a typical example of their work, von Seelhorst, *et al.* tested the effect of varying moisture contents, 45, 58, 71, and 84 per cent of the total water-holding capacity; the greatest yield and the maximum gain in nitrogen occur in the presence of the highest moisture content. Fred, Whiting, and Hastings, 1926, similarly reported that in Miami silt loam the optimum for alfalfa is about three fourths of the total water-holding capacity. Plate 35 illustrates the effect of different moisture contents upon the development of tops, roots, and nodules. Wilson, 1917, studied particularly the correlation of nodule numbers and moisture content of the soil. His results with soybeans on a silt-loam soil are given:


1
2
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Moisture in soil per cent on dry basis	*Number of nodules* on 100 plants
25	3
35	192
45	477
55	807
65	1200
75	1407

Wilson suggested two possible explanations of the beneficial influence of moisture: (1) the greater the water content of the soil, the better the chance of infection; (2) the more dilute the solution of soil solutes, the less hindrance to the bacteria through the toxicity of any compound. Moore, 1905, had made similar suggestions. It is also conceivable that the degree of moisture in the soil may exert a direct effect upon the higher plant, which in turn might affect infection. It is known that the development of root hairs, for example, is in part dependent upon the moisture of the environment.

In summary, it may be said that a relatively high water-content of the soil, but not actual water-logging, is desirable for maximum nodulation and nitrogen assimilation.

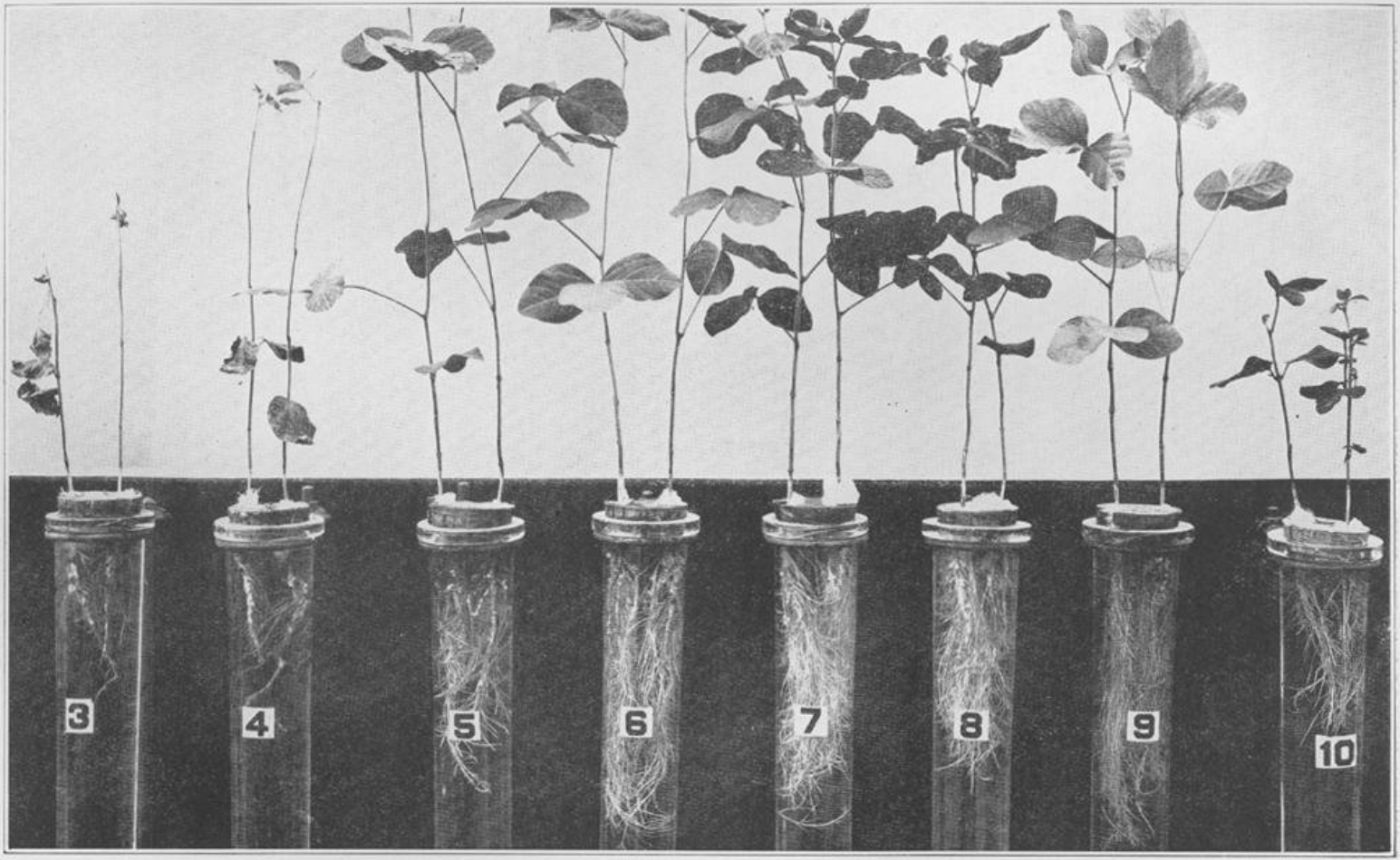
Light. By virtue of its function in photosynthesis, light is one of the agents indirectly affecting nodulation through its action upon the host plant. It is thought that lack of light and the resulting deficiency in carbohydrate are responsible for poor growth of plants in the greenhouse during winter months. Leonard, 1926, pointed out that soybean plants grown during the short days of winter or plants with the leaves clipped (and therefore the carbohydrate synthesis retarded) show poor nodule formation. He planned an experiment in which successive series of pots were planted on the first of each month for nearly a year. Light was the chief variable, since other conditions were kept as nearly constant as possible. The results indicate that the minimum number of nodules occurs upon plants grown in the short-day months of November, December, and January. The dry weights of the plants also are low during the dark months. These results, together with some additional experiments on the effect of reduced chlorophyll (leaves clipped off) and insufficient CO_2 supply, suggested that under the conditions specified, the lack of sufficient carbohydrate formation is the limiting factor for plant growth and nodule development. According to a report by Eaton, 1931, there is direct proportion in the soybean plant between the length of day and the weight of tops, roots, and nodules. All three are decreased as the days shorten. A positive correlation is also found between the percentage of polysaccharides and the weight of nodules; the correlation between the percentage of total sugars and the weight of nodules, however, is much less perfect. On the absolute basis of amount of carbohydrate to growth and nodule development there is found very close correlation. It is recognized in view of the recent report (Burk, 1927a) on the possible exothermic nature of nitrogen fixation, that the old necessity of carbohydrate as a source of energy for the process is untenable. Never-

theless, carbohydrate is still the source of carbon for the synthesis of complex nitrogenous organic compounds which are the end products of the process. A positive, though not exact, correlation is therefore understandable. Another aspect of the beneficial effect of light is indicated in the recent report of Sears, Myers, and Clark, 1929, who have found that adequate lighting in some measure overcomes the unfavorable effect of nitrates upon nodule formation.

So much for the fact that adequate lighting is necessary for the best growth of plants and the normal formation and functioning of nodules. What is the effect of the reduced intensity of light or of actual darkening? As early as 1888-89 Vines observed that no nodules appeared upon *Vicia Faba* plants grown in shade. He suggested that only healthy plants could form nodules. Prazmowski, 1890, did observe nodules upon plants actually kept in the dark, but he admitted that such nodules remain smaller than normal. Thornton, 1930a, subjected to darkness young alfalfa seedlings in the process of nodule formation. He found that new nodules fail to form, although plenty of bacteria are present, and that the few nodules already formed cease to grow. Cytological examination of these latter revealed that the bacteria had become active parasites and were attacking the cytoplasm and even the nuclei of their host. This behavior Thornton attributed to the need for energy-yielding foodstuff, the usual supply of carbohydrate being cut off through lack of photosynthesis. Wilson, 1931c, attempted an even more severe test of the relation of light to nodulation, when he studied infection of seedlings grown from the seed in entire absence of light (*i.e.*, of completely etiolated seedlings) in medium with and without supplementary sugars. After 36 days the plants were examined and it was found in the cases of the vetch and pea (rarely) that macroscopic nodules had formed. The plants grown without sugar showed very few nodules (1 on 40 plants); those with levulose none; those with dextrose only in 2 per cent concentration; those with sucrose in 0.5 and 1 per cent concentration as many as 4 nodules per vetch plant. No nodules were ever formed on etiolated red clover and alfalfa seedlings. Obviously the problem is complex but it would seem that infection and at least early stages of nodule formation are possible in the absence of light, provided that sugar suitable in kind and amount is available in the nutrient medium. Kind of plant is also important.

Quite apart from the effect of light on the higher plant is the question of light and its direct action upon the bacteria and the nodule. In Chapter 7 will be found a discussion of the relation of sunlight to the longevity of the rhizobia. Upon the growing nodule, light produces no marked injury. In agar culture or even in the field, it is not uncommon to find some actually aerial nodules. These may be slightly green, presumably with chlorophyll. The effectiveness of such exposed nodules has not been studied. Prucha, 1915, observed a slight decrease in the number of nodules developing upon roots directly exposed to light. Such nodules as were formed were apparently normal, however.

Temperature. The temperature most suitable for the host plant best promotes nodule formation. Temperatures appreciably higher or lower than this favorable range are distinctly injurious. As early as 1891 Laurent called attention to the harmful effects of low temperatures, and in 1897 Zinsser observed the same concerning plants kept at about 15°C. The most extensive investigation yet made on the subject is that of Jones and Tisdale, 1921. Briefly their experiments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were as follows: plants of alfalfa, red clover, pea, and soybean were grown in soils held at a series of temperatures 3° apart ranging from 12° to 36° C. The air about these plants was held between 14° and 20° C. It was found that the plants differ somewhat in the extremes of temperature which they will tolerate. Nodules may be formed at all temperatures within the range studied, but at the extreme upper and lower limits at which the plants survive, the numbers of nodules are reduced. The maximum weight of nodules was obtained on soybean plants at a soil temperature of 24° C.; the optimum for the other plants was approximately the same.

Reaction. The aerobic nitrogen-fixing bacteria, such as *Azotobacter* and *Rhizobium,* are profoundly affected by the reaction of their working environment. This fact has been emphasized in Chapter 7 in a discussion of the rhizobia apart from their host plants. It was there stated that certain differences exist between the rhizobia belonging to different species (Fred and Davenport, 1918). Probably these variations have arisen through long adaptation of the rhizobia to a given host.

The formation of nodules is influenced by soil acidity in a number of ways. The effect may be directly upon the bacteria before or after their entrance into the host plant, or they may possibly be affected indirectly through changed metabolism of the host plant. Generally the pH value at which nodulation is inhibited is not so low as that at which the free bacteria are adversely affected. The mechanism of the injurious effect of acidity is not clear. Fellers, 1918b, reported poor nodulation of soybeans in an acid soil, even though large numbers of the proper bacteria were known to be present. The results of Karraker, 1927, indicate a direct effect of the acid soils upon the bacteria, while Wisconsin workers are inclined to place more emphasis upon the indirect effect of acidity upon the host plant (Bryan, 1922 and 1923a). Doolas, 1930, suggested that the injury from acidity may be local, and that it may depend also upon other external factors which govern the growth of the plant. Scanlan, 1928, expressed a similar opinion. He found that the addition of calcium acetate to acid soil greatly favors nodulation, although this salt has in itself little effect upon reaction.

The fluctuations in nodule production upon leguminous plants in soil and liquid substrates of varying reaction have been studied by Joffe, 1920; Bryan, 1922 and 1923a; Virtanen, 1927, 1928a and b; Sewell and Gainey, 1930, and others. Joffe found that alfalfa in soils ranging from pH 3.0 to 7.1 shows an increasing yield with increase of pH value. Nodules appear within the entire range suitable for growth of the alfalfa plant, but the greatest number of nodules and the greatest gain in nitrogen occur in plants growing in soils of neutral or nearly neutral reaction. Bryan, 1922, made similar observations in his studies of alfalfa, alsike clover, red clover, cowpeas, and soybeans, raised in nutrient solutions of varying reaction. His results with soybeans, shown below and in Plate 36 are typical. According to the figures of this experiment, the limits for nodule production on the soybean are approximately pH 4.9 to 8.0 and for growth of the host about pH 3.9 to 9.6. Similar tests with the cowpea show an even greater resistance to extremes of acidity and alkalinity. It is worthy of note that the pH of the plant sap generally differs from that of the external substrate; see, for

Growth and nodule production in nutrient solutions of different reaction (soybeans)

Reaction of media pH	Growth of plants	Leaves pH	Roots pH	No. of nodules per plant
3.30	Dead	5.60	4.68	0
3.97	Poor	5.90	5.09	0
4.95	Fair	6.08	5.29	30
6.50	Good	6.11	5.61	77
7.40	Good	6.12	5.75	68
8.20	Good	6.11	5.85	21
8.70	Fair	6.14	6.29	3
9.60	Poor	6.15	7.12	0

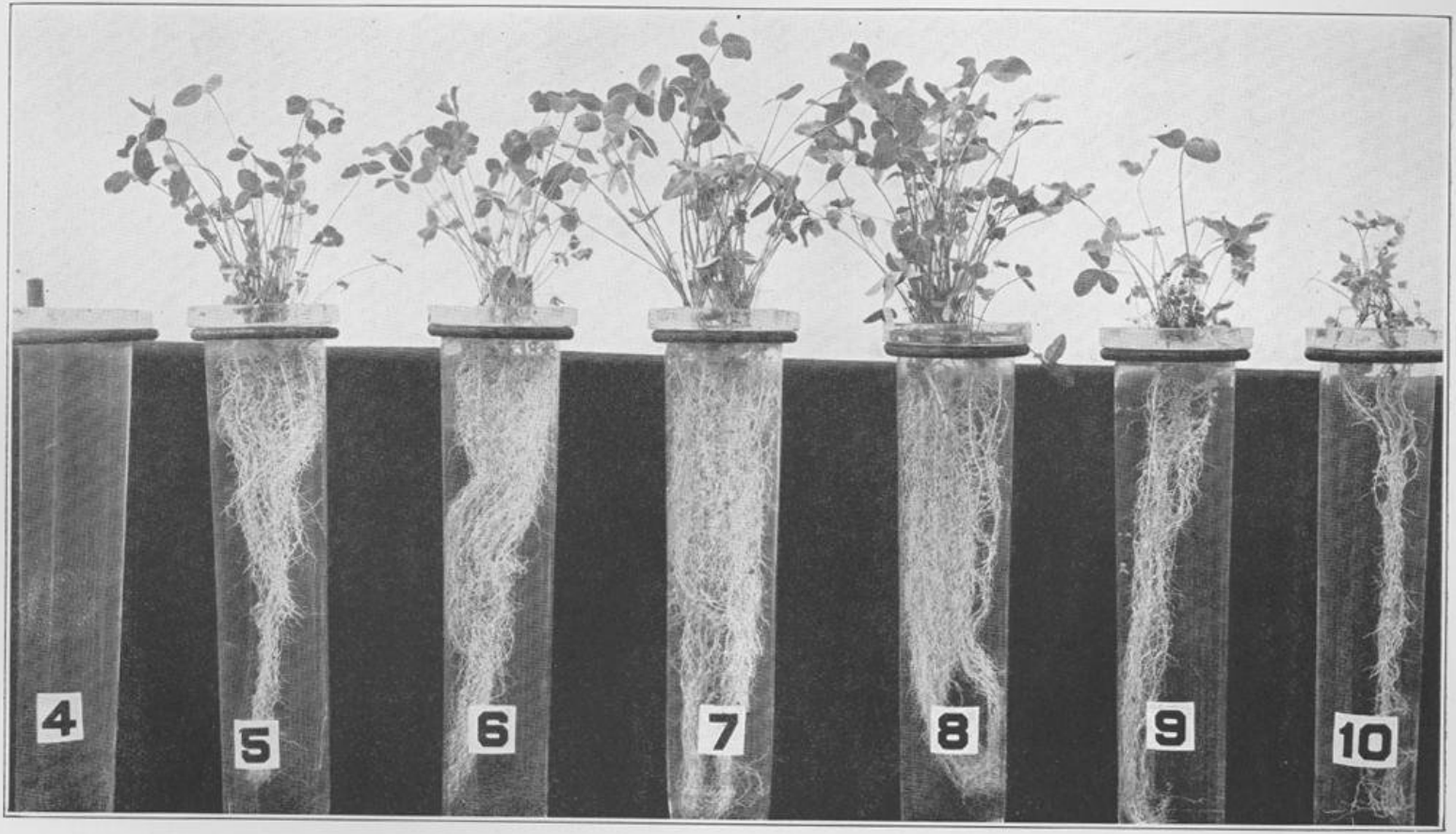
example, the data above, indicating for the soybean a pH in the root slightly higher than the external pH in the lower range, and reaching neutrality only in the extreme alkaline limit of the experiment. These values are typical of the general statement that cell sap of a plant in acid soil is less acid than an extract of the soil. This maintenance of comparative stability of reaction is a function of the life of the plant, in a sense comparable to the regulation of the pH of the blood of an animal. It is, of course, the pH of the root, rather than of the outside soil, with which the rhizobia in the nodule must cope. Providing they are able to invade the plant at all, they stand a fair chance of finding a suitable environment for their life work.

In his studies of alfalfa and clover, Bryan, 1923a, used sand instead of liquid culture. He obtained growth of Alsike clover between pH 5.0 and 10.0, although above pH 8.0 the plants were obviously not healthy. The maximum plant growth and best nodule production were obtained at pH 6.0 to 8.0 as indicated in the following data and Plate 37.

Growth and nodule formation in sand with nutrient solutions of different reactions (Alsike clover).

Reaction of media pH	Growth of plants	Nodulation
3	Dead	----
4	Dead	----
5	Fair	Good
6	Good	Good
7	Good	Good
8	Good	Good
9	Yellow	Good
10	Yellow	Fair

Virtanen, 1927, pointed out that the limits of growth, as expressed in terms of pH, vary in different soils as well as with different leguminous plants. Red clover produces nodules at a pH of 4.6, a lower value than can be obtained with peas (pH 5.1). The following year Virtanen, 1928a and b, attempted to explain the depressing effect of acid soils by their effect upon the plant itself. He was able to demonstrate decreased total nitrogen content of the plant and to a lesser degree the decrease of phosphorus and potassium. Sewell and Gainey, 1930, investigated the growth and nodulation of alfalfa within the range pH 4.5 to 7.0. Providing the nutrient salts are adequate in amount and kind, the reaction of the


4
5
6
7
8
9
10

soil is a minor factor. With deficiency of available nutrients, however, the effect of the more acid reactions may be serious.

Olsen, 1925, arranged an experiment with an acid soil rich in humus, to which he added $CaCO_3$ in amounts to obtain a range of reactions from pH 4.0 to 8.5. These soils were used for pot culture of *Medicago sativa* and *Medicago lupulina*, with the following results.

Medicago sativa

pH value	4.0	5.0	6.0	6.5	7.0	7.5	8.5
Relative weight of dry matter	24.0	51.6	88.6	100.0	95.2	89.6	66.3

Medicago lupulina

pH value	4.02	5.04	6.94	7.50
Relative weight of dry matter	13.4	62.4	100.0	88.7

He found root nodules throughout the series, although in the more acid soils, the nodules were poorly developed and few in number. Best growth was obtained at pH 6.5 to 7.0. Olsen also determined the percentage distribution of phosphorus, calcium, and nitrogen. He found the nitrogen fairly constant throughout the series, whereas the calcium content was increased in those plants grown in soils of pH exceeding 7.0.

In general the evidence relating to acidity warrants the conclusion that any reaction at which the plant will grow will permit nodule formation. Reactions at or near neutrality, however, are most favorable for normal plant growth and nodule formation.

THE EFFECT OF CERTAIN INORGANIC SALTS

The inorganic salts essential to the life of bacteria and the higher plants are in the main the same, but the required proportions of salts and the concentrations that prove toxic may be different. Generally speaking, the bacteria are remarkably resistant to fluctuations in the concentration and often may thrive in solutions of osmotic pressure far greater than the higher plant can withstand. Thus it is that certain concentrations of inorganic salts may show no apparent injury to the bacteria, yet may prohibit nodule formation. In such cases it is a question whether the effect is due to direct action upon the bacteria, making them incapable of infection, or to indirect action upon the host plant, making it by some change in metabolism unfavorable for nodule development. Often it is impossible to distinguish between these two effects; *e.g.*, the proper addition of a phosphate salt may greatly favor root development and thus increase the chances for infection, or it may cause the bacteria to multiply more rapidly and so in another way increase the chances for infection.

Calcium, magnesium, and other salts. Early in the study of plant nutrition it was observed that lime applied to the soil greatly stimulates the growth of plants,

particularly of the Leguminosae. In consequence there have appeared a great number of both field and laboratory studies dealing with the beneficial effect of calcium, magnesium, and other salts on nodule formation and plant yield. The benefit of lime is noted with all leguminous crops except certain acid-resistant species like the lupines and serradella. In many cases the addition of even small amounts of calcium salts brings about a great stimulation of growth, increased number and size of nodules, and a well-defined gain in protein content of the plant. The reports on this subject are very numerous; a few of the more important references may be cited—Laurent, 1901; Marchal, 1901; Flamand, 1904; Moore, 1905; Prucha, 1915; Fellers, 1918b; Wilson, 1917; Albrecht and Davis, 1929a and b; and Scanlan, 1928. Some of these, as well as other investigations, deal with a number of metallic salts, the actions and interactions of which are difficult to consider separately. For example, Laurent, 1901, and Marchal, 1901, found calcium and potassium phosphates especially beneficial to the pea, from which observation it is impossible to say whether the metallic ions or the phosphate are responsible. Moore, 1905, found that calcium and magnesium salts in general stimulate nodule production except in the case of lupines; and Prucha, 1915, stated that small amounts of either calcium carbonate, magnesium sulphate, or potassium acid phosphate favor nodulation of field peas in sandy soil. Ammonium, ferric, and potassium chlorides have the opposite effect. In 1917 Bear reported the beneficial effect of calcium carbonate on the nodulated soybean, as evidenced by the increased nitrogen content of both stems and roots of the plants. Wilson, 1917, who studied the effect of a large number of salts, listed calcium oxide, chloride, and carbonate as beneficial. The phosphates of sodium, potassium, and magnesium also proved stimulatory in small quantities such as 0.05 gm. per 100 gm. of soil. Of the 22 chlorides tested, only those of nickel, cobalt, and lithium appeared to be specifically toxic; ammonium chloride was found to be depressing, but for special reasons to be discussed later under the influence of nitrogenous salts. In other words, the chlorides as a class are not toxic, unless made so by some specific metallic ions. Hendry, 1918, showed that sodium chloride in concentrations of 0.2 and 0.8 per cent in liquid added to quartz sand cultures causes a well-defined injury to bean and cowpea plants as well as a decrease in the number and size of the nodules. Larger amounts, 1.5 per cent NaCl, may entirely prevent nodule formation. Ground oyster-shells and burnt lime were found by Fellers, 1918b, to have a favorable effect upon nodulation, protein content, and total yield of the crop. Alway and Nesom, 1927, obtained great benefit from the liming of acid field soil prior to sowing alfalfa.

Contrary to the above reports, Perkins, 1924a and b, failed to note any improvement in nodulation from the use of calcium, potassium, and phosphatic salts, and hence concluded that the elements essential to plant growth do not directly affect infection and nodulation. Distinctly harmful effects from large applications of calcium oxide or calcium carbonate have been reported by Salfeld, 1894a and b and 1900; Salfeld and Wolff, 1898; Pfeiffer and Blanck, 1914; Pfeiffer and Simmermacher, 1919; Creydt, 1915; von Seelhorst, Geilmann, and Thiele, 1915; Merkenschlager, 1921; and Boas and Merkenschlager, 1923. The so-called acid-loving plants, lupine and serradella, are particularly sensitive to overdoses of lime

(Hellriegel and Wilfarth, 1888, and Densch and Steinfatt, 1930). Yellow lupine particularly suffers from the so-called calcium sensitivity, a condition which in the analysis of Reincke, 1930, appears to be a complication of chlorosis and nitrogen hunger. In a 1931 report Reincke suggested that the chlorosis is due to the effect of absorbed calcium upon transportation of iron. Nitrogen fertilization along with application of calcium increases chlorosis.

A study of these reports will reveal the decided lack of agreement as to the cause of depressed nodulation. Some maintain that there is an indirect effect resulting from injury to the bacteria, whereas others claim that the higher plant suffers directly and is thus unable to form nodules in response to infection. Albrecht and Davis, 1929a, pointed out that there is probably truth in both explanations. They observed that the presence of calcium has a marked effect upon the viability of rhizobia. The effect upon the plant is also important, as shown by histological differences in plants grown with and without adequate calcium in the nutrient supplied. This phase of the nodule problem has not before been considered; it seems a line worthy of extension. The plant reaction to various treatments, which are now known merely to stimulate or depress nodulation, may prove of great interest.

The effect of calcium is peculiarly localized as shown by Karraker, 1927, and Albrecht and Davis, 1929a. The roots of a single plant may be divided, part in acid soil and part in well-limed soil; the difference in nodulation on the two parts is as great as on individual plants grown in the respective soils.

Sulphur and the sulphates. From the investigations of Pitz, 1916; Reimer and Tartar, 1919; and Neller, 1926, it appears that sulphur and calcium sulphate exert a stimulating effect on alfalfa, clover, and probably other Leguminosae. In some cases these fertilizers bring about an increase in the root system and in the formation of nodules, thus enhancing nitrogen fixation. Quite the opposite effect is reported by Wilson, 1917, who studied some 21 sulphates. He found that sulphates in general depress nodulation unless the depression is offset by stimulating action of the metallic ion. Such is the case of aluminum, chromium, and potassium sulphates used by Wilson. The reports are thus conflicting and indicative of the need of further study.

Phosphorus and the phosphates. The rôle of phosphorus in nodulation is more than commonly complex because of its independent effects upon the bacteria and the plant. Phosphorus is an essential element for the growth of bacteria, and certainly the rhizobia present no exception to this rule. As detailed in Chapter 7, the phosphates are reported to be distinctly stimulating to multiplication of the rhizobia (Truesdell, 1917, and Thornton, 1929c) and are particularly conducive to the development of motile forms. It is to the motile forms and their increased ability to spread through soil and infect the plant that Thornton and Gangulee, 1926, attribute the advantage from the milk-phosphate process of inoculation as recommended by the Rothamsted Experimental Station. In South Carolina there has been developed a new method for inoculation, an important feature of which is the coating of the seed with a sugar syrup and basic slag solution (Yeager, 1929). It is probable, although the point has not been particularly studied, that the phosphate of the slag is in part responsible for the success of the method.

Marchal, 1901; Laurent, 1901; Wohltmann and Bergené, 1902; Dehérain and Demoussy, 1902; Löhnis, 1902; Flamand, 1904; Müller, 1905; and Prucha, 1915, are among the early workers who noted that applications of phosphates within a certain range increase the number of nodules. Prucha, 1915, secured much better nodulation of the field pea in soil to which small amounts of potassium acid phosphate and monobasic calcium phosphate had been added. Wilson, 1917, tested a number of phosphate salts and found them beneficial to the soybean. Fellers, 1918b, reported that application of acid phosphate to limed plots was effective in increasing nodulation of the soybean but exerted little or no effect upon unlimed soil. High concentrations of phosphates in Crone's solution were found by S'Jacob, 1927, to increase the number of nodules on peas and also to affect their location on the roots. The latter observation is new and interesting in that normal concentrations of phosphates seem to permit well-distributed nodulation, whereas high concentrations favor larger nodules placed on the upper tap and lateral roots. Scanlan, 1928, found phosphates beneficial on one type of soil but ineffective on another. Helz and Whiting, 1928, from field tests with soybeans concluded that ". . . phosphorus fertilizers increased nodulation when used in amounts which were not inhibitory to germination." Thornton, 1929c, in a study of the effect of fresh straw on the growth of leguminous plants, noted that dibasic potassium phosphate supplied with chaff increases nodulation of both the soybean and broad bean. With the broad bean, applications of phosphate alone are also effective, but not so with the soybean. Sewell and Gainey, 1930, demonstrated that in an acid soil deficient in calcium, the nodulation of alfalfa is benefited more by applications of superphosphate than by lime. The two used together were more effective than either alone.

The effect of phosphates in increasing plant growth is well known; the work of Truesdell, 1917; MacTaggart, 1921; and Graul and Fred, 1922, are illustrative of the stimulation of the Leguminosae. Of still more interest is the fact that under the influence of phosphates, leguminous plants not only increase in size and dry weight, but also in percentage of nitrogen (Truesdell, 1917; Fellers, 1918b; Graul and Fred, 1922). Such results are not always obtained, however, and the phenomenon is not well understood.

Nitrates and ammonium salts. Of the numerous factors which may influence nodule production, the amount and nature of the combined nitrogen of the soil are preëminent. It is common knowledge that if the soil is rich in nitrate nitrogen or other form of soluble nitrogen, the number and size of nodules is reduced. This fact was known even before the actual function of nodules or their causal agent was discovered. As early as 1864 Rautenberg and Kühn observed that *Vicia Faba* grown in Knop's solution containing ammonia or nitrate forms no nodules, although in a nitrogen-free solution numerous nodules are formed. De Vries, 1877, made a similar observation for red clover, and Frank, 1879, found that an extract of horse manure prevents nodulation of peas grown in pots of soil.

After Hellriegel and Wilfarth had demonstrated the relation of the root nodules to nitrogen fixation, much consideration was given to the question of the effect of ammonium salts and nitrates upon nodule formation, since these nitro-

TABLE 17

Effect of nitrates and ammonium salts on nodule formation

Investigator	Plant	Salts tested	Effect
Rautenberg and Kühn, 1864	*Vicia Faba*	Nitrates and NH_4 salts	Prevent nodule formation
Hellriegel and Wilfarth, 1888	Peas	$Ca(NO_3)_2$	Decreases number of nodules
Vines, 1888-9	*Vicia Faba*	KNO_3	Decreases number of nodules
Beijerinck, 1890	*Vicia Faba*	$Ca(NO_3)_2$ and $(NH_4)_2SO_4$	No effect on nodules
Prazmowski, 1891	Peas	$Ca(NO_3)_2$	Decreases number of nodules
Frank, 1892a	Peas, red clover, and lupines	$Ca(NO_3)_2$ and $(NH_4)_2SO_4$	Hinder N-fixation in lupines, but favor it in clover and peas
Nobbe and Hiltner, 1893	*Vicia villosa*	N-containing solution	Depresses nodule formation
Nobbe and Hiltner, 1899c	*Robinia Pseudacacia*	$Ca(NO_3)_2$	Depresses nodule formation
Hiltner, 1900a	Peas	Nitrate	5 mg. NO_3-N:1 liter of solution prevents nodule formation
Laurent, 1901	Peas	Nitrate and $(NH_4)_2SO_4$	Prevent nodule formation
Marchal, 1901	Peas	$NaNO_3$, KNO_3, $Ca(NO_3)_2$, NH_4NO_3, $(NH_4)_2SO_4$	Alkaline earth nitrates inhibit nodules at 1 to 10,000; $(NH_4)_2SO_4$ and NH_4NO_3 at 1 to 2000
Malpeaux, 1901	Red clover, alfalfa, peas, lupines, and beans	KNO_3	Decreases number of nodules
Wohltmann and Bergené, 1902	Peas	NH_4NO_3	Decreases number of nodules
Nobbe and Richter, 1902	Soybeans	Nitrate	Decreases number of nodules
Flamand, 1904	*Pisum sativum*, *Vicia narbonensis*, *Faba equina*	KNO_3, $NaNO_3$, $Ca(NO_3)_2$, $(NH_4)_2SO_4$, NH_4NO_3	Decrease number of nodules. For data see text
Ritter, 1911	Lupines	$(NH_4)_2SO_4$ and KNO_3	Inhibit nodule formation
Ewart, 1915	*Vicia Faba*	KNO_3 and $NaNO_3$ (only 100 lbs. NO_3 per acre)	No effect on nodule formation
Prucha, 1915	Peas	KNO_3 and $Ca(NO_3)_2$	Decrease number of nodules. For data see text
v. Seelhorst, Geilmann, and Thiele, 1915	Lupines	$NaNO_3$ and $(NH_4)_2SO_4$	Depress nodule formation

TABLE 17 (Continued)

Effect of nitrates and ammonium salts on nodule formation

Investigator	Plant	Salts tested	Effect
Lipman and Blair, 1916a	Soybeans	$NaNO_3$ and $(NH_4)_2SO_4$	Depress nodule formation
Fred and Graul, 1916a	Alfalfa, soybeans, vetch, crimson clover	$(NH_4)_2SO_4$, $NaNO_3$ and $Ca(NO_3)_2$	Depress nodule formation. For data see text
Wilson, 1917	Soybeans	Nitric acid and nitrates of Ca, K, Na, Al, Ba, Fe, Pb, Cd, Ce, Sr, Li, U, Ni, Hg. Ammonium salts of H_2SO_4, H_2CO_3, HNO_3, HCl, and H_3PO_4	Aluminum nitrate, nitric acid, $(NH_4)_2CO_3$ and $(NH_4)_2HPO_4$ without effect on nodules; all others decrease number of nodules
Hills, 1918	Alfalfa	KNO_3, $NaNO_3$, and $Ca(NO_3)_2$	10 mg. NO_3-N per 100 cc. as KNO_3 or $Ca(NO_3)_2$ and 25 mg. as $NaNO_3$ prevent nodule formation
Fellers, 1918b	Soybeans	$NaNO_3$ (100-200 lbs. per acre)	100 lbs. per acre decrease number of nodules
Hartwell, 1920	Soybeans	$NaNO_3$ (150-450 lbs. per acre)	150 lbs. per acre decrease the weight of nodules
Albrecht, 1920	Soybeans and cowpeas	$NaNO_3$ (10-250 lbs. per acre)	No effect until 150 lbs. per acre applied; then slight decrease in size and number of nodules
Strowd, 1920	Soybeans	$Ca(NO_3)_2$	2.5 mg. NO_3-N per 100 gm. sand decrease nodule numbers
Bryan, 1922	Soybeans	$Ca(NO_3)_2$	Prevents nodule formation
Perkins, 1924a	Soybeans	$NaNO_3$ (50-3000 lbs. per acre)	1000 lbs. per acre prevent nodule formation
Giöbel, 1926	Alfalfa and soybeans	$NaNO_3$ (150-2600 lbs. per acre)	300 lbs. per acre decrease weight of nodules of soybeans, and 1000 lbs. per acre decrease number of nodules of alfalfa
Helz and Whiting, 1928	Soybeans	$NaNO_3$, $Ca(NO_3)_2$, and $(NH_4)_2SO_4$	Decrease nodule formation
Ohkawara, 1928	Lupines and serradella	KNO_3, $NaNO_3$, $Ca(NO_3)_2$, and $(NH_4)_2SO_4$	Depress nodule formation. For data see text
Sears, Myers, and Clark, 1929		Nitrate	Lowers number of nodules
Löhnis, 1930b	*Trifolium pratense*	KNO_3	0.07 per cent decreases nodule formation in agar cultures
Weber, 1930	Victoria pea	$NaNO_3$	1 gm. $NaNO_3$ to 10 Kg. sand decreases nodule formation

genous compounds are known to be present in soil. The great number of reports on this question forbids a detailed account of each result; Table 17 therefore is arranged to sketch briefly the accumulated evidence that nitrate and ammonium salts are depressing to nodulation. In a number of the papers reviewed in the table, the effect of nitrates and ammonium salts is given as a simple statement, unsupported by experimental details. Several of the reports consider the question more thoroughly and may profitably be discussed in greater detail. Flamand, 1904, raised *Pisum sativum, Vicia narbonensis,* and *Faba equina* in Sach's solution with an addition of sodium, calcium, potassium, or ammonium nitrates, ammonium sulphate, or potassium cyanide. Nodule formation in *Pisum sativum* is suppressed by 1:2000 solutions of calcium nitrate and by 1:10,000 solutions of potassium, sodium, or ammonium nitrate, or ammonium sulphate. With *Vicia narbonensis* the salts are effective in the following concentrations: potassium nitrate 1:1000; sodium nitrate 1:2000; calcium nitrate 1:1000; ammonium nitrate, ammonium sulphate, and potassium cyanide 1:20,000. In cultures of *Faba equina,* nodules are inhibited by potassium nitrate and ammonium sulphate 1:10,000; sodium nitrate and ammonium nitrate 1:2000; calcium nitrate 1:20,000; and potassium cyanide 1:100,000. Prucha, 1915, tested the effect of 0.25 to 2 gm. of potassium nitrate, calcium nitrate, and ammonium chloride to 300 gm. of soil upon formation of nodules in the pea. Even the least amount of ammonium chloride and potassium nitrate prevent nodulation, while with calcium nitrate 0.5 gm. is necessary for the same result.

Fred and Graul, 1916a, found 15 mg. of nitrate nitrogen as ammonium nitrate per 100 cc. of nutrient solution used for watering plants in sand culture sufficient to prevent the nodulation of alfalfa and vetch. In soil, however, alfalfa plants are still able to form a few nodules in the presence of 30 mg. of nitrate nitrogen as sodium nitrate per 100 cc. of watering solution. Ammonium sulphate is somewhat less depressing than sodium nitrate. Similar experiments were carried out with sodium and calcium nitrate on soybeans and crimson clover with essentially the same results. It is significant that larger amounts of nitrate are required in soil than in sand or water culture to produce the inhibition. Wilson, 1917, tested the effect of nitric acid and of 17 alkaline earth and metallic nitrates, used at the rate of 0.05 to 0.1 gm. per 208 gm. of dry soil, upon nodulation of the soybean. The nitric acid and aluminum nitrate applications were without effect, but all others depressed the number of nodules by half or more. In a second experiment the amount of each salt necessary to prevent nodule formation was determined, and in most cases was found to be considerably less than the amount toxic to the plant. Of the several ammonium salts tested, all except ammonium carbonate were depressing to nodule formation.

Albrecht, 1920, raised soybeans and cowpeas in pots of nitrogen-poor soil to which were added varying amounts of nitrate nitrogen, as sodium nitrate. Application equivalent to 150 lbs. of nitrate per acre induced slightly more, but smaller, nodules on the soybean, whereas 250 lbs. per acre had little effect upon the cowpea. Giöbel, 1926, studied the effect of nitrate on the soybean and also on alfalfa. When the plants were grown in soil with either initial or gradual addition of nitrate to the equivalent of 150 to 900 lbs. of sodium nitrate per acre, no effect is noted on the nodulation of soybeans; on alfalfa there is no effect from

applications as high as 500 to 2600 lbs. per acre. In sand culture, however, alfalfa proves more sensitive, the nodule numbers being decreased by three-fourths under the influence of 1200 to 1600 lbs. per acre application. Soybeans in sand cultures also show greater susceptibility to nitrate. Giöbel, however, did not remark the different results with soil and sand, but drew his conclusions rather from the sand-culture results. Nevertheless, the facts as they appear in his data are worthy of emphasis and are extremely interesting in the light of earlier work on the subject by Fred and Graul, 1916a.

Ohkawara, 1928, tested the influence of sodium, potassium, or calcium nitrate and ammonium sulphate on sand cultures of lupines and serradella. With these plants it appears that nodulation is prevented by 0.2 per cent of nitrates but is stimulated by lesser amounts, such as 0.02 or 0.05 per cent. Ammonium sulphate likewise in 0.1 per cent concentration is inhibitory and in 0.01 or 0.02 per cent stimulatory.

Concerning the mechanism of depression of nodulation by the nitrates, there is little agreement. A number of hypotheses have been offered which are at least worthy of consideration. Hiltner, 1900a, pointed out that root invasion is influenced to a great extent by the nutritive condition of the leguminous plant. A plant which is receiving combined nitrogen in addition to the other elements essential for plant growth is by virtue of its vigor immune to invasion by the rhizobia. Hiltner further recognized the so-called toxic effect of nitrates upon the bacteria themselves and considered it a possible secondary factor in the prevention of invasion. A similar view was expressed by Zipfel, 1911. That the effect of the nitrates upon the bacteria outside the plant is not to be considered a vital factor is evident from the report of Laurent, 1901. He was able to show that applications of nitrate sufficient to prevent nodulation were not injurious to the bacteria, as shown by their ability to form nodules on a subsequent series of plants. Wilson, 1917, likewise reported survival of *Rh. japonicum* through concentrations of nitrate inhibitory to nodule formation, and Prucha, 1915, found no evidence that culture upon the usual nitrate media had any effect upon the infecting power of *Rh. leguminosarum.*

In 1920 Strowd showed that the concentration of nitrates in the sap of leguminous plants may attain a high figure, sufficiently high to cause injury to the rhizobia as shown by Hills, 1918, in experiments with the bacteria alone (see p. 81, for the statement that nitrates are stimulatory in low concentrations but in amounts approaching 100-150 mg. per 100 gm. of soil are decidedly toxic). Strowd then suggested that depression of nodulation by nitrates is related to injury of the bacteria within the plant, rather than to prevention of invasion as supposed by Hiltner. There may even be an exaggerated toxicity of nitrates within the plant as hinted by Laurent, 1891. He found that $NaNO_3$ and KNO_3, when added to pea or lupine bouillon in concentrations of 1:500 or 1:1000, prevent growth of rhizobia. Laurent suggested that some combination of the nitrate with the constituents of the plant extract is involved, since neither the plant extracts nor the nitrates alone have such an effect.

Mazé, 1898, has explained the relation of nitrate nutrition of the plant to root invasion in still a different way. When the plant is receiving plenty of nitrate, the carbohydrate which results from photosynthesis may be assimilated at once.

The result is a vigorously-growing plant with well-balanced carbon: nitrogen metabolism; the sap of such a plant would be low in carbohydrate. A plant deficient in nitrogen, on the other hand, would contain an excess of carbohydrate circulating in its sap. A part of that excess would, according to Mazé, be excreted from the roots of the plant and would serve to attract the nodule bacteria and induce them to invade the root hairs. The apparent immunity of the plant receiving adequate nitrogen as nitrate is then referable to lack of the attractive carbohydrate secretion. According to unpublished results obtained at this station, the rapid assimilation of the synthesized carbohydrate in the plant well supplied with nitrate nitrogen results in a locking up of carbon compounds as suggested by Mazé. Deficiency of carbohydrates for the rhizobia may then account for the depressing effect of nitrates on nodule formation.

Giöbel, 1926, offered an explanation for the seeming inactivity of what few nodules are formed upon plants supplied with nitrates. It seems probable that the nitrogen is fixed in the nodule as an organic compound. Its diffusion from the nodule is therefore less rapid than would be the case of a rapidly transferable ion like nitrate. There is then a tendency toward accumulation in the nodule, particularly when the rest of the plant is well supplied by nitrate taken up from the soil. The very fact of accumulation of the organic nitrogen in the nodules then reacts upon the process of nitrogen fixation and eventually brings it to a standstill.

THE EFFECT OF SIMPLE ORGANIC NITROGENOUS COMPOUNDS

In only a few instances have the organic nitrogenous compounds been tested for their effect upon nodule formation. Flamand, 1904, added peptone, urea, and oxamide to Sach's solution. Under such conditions nodulation is inhibited by the following concentrations: for *Pisum sativum,* peptone 1:1000; urea less than 1:20,000; oxamide 1:20,000; for *Vicia narbonensis,* peptone 1:1000; urea 1:20,000; oxamide 1:20,000; for *Faba equina,* peptone 1:10,000; urea 1:20,000; and oxamide 1:10,000. According to Prucha, 1915, Witte's peptone, 0.5 gm. to 300 gm. of soil, prevents nodule formation on the pea, and according to Wilson, 1917, 0.5 gm. to 208 gm. of soil cause reduction of the number of nodules on the soybean. Helz and Whiting, 1928, have shown that dicyano-diamide in amounts equivalent to 50 lbs. per acre suppresses nodule formation in the soybean.

THE EFFECT OF COMPLEX ORGANIC NITROGENOUS COMPOUNDS

It is conceivable that the organic nitrogenous constituents of soil exert some influence upon nodule formation. Unfortunately many of the investigations to date have not considered the accessory effect of inorganic nitrogenous compounds also present in rich soils. In 1887 Tschirch noted that more nodules are formed in a poor soil than in a rich one. Beijerinck, 1888, stated that few nodules or none at all are to be found upon leguminous plants growing in humus-rich soils. Wohltmann and Bergené, 1902, concluded from their experiments with peas that the humus content of the soil must be very high if the nodulation is to be adversely affected. Nobbe and Richter, 1902, reported that soybeans form better nodules

in a soil-sand mixture than in a rich humus garden soil, and Penny and MacDonald, 1910, stated that large nodules are not formed in soils rich in nitrogen. A recent paper by Welton and Morris, 1930, reports observations on the growth of soybeans in a silt loam-sand mixture, silt loam, and silt loam-manure mixture. The number of nodules is found to decrease with increasing soil fertility, but the weight of tops parallels the richness of the soil in which the plants are grown. There is also a decrease in the percentage of dry matter and of total carbohydrates as the richness of the soil increases. Clearly some of the above reports confuse the action of complex organic compounds and inorganic nitrogen in a so-called "rich" soil.

THE EFFECT OF COMPOUNDS PRINCIPALLY OF CARBOHYDRATE NATURE

The many compounds poor in nitrogen but rich in carbon which are annually returned to the soil in the form of plant residues have received small notice in relation to their effect upon nodulation.

Straw and chaff. Thornton, 1925, found that unrotted straw promotes the formation of nodules on clover. In 1929c the same author reported that a half pound of fresh chaff in a pot containing 23 pounds of a soil-sand mixture nearly doubles the number of nodules per gram of root. Rotted straw, however, has no effect at all, whereas a combination of chaff and dibasic potassium phosphate is particularly effective. These observations were made with both soybean and broad bean in pot experiments. There are perhaps two possible explanations of the effect of unrotted straw. Gray, 1929, found that fresh chaff markedly stimulates multiplication of rhizobia in sterilized soil; hence greater chance of infection. In unsterilized soil the addition of chaff would induce a sudden wave of biologic activity with consequent locking up of available nitrate, which in turn would favor nodule formation.

Sugar, starch, and organic acids. At least three investigators have tested the sugars and organic acids for their effect upon nodule formation. Ritter, 1911, found that sucrose is without influence upon nodulation of the lupine. Prucha, 1915, demonstated that peas grown in soil with 1 gm. of starch per 300 gm. of soil produce more nodules than do control plants; larger amounts of starch do not further increase the number of nodules. Sucrose is reported to have no ill effect in amounts to 2 gm. per 300 of soil, but no counts of nodules are given. Tannic acid in concentration of 0.5 gm. per 300 gm. appears to be stimulating, but larger quantities are without influence. Wilson, 1917, obtained evidence that sugars such as maltose, fructose, lactose, and sucrose are distinctly stimulatory to nodule formation by the soybean. Glucose was without effect in his experiments. Glycerol, starch, and various organic acids, for example citric, oxalic, lactic, and tartaric acids and their salts, are distinctly beneficial and calcium saccharate especially so.

THE EFFECT OF COMPLEX ORGANIC FERTILIZERS

Lewis and Nicholson, 1905, secured more abundant nodulation upon plants grown in sand culture in the presence of well-rotted stable manure than in sand

alone. Thornton, 1929c, reported a similar stimulation of the soybean by the action of manure. Albrecht, 1920, investigated the influence of green manure, as represented by the addition of clover tops (amount equivalent to 1000-1500 lbs. per acre) to pots of nitrogen-poor soil. Soybean and cowpea plants grown in the soil developed fewer nodules but somewhat larger ones, well distributed on the root systems. Schindler, 1885, referred to compost as depressing nodule numbers and interfering with the development of *Trifolium pratense, Vicia villosa, Anthyllis vulneraria, Ornithopus sativus,* and *Phaseolus vulgaris.* According to Lipman and Blair, 1916a, blood meal when added to sand in amounts from about 0.7 to 5.5 gm. per 20 lbs. of sand exerts a neutral or slightly stimulating effect on the total nitrogen content of the soybean. Wilson, 1917, remarked that 1 gm. of dried blood meal in 208 gm. of soil is without effect on nodule production.

Commercial fertilizers if added in large amounts may retard nodule formation especially if the fertilizer mixture is rich in nitrogenous compounds. As shown by Helz and Whiting, 1928, a fertilizer mixture consisting of 1-8-2 (N, P, and K, the nitrogen being in the form of Cyanamid) when applied at the rate of 200 lbs. per acre, has little, if any, effect on number of nodules, whereas a 2-8-4 fertilizer, also 200 lbs. per acre, injures nodulation. Harper and Murphy, 1928, observed no effect upon the degree of nodulation of soybeans with application of 200 lbs. of 16 per cent superphosphate per acre. On the contrary, Sewell and Gainey, 1930 reported the opposite effect of superphosphate with alfalfa.

It is apparent from the foregoing discussions that many factors, acting independently and in combination, are involved in the complex process of nodule formation. In spite of the numerous contributions to this problem, there is much still to be learned. Our present knowledge of the effect of organic material is particularly sketchy.

CHAPTER 12

ECONOMIC IMPORTANCE OF LEGUMINOUS CROPS

"They not only work for nothing and board themselves, but they pay for the privilege."
—DAVENPORT

The very fact that leguminous crops have found a place in every known system of farming is indisputable proof of their importance in the agricultural economy of the world. It may be said with reason that the extent to which the Leguminosae are cultivated is the determining factor in the development and maintenance of a high level of agriculture. If the Leguminosae are raised in abundance, there is seldom need for artificial building up of soil productivity. Naturally if the soil is deficient in calcium, phosphorus, potassium, or other inorganic constituent, the use of leguminous crops will not supply the lack. How important the Leguminosae have been in building up the natural fertility of virgin soils is an intriguing question for speculation, but one not easy to answer. Certainly within historic times the Leguminosae have constituted a considerable factor in the economics of food production and in the complex systems of handling cultivated soils. Who knows to what extent they have influenced the trend of civilization itself?

Generally speaking, the farmer grows two types of crops, gramineous and leguminous. No better expression of the primary difference between them can be given than that of Schultz-Lupitz, 1881. He designated all cultivated plants as the leguminous or *nitrogen accumulators,* and the non-leguminous or *nitrogen consumers.* The continued growth of the latter will, of course, deplete the nitrogen supply of the soil, unless compensated by fertilizers. The Leguminosae, on the contrary, will continue indefinitely to produce large crops and at the same time to maintain or increase the nitrogen supply of the soil. It is now well known that this peculiar quality of the Leguminosae is not a function of the plants alone, but a result of their association with the bacteria of their root nodules. This will be apparent in the following discussion of the plants with and without bacteria. The extensive literature on this phase of the subject includes both field and greenhouse studies with nearly all of the principal leguminous crops.

Comparison of plants with and without nodule bacteria. The early papers of Boussingault, 1838; Lawes, Gilbert, and Pugh, 1861; Schultz-Lupitz, 1881; Atwater, 1884 and 1885; and others reported the bare fact that by some means leguminous plants acquire more nitrogen than can be accounted for by straight absorption from the substrate. The following year Hellriegel, 1886, achieved fame by announcing conclusive proof that the apparent nitrogen-fixation in the

leguminous plant occurs only when the plant is furnished with root nodules. This initial discovery has been confirmed and extended by numerous investigators: to mention only a few of the early workers, by Hellriegel and Wilfarth, 1888; Atwater and Woods, 1889, 1890, 1891 a and b, and 1892; Lawes and Gilbert, 1890 and 1891; Schloesing and Laurent, 1892a and b; Bréal, 1888a and b and 1889a and b; Kossowitsch, 1892; and Aeby, 1896. Since then there have appeared a host of other papers showing the indisputable benefit of nodulation. Inasmuch as nodulation is thus shown to be inseparably associated with fixation of nitrogen by the plant, it is logical to suppose that the nodule should be the seat of fixation. There is actually some evidence that such is the case. It is certain that the nodule during the period of most active nitrogen assimilation is much richer in nitrogen than the adjacent root tissue. The figures of Table 20 indicate this clearly and show that there is also a decrease in nitrogen content as maturity approaches, suggesting transfer of nitrogen to the plant. There is, however, no direct proof that the bacteria are the agents of nitrogen assimilation. As pointed out in Chapter 6, the older reports on positive fixation in cultures of the bacteria are open to question because of methods of analysis or impurity of the cultures used. The three most recent and extensive works on the subject—by Hopkins, 1929; Allison, 1929; and Löhnis, 1930a—have reported negative results. These negative findings are not, of course, conclusive evidence that the bacteria within the nodule do not fix nitrogen. Perhaps they assist in the process, or perhaps, as Beijerinck, 1918, suggested, they stimulate the plant and enable it to take in free nitrogen.

Chimerical is it may sound, a number of workers reported that leguminous plants may in some cases benefit from association with rhizobia even though no nodules are formed. Moore, 1905, claimed that it is possible for the bacteria to enter the roots and benefit the host plant without any external evidence of nodule formation. Joshi, 1920, reported that the rhizobia cause increased growth of roots and of the plant as a whole on both Leguminosae and Gramineae. He was unable to find rhizobia inside the root tissues and so concluded that the bacteria act from outside of the plant. He hypothesized two functions for the bacteria: in the first place, they fix nitrogen and thus increase the food supply, and in the second place, they exert a direct stimulating effect upon the higher plant. Unfortunately the inadequacy of Joshi's data makes his observations questionable.

Recently Leonard and Reed, 1930, and Gutschy, 1931, have pointed out that the non-nodule-forming Leguminosae may attain a nitrogen content comparable to that of their nodule-forming relatives. The former studied the yields, total nitrogen, and green manuring value of plants of the genus *Cassia,* as representative of the non-nodulated Leguminosae, and *Sesbania macrocarpa, Crotalaria spectabilis,* and the soybean of the nodulated types. There was found no decided difference among them. To the writers these data seem highly significant, and it is to be hoped that this work will be carried further.

Effect of the rhizobia on the host plant. Frank, 1890b, described six ways in which the rhizobia may influence the host plant.

1. Increase in growth of the various parts of the plant, especially leaves and stem

TABLE 18

Effect of nodule bacteria on nitrogen content and yield of various leguminous crops

Pounds per acre

Location	Plant	Investigator	Nitrogen			Yield		
			With bacteria	Without bacteria	Gain	With bacteria	Without bacteria	Gain
Wisconsin	Alfalfa	Graul and Fred, 1922	86.7	46.2	40.5	2866.2	1714.8	1151.4
Illinois	Alfalfa	Hopkins, 1902	62.0	21.8	40.2	2300.0	1180.0	1120.0
Minnesota	Alfalfa	Arny and Thatcher, 1915	----	----	----	3022.0	1293.0	1729.0
England	Alfalfa	Thornton, 1929a	74.0	42.6	31.4	2654.4	1624.0	1030.4
Wisconsin	Pea	Whiting, Fred, and Stevens, 1925	129.9	99.5	30.4	6098.4	3630.0	2468.4
Michigan	Soybean	Smith and Robison, 1905	113.5	75.9	37.6	3924.0	3572.0	352.0
Wisconsin	Soybean	Fred and Graul, 1919	164.9	35.0	129.9	5160.0	2296.2	2863.8
Wisconsin	Soybean	Fred, 1921	57.1	7.5	49.6	2598.0	811.0	1787.0


RED CLOVER
UNINOCULATED
RED CLOVER
INOCULATED

2. Increase in chlorophyll
3. Increase in CO_2 assimilation
4. Increase in nitrogen content
5. Increase in total plant substance (*i.e.* dry weight)
6. Increase in albumin in the form of bacteroids.

The first five effects are relative and represent a gain in compounds already present, while the sixth is a totally foreign product not found in the nodule-free plant.

It seems to the authors that Frank's list of effects does not lay enough emphasis upon the nitrogen fixation, which is undoubtedly the chief effect of the rhizobia upon their host. In a sense the other effects are secondary and dependent upon the enhanced nitrogen-content of the host. It is well known that a gain in nitrogen will in turn mean a more vigorous and darker green plant, better able to secure nutrients from the soil and to undertake photosynthesis (involving CO_2 assimilation, of course). Fortunately the resultant effect is usually better plant growth, which, in the farmer's terms, means larger yield. Plate 38 illustrates what nodulation will do for the growth of red clover. The increase in yield is sometimes very striking, as indicated by a few examples presented in Table 18. To the casual observer this larger yield appears all important, but there are other points to be considered. In many cases the well-nodulated plants show no increase in yield, yet do show a distinct increase in percentage of nitrogen. Furthermore, the quality of the crop may be distinctly improved. This latter phase is important, for example in the case of canning peas, which Whiting, Fred, and Stevens, 1925; Whiting, 1925; and Whiting and Fred, 1926, have particularly studied. Twelve large-scale field experiments, usually with 5-acre plots,

TABLE 19

Effects of nodulation upon crops of canning peas

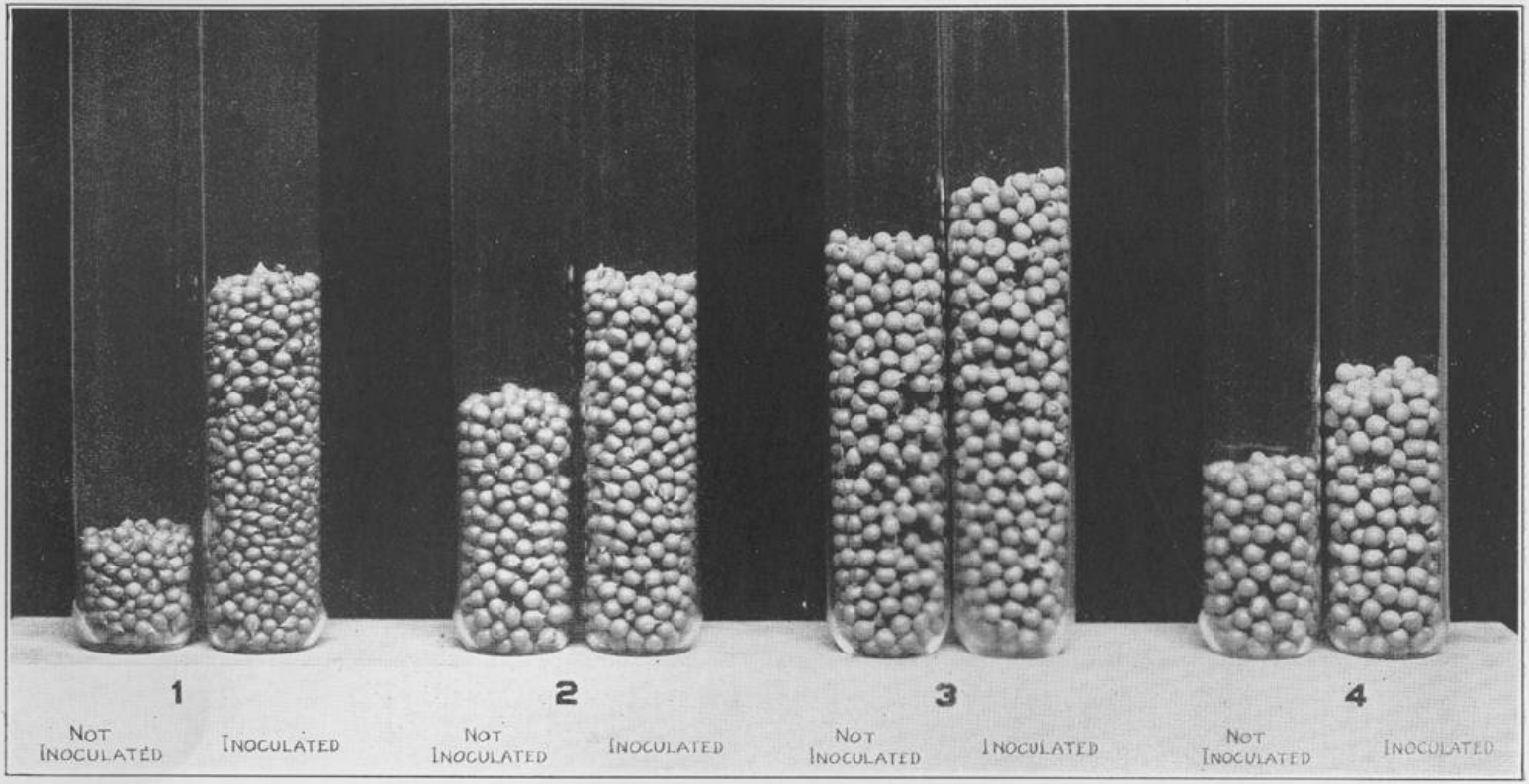
Trial	Variety	Increase in height of vines	Increase in yield of vines	Increase in protein of vines	Increase in yield of peas
		Inches	Per cent	Per cent	Per cent
1	Alaska	7	68.00	55.67	96.00
2	Alaska	6	9.09	49.68	6.57
3*	Alaska	5	52.90	10.52	----
4	Alaska	8	73.07	34.86	41.83
5	Alaska	11	47.36	32.30	35.29
6	Alaska	11	107.14	70.00	53.17
7*	Alaska	0	-----	-----	-----
8	Yellow Admiral	11	21.0	62.34	14.52
9	Perfection	3	31.05	33.48	48.11
10*	Perfection	8	25.64	18.46	6.03
11*	Horsford	2	11.94	25.59	11.32
12*	Horsford	0	7.70	2.12	3.45

*Peas had previously grown on these fields.

were carried out with several varieties of peas, with and without inoculation, and raised on several soil types. Some of the more important effects of inoculation are summarized in Table 19. From the canner's point of view, there is the additional advantage of more uniform growth and ripening. It is thus possible to time the harvesting to secure a higher percentage of the smaller-sized or high-priced peas. The distribution of the different sizes or grades obtainable from nodulated and non-nodulated plants is shown in Plate 39. The percentage of nitrogen also is considerably higher in the peas from nodulated plants (Chart 4). And lastly there is a slight lengthening of the period of ripening which to the canner means a longer maintenance of fancy condition in the field, an important consideration during the rush of the canning season. A similar prolonging of the growing period was reported as early as 1905 by Steglich in the case of nodulated serradella.

There are a number of minor ways in which nodule-bearing plants differ from the normal. In 1915 Arny and Thatcher carried out some analyses of the mineral constituents of nodulated and nodule-free plants. With alfalfa and sweet clover they found an increase in total ash in the roots of nodulated plants, whereas in the tops the reverse is true. However, an examination of the ratios of total dry matter in tops and roots shows proportionately more dry weight in the tops. Stone, Gilmore, and Fraser, 1906 on the contrary, have reported greater root-development in the case of nodulated plants. In 1890 Lawes and Gilbert noted that nodulated pea plants possess a greater number of leaves and more leaf surface. According to Weber, 1920, the lupine plant with nodules contains a high alkaloid content, as compared with a normal nodule-free plant nourished with inorganic nitrogen. The increase in vigor of the nodulated plant has a tendency to render it more than commonly resistant to plant diseases (Linsbauer, 1914). Fellers, 1918b, has shown that it is possible to alter the oil content of soybeans by changing the nutrition of the plant. His results indicate that the increase in the protein content of soybean seeds takes place at the expense of the oil content, and thus well-nodulated soybean plants usually show a decrease in oil content.

Distribution of nitrogen in different parts of the leguminous plants. The percentage of nitrogen in the tops, roots, and nodules varies with the species of plant, its stage of growth, and the type of soil upon which it has grown. Studies of the nitrogen distribution are obviously of great importance and have engaged the attention of a number of workers (for example, Thompson, 1917; Albrecht, 1920; and Snider and Hein, 1926). Troschke, 1884, was the first to emphasize the decided difference in nitrogen content as well as other elements between the roots and nodules. His detailed analyses of the lupine, expressed as percentage on the dry basis, are given below.


1
NOT INOCULATED
INOCULATED
2
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3
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4
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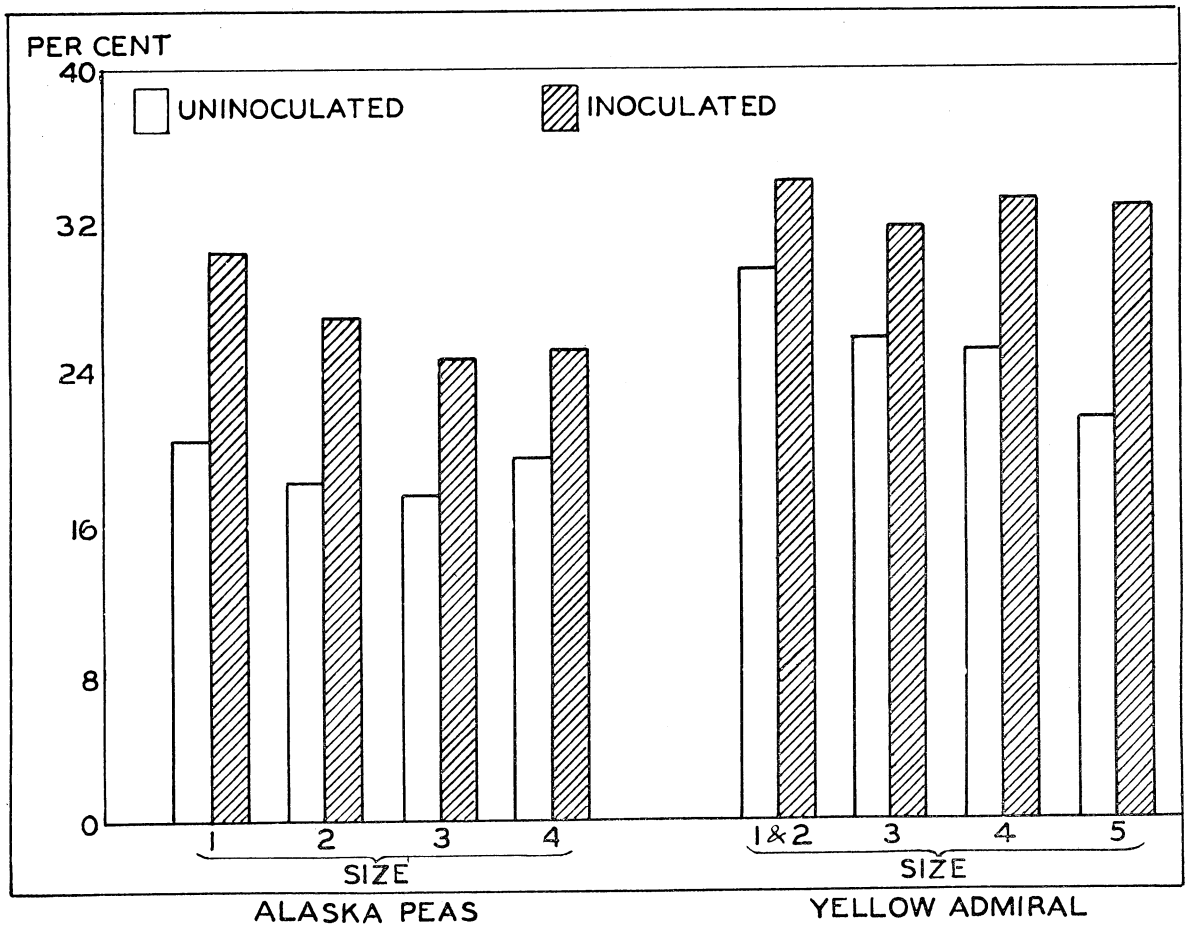

PER CENT
40
32
24
16
8
0
UNINOCULATED
INOCULATED
1
2
3
4
SIZE
ALASKA PEAS
1&2
3
4
5
SIZE
YELLOW ADMIRAL

Composition of roots and nodules

ORGANIC

	Roots	Nodules
	Per cent	Per cent
Ash	4.07	7.51
Crude fat	1.31	5.33
Crude fiber	52.95	9.43
Total nitrogen	1.13	7.25
Crude protein	7.06	45.31
Albumin	5.02	31.59
Nitrogen-free extract	34.61	32.42

INORGANIC

	Ash	Ash
	Per cent	Per cent
Potassium	12.80	16.90
Sodium	24.11	25.87
Calcium	11.23	10.03
Magnesium	11.61	10.82
Iron oxide	0.34	1.82
Manganese oxide	0.68	0.69
Phosphoric acid	8.84	16.19
Sulphuric acid	24.27	11.74
Silicic acid	3.28	3.11
Chlorine	3.48	4.45

According to these figures, the nodule is high not only in total nitrogen but also in fat, albumin, phosphoric acid, and iron oxide.

The results of total-nitrogen analyses, taken at several periods in the life of the plant, show changing values for the nodule, whereas the nitrogen of the root remains fairly constant, or decreases slightly with age (Table 20). In general, the percentage of nitrogen in the nodule is high in the period preceding blossoming, and decreases with the formation of seed, until at maturity of the seed, it reaches approximately the level of the root tissue. For greater detail concerning the forms of nitrogen in the nodule, the reader is referred to the papers of Stoklasa, 1895; Strowd, 1921; Parisi and Masetti-Zannini, 1926; Stallings, 1926; Wozak, 1929; and to Chapter 10 of this monograph.

As to the amount of nitrogen that may be fixed by an individual plant, we refer to the papers of Hopkins, 1904; Alway and Pinckney, 1910; Brown and Stallings, 1921; and Wright, 1925b. More important from the economic point of view is the gross fixation of nitrogen which may be expected of an entire leguminous crop.

The gain in nitrogen by various leguminous crops. The actual amount of nitrogen taken from the air by a leguminous crop is dependent upon many factors, *e.g.*, kind of leguminous crops, efficiency of the rhizobia, nature of the soil, supply of moisture, temperature, presence of adequate supplies of phosphorus, calcium, etc., the amount of nitrate nitrogen, and other factors of less importance. There are two methods of approach to the determination of the amount of nitrogen

TABLE 20

Percentage distribution of nitrogen in roots and nodules of various leguminous crops

Name of plant	Author	Age of plant	Nitrogen content	
			Roots	Nodule
			Per cent	Per cent
Lupine, blue	Troschke, 1884	Pods well formed	1.13	7.25
Acacia	Bréal, 1888b	No record	2.30	3.25
Lentil	Bréal, 1888b	Before blooming	1.80	7.0
Lentil	Bréal, 1888b	After blooming	----	5.6
Lupine, yellow	Stoklasa, 1895	Blossoms formed	1.64	5.22
Lupine, yellow	Stoklasa, 1895	Pods well formed	1.84	2.61
Lupine, yellow	Stoklasa, 1895	Seed ripe	1.42	1.73
Cowpea	Hopkins, 1904	Pods well formed	1.48	5.92
Soybean	Smith and Robison, 1905	Pods well formed	0.91	4.19
Cowpea	Smith and Robison, 1905	Pods well formed	0.89	3.90
Hemp, wild	Kellerman and Robinson, 1908	No record	1.50	6.40
Lupine	Knisely, 1901	Full bloom	0.92	5.17
Lupine	Knisely, 1901	Pods well formed	0.83	4.29
Lupine	Knisely, 1901	Pods very large	0.66	3.70
Lupine	Heinze, 1907	No record	0.99	6.18
Serradella	Heinze, 1907	No record	1.52	7.12
Horse bean	Sani, 1910	No record	2.39	5.71
Sann-Hemp	Joshi, 1920	First week	1.95	10.81
Sann-Hemp	Joshi, 1920	Second week	1.65	9.46
Sann-Hemp	Joshi, 1920	Third week	1.45	6.68
Sann-Hemp	Joshi, 1920	Fourth week	1.19	5.34
Sann-Hemp	Joshi, 1920	Fifth week	1.14	3.06
Sann-Hemp	Joshi, 1920	Sixth week	1.05	3.36

TABLE 20 (Continued)

Percentage distribution of nitrogen in roots and nodules of various leguminous crops

Name of plant	Author	Age of plant	Nitrogen content	
			Roots	Nodule
			Per cent	Per cent
Soybean	Fred, 1921	Pods well formed	1.31	5.67
Pea, (canning)	Fred and Bryan, 1922a	Seed ripe	----	1.49
Horse bean	Parisi and Masetti-Zannini, 1926	Before blooming	2.99	7.28
Lupine, white	Parisi and Masetti-Zannini, 1926	Before blooming	----	4.17
Soybean alone*	Stallings, 1926	First bloom	2.74	7.85
Soybean with wheat*	Stallings, 1926	First bloom	2.41	7.79
Soybean alone*	Stallings, 1926	Pods half mature	2.14	9.81
Soybean with wheat*	Stallings, 1926	Pods half mature	2.21	9.58
Soybean alone**	Stallings, 1926	First bloom	1.60	4.04
Soybean with wheat**	Stallings, 1926	First bloom	1.37	3.99
Soybean alone**	Stallings, 1926	Pods half mature	1.66	3.83
Soybean with wheat**	Stallings, 1926	Pods half mature	1.62	3.71
Soybean alone***	Stallings, 1926	First bloom	2.64	4.34
Soybean alone***	Stallings, 1926	Pods half mature	2.91	4.51
Pea	Virtanen, 1928a	Seed ripe	4.17	4.23
Clover, red	Virtanen, 1928a	Before blooming	3.60	4.31
Clover, alsike	Virtanen, 1928a	Blossoms formed	3.61	8.05
Clover, white	Virtanen, 1928a	Blossoms formed	3.62	5.63
Horse bean	Wozak, 1929	Young	3.46	8.79
Horse bean	Wozak, 1929	Old	1.27	6.29
Pea	Wozak, 1929	Young	3.60	8.89
Pea	Wozak, 1929	Old	2.15	4.34
Bean	Wozak, 1929	Young	2.68	7.93
Bean	Wozak, 1929	Old	1.06	4.70
Lupine	Wozak, 1929	Young	2.02	7.82
Lupine	Wozak, 1929	Old	1.00	5.52
Vetch	Wozak, 1929	Young	3.10	7.81
Vetch	Wozak, 1929	Old	1.89	4.78
Clover, red	Wozak, 1929	Young	3.82	10.61
Clover, red	Wozak, 1929	Old	2.22	7.54
Alfalfa	Wozak, 1929	Young	3.22	10.64
Alfalfa	Wozak, 1929	Old	2.12	5.08

*A productive soil. **An unproductive soil. ***Nitrogen-free sand.

TABLE 21

The amount of nitrogen taken from the air by various leguminous crops

Calculated for one acre per annum

Name of investigator	Place	Crop	Total nitrogen
			pounds
Arny and Thatcher, 1915	Minnesota	Alfalfa	113*
Arny and Thatcher, 1917	Minnesota	Sweet clover	117
Hopkins, 1902	Illinois, (1 crop)	Alfalfa	40
Whiting, 1915	Illinois, (4 tons)	Alfalfa	132
Lipman and Blair, 1917	New Jersey	Alfalfa[1]	162*
Fred and Graul, 1916b	Wisconsin	Alfalfa	64*
Graul and Fred, 1922	Wisconsin	Alfalfa	94*
Shutt, 1906, 1910	Canada	Clover, red	75–150
Shutt, 1912	Canada, (av. 10 yrs.)	Clover, red	51
Whiting, 1915	Illinois, (4 tons)	Clover, red	106
Graul and Fred, 1922	Wisconsin	Clover, red	100
Fred, Wright and Frazier, 1921	Wisconsin	Pea[2]	132
Duggar, 1898	Alabama	Vetch, hairy	79
Whiting, 1915	Illinois	Cowpea	86
Bréal, 1889a	France	Bean	87
Fred, 1921	Wisconsin, (25 bu. crop)	Soybean	57
Fred and Graul, 1919	Wisconsin	Soybean	108
Albrecht, 1920	Illinois	Soybean and cowpea	107
Moore, 1905	16 States of the U.S.A.	Mixed leguminous crops	122
Mertz, 1918	California	Mixed leguminous crops	94
Hartwell and Pember, 1911	Rhode Island	Mixed two crops	120

In several cases the high figures represent the gain in nitrogen from the growth of two or three crops.
*Average figure.
[1]Estimated a non-leguminous crop used as control.
[2]Heavy rate of seeding.

which a leguminous crop takes from the air; *i.e.,* by analysis of the crop alone (field results) or by analysis of seed, entire plant, and soil (pot experiments from which calculation to the field basis is made).

A. *Analysis of the Crop Alone.* If it be assumed that a nodule-bearing leguminous crop takes the same amount of nitrogen from the soil as the same crop without nodules, then the difference between the ultimate analyses of the two represents the amount of nitrogen assimilated from the air. Such an estimate may be approximate but is obviously not exact, as there is no guaranty that nodulated plants draw to the same extent upon the nitrogen of the soil. Furthermore the "crop" usually consists of the tops only; the nitrogen of the roots and any that may have been given off to the soil by decay or excretion would thus be missed.

B. *Analysis of Seed, Soil, and Crop.* This procedure is much more time-consuming than that just considered. Obviously it can be done only on a limited scale, generally upon pot experiments raised under greenhouse conditions. Translation of the results to terms of the field is therefore open to the criticism that field conditions are different, and that the results of greenhouse experiments may not necessarily hold in the field.

In any case, the reports upon nitrogen fixation now in the literature are based upon one or the other of these calculations. While they are thus not exact, they are still of value; they are at least conservative estimates of the amount of nitrogen that may be fixed. A representative selection of these reports is set forth in Table 21, from which it appears that the amount of nitrogen fixed in a single crop averages less than 100 lbs. per acre per annum. In fact, 100 lbs. is rather the exception than the rule. Some of the fabulously high figures in the literature may be traced to confusion of statement and meaning. For example, Giöbel, 1926, gave a number of figures from the early reports, which purport to show the gain in nitrogen in a leguminous crop. In reality these reports refer to the total nitrogen-content of the crop and not to the amount taken from the air. In 1912 Shutt reported the results of a 10-year experiment on the nitrogen enrichment of soil by the continuous growth of red clover. The experimental plot was chosen originally for its low nitrogen and humus content and was treated at the beginning of the experiment with super-phosphate and potash. Once during the experiment a dressing of lime was required, as the plot showed a tendency toward acidity. The crop was cut as required during the growing season (never allowed to go to seed) and the material left upon the ground. Every second year the plot was plowed and resown. The nitrogen-enrichment of the soil as summarized by Shutt at the close of the 10-year experiment is as follows:

	Date of collection	Nitrogen	
		Percentage in water-free soil	Pounds per acre to a depth of 4 inches
Before experiment	13–5–02	.0437	533
After two years	14–5–04	.0580	708
After four years	15–5–06	.0608	742
After five years	30–5–07	.0689	841
After six years	23–5–08	.0744	908
After seven years	4–5–09	.0750	915
After nine years	5–5–11	.0824	1,005
After ten years	22–5–12	.0856	1,044
Increase in nitrogen due to ten years' growth	———	.0419	511

The total increase will be seen to be approximately 500 lbs. or 50 lbs. per acre per year.

Lipman, 1919, and Löhnis, 1925, have made some interesting calculations concerning the economic importance of symbiotic nitrogen fixation. According to the figures of Lipman, the symbiotic bacteria of the Leguminosae fix annually above 1,750,000 tons of nitrogen in the United States alone. Löhnis, basing his calculations on the assumption of 60 pounds per acre and approximately 37,000,000 acres of leguminous crops, arrived at the more conservative figure of slightly over 1,000,000 tons of nitrogen per year. Even this figure the writers feel to be too generous. Lipman further estimated that there is an average loss of 60 pounds of nitrogen per acre per annum for all arable land in the United States. This means a gross loss of 9 million tons. The nitrogen added yearly by way of manures and artificial fertilizers, leguminous crops, atmospheric precipitation, and free-nitrogen fixation offsets 5 or 6 million tons of this, leaving a deficit of 3 to 4 million tons each year.

Nitrogen balance after a leguminous crop. Because of the well-established fact that under certain conditions the Leguminosae can draw all their nitrogen from the air, many writers have assumed that continuous cropping with leguminous crops will not deplete the soil. It is forgotten that in the field the leguminous plant draws to some extent upon the soil nitrogen and unless the residue after harvest contains sufficient nitrogen to make up the loss, fertility suffers. There

The amount of nitrogen in total crop and in roots

Plant	Pounds per acre		
	Tops	Roots	Total
Horse bean	171	32	203
Soybean vines and seed	165	9	174
Soybean vines	75	13	88
Cowpea vines	95	22	177
Vetch vines	153	27	180
White lupine vines	87	10	97
Yellow lupine vines	133	16	149
Blue lupine vines	127	11	138
Red clover vines	138	44	182

is in the literature a great deal of data on this point. As early as 1890, Woods investigated the total nitrogen content of the tops and roots of some of the common leguminous plants.

The distribution of nitrogen in the tops and roots at different stages of growth has been studied by a number of investigators, Penny, 1905; Mielck, 1913; Albrecht, 1920; Brown and Stallings, 1921; Graul and Fred, 1922; Erdman and Fife, 1928; Luekel, Barnette, and Hester, 1929; and Wozak, 1929. The book of Pieters, 1927, is to be particularly recommended for an interesting survey of the question of the distribution of nitrogen. Of the recent papers, that of Brown and Stallings, 1921, is noteworthy as containing an extensive study of the nitrogen distribution in alfalfa and red clover. The percentage of nitrogen in the roots appears to be higher in plants grown on poor soil. Expressed as percentage of total nitrogen of the plant, that in the roots varies with the age of the plant and also with the kind of plant. For an example of the difference between clover and alfalfa—at maturity clover roots contain 27 per cent of the plant nitrogen, whereas alfalfa roots contain 46 per cent. Thus Erdman and Fife, 1928, showed that in the case of Manchu soybean plants 67.3 per cent of the nitrogen assimilated from the air is in the tops and 32.7 per cent in the roots. Williams, 1928, demonstrated that the roots of soybean plants, 30 to 40 days after planting, contain 35 to 45 per cent of the plant nitrogen. At maturity the percentage in the roots is only 10 per cent of the whole. According to the figures of various workers, the proportion of nitrogen in the roots of annual Leguminosae ranges from 5 to 20 per cent and that of the biennial or perennial plants from 24 to 35 per cent. In exceptional cases, it may be as high as 74 per cent. The striking difference between the small-seeded biennial or perennial Leguminosae and the large-seeded annuals is clearly portrayed in the discussion by Pieters. The following figures will illustrate:

		Dry weight	Nitrogen	
		In roots	In roots	In tops
		per cent	per cent	per cent
Small-seeded	Red clover	33.48	2.34	2.70
	Alfalfa	33.52	2.03	2.56
	Sweet clover	26.48	2.04	2.41
	Crimson clover	24.38	2.29	2.85
Large-seeded	Vetch	17.30	2.16	3.34
	Cowpea	14.45	1.45	2.70
	Velvet bean	13.20	1.27	2.34
	Soybean	12.18	1.91	2.58

It is clear that the annual leguminous crops have root systems comparatively small in proportion to the mass of tops. The percentage of nitrogen in the roots of the annuals is also slightly less than in the longer-lived Leguminosae. In actual percentage of nitrogen also the roots of the Leguminosae are inferior to the tops, which in the plants cited above, range from 2.41 to 3.34 per cent. If the tops are entirely removed as crop, it is clear that the major portion of the nitrogen will be removed. It is then a fallacy to suppose that continuous cropping of Leguminosae, with entire removal of the parts above ground, is beneficial.

Swanson, 1917; Swanson and Latshaw, 1919; and Wright, 1920, have emphasized this point and have shown that there may be actually a decrease in the combined nitrogen of the soil. The loss, however, may be more apparent than real. Under the usual systems of farming, the hay crop is fed upon the farm. Manure is returned to the fields, and so for the farm as a whole the balance sheet may show an actual gain.

The associated growth of leguminous and non-leguminous plants. In nature leguminous and non-leguminous plants are found growing side by side, often in such close association that their root systems are intermingled. To all appearances this intermingling is far from harmful. The early writings on agriculture indicate that the practice of raising mixed crops of leguminous and non-leguminous plants has been handed down from ancient times. In a description of farming methods in the Orient, King, 1911, referred to the common practice of intertillage in China and Japan. Intertillage is a system of multiple cropping, whereby a legminous plant such as the soybean is grown in alternate rows with barley, millet, or corn. Although unsupported by scientific knowledge, the early farmers of western Asia and the Roman Empire recognized the value of the leguminous crops and used them liberally in their systems of crop rotation. Among the early scientific reports on the beneficial effect of leguminous plants on cereals, the paper by La Flize, 1892, deserves mention. La Flize judiciously recommended that the grain crop be raised in association with some member of the Leguminosae. He himself demonstrated the favorable effect of peas or vetch sown with barley, especially in case the crop is raised on land of low productivity. La Flize hinted that the cereal crop actually makes use of the nitrogen fixed by the leguminous crop. In 1902 Nobbe and Richter reported that the yield from pots with oats alone may amount to 3.353 gm. per plant, and from pots of mixed oats and soybeans 4.020 gm. per oat plant. Lipman, 1910a and 1912, devised an ingenious method for raising leguminous and non-leguminous plants side by side but separated by means of a porous partition. In practice he used small porous pots planted to cereals, sunk into larger pots planted to Leguminosae. While his experiments are not conclusive, they seem to indicate passage of nitrogenous compounds from the leguminous to the non-leguminous plants. Lyon and Bizzell, 1910 and 1911, demonstrated that timothy grown in association with a leguminous crop such as alfalfa, possesses a higher nitrogen content than does timothy grown alone; *viz.,* timothy with alfalfa, 15.56 per cent protein in the dry matter in contrast with 12.75 per cent in timothy alone. Somewhat similar results were secured by Evans, 1916, in a study of the protein content in the leaves of timothy, red top, and Kentucky blue grass in mixture with and without clover. Almost without exception the grasses grown with clover show a higher nitrogen content. Bagge, 1927, contended that associated growth of leguminous and non-leguminous crop regularly brings about an increase in the nitrogen content of the non-leguminous member. Kellerman and Wright, 1914, made an even more startling statement when they asserted that *both* the leguminous and non-leguminous plants acquire a higher nitrogen content by growing together.

Virtanen has perhaps done the most extensive recent work along this line. In 1929a he reported that oats grown in a nitrogen-free quartz sand may profit by the presence of a leguminous plant such as clover or the pea. That the benefit is

due to nitrogen fixation by the leguminous associate is indicated by the observation that the oats derive no benefit unless or until the leguminous plant bears nodules. In later papers, Virtanen and Hausen, 1930a and b, presented further evidence that oats may take up nitrogen from nodule-bearing pea plants. For maximum benefit there should be not more than one or two oat plants for each pea plant. Similar results are obtained if the peas are replaced by clover and the oats by meadow foxtail. There is not yet a clear explanation of the transfer of nitrogen from the leguminous to the non-leguminous plant. Stallings, 1926, suggested that ammonia may be the form of the nitrogen in question. This he judged from his observation that the ammonia content is, as a rule, considerably higher in wheat grown in association with the soybean than in normal wheat. A particularly interesting experiment is reported by Lipman and Blair, 1928, who raised Indian corn with various catch crops on adjacent plots continuously for 18 years. On one set of plots, vetch, a mixture of clovers, and alfalfa were used as leguminous catch crops with corn; on another plot rye was the catch crop. The average yield for 18 years was:

	Corn per acre bushels
Corn with leguminous crop	37.9
Corn with rye	26.0

The danger of over-emphasizing the practical value of the associated growth of Leguminosae and Gramineae must not be overlooked. Westgate and Oakley, 1914, pointed out that the association is not in all cases beneficial. Under certain conditions there may even be a decrease in the nitrogen content of both leguminous and non-leguminous participants. Moreover, there are other considerations involved in such a complex situation as obtains in an association of leguminous and non-leguminous plants. According to Ellett, Hill, and Harris, 1915; Mooers, 1927; McClelland, 1928; and others, the combination of leguminous and non-leguminous plants may be mutually harmful. Crowding and competition for the available plant food and moisture are factors to be considered. The non-leguminous members will naturally take up the available nitrate nitrogen and so force its leguminous neighbor to draw from the air. Thus the associated growth may eventually lead to greater nitrogen fixation, but other factors affecting plant growth may at the same time operate to limit total growth, or in other words, yield.

Availability of nitrogen from leguminous crops and the value of leguminous crops. Plant residues returned to the soil are promptly attacked by soil microörganisms, which multiply enormously in response to the additional food. If now the plant material provides carbohydrate and protein in proper proportion, the decomposition is accomplished rapidly without loss to the soil itself. If, however, there is a large overbalance of carbohydrate, the attacking microörganisms will draw upon the available nitrogen of the soil and to a large extent lock it up in their own bacterial and fungal protoplasm. The result is an immediate decrease in the nitrogen available for plant nutrition.

In view of the relatively high nitrogen content of the Leguminosae, it is to be expected that they constitute a desirable form in which to furnish organic and

nitrogenous matter to the soil. Generally their tissue is promptly and completely decomposed and soon becomes available to succeeding crops. No attempt will be made to discuss the various steps involved in the decomposition and mineralization of the nitrogen nor to specify the organisms involved in the conversion of plant tissue to plant food. For a discussion of these subjects the reader is referred to the papers of Löhnis, 1926a and b; Martin, 1927 and 1929; and Starkey, 1929a, b, and c. The speed with which plant residue, particularly green plant tissues or so-called green manure, is made available through decay has occasioned much study. Lipman and Blair, 1928, have demonstrated that several factors control availability; *i.e.*, quality and quantity of the green manure, the time of application, the quality of the soil, and the kind of crop which follows. Pieters, 1927; and Löhnis, 1926a, have added to the list such factors as kind and age of the plant, moisture content of the plant and of the soil, temperature, and the nature of the microörganisms in the soil. These considerations are also discussed by Hutchinson and Milligan, 1914; Joshi, 1919; Bal, 1922; and Whiting and Schoonover, 1920a. Wright, 1915, particularly studied the question of the maturity of the plant tissue at the time of manuring and showed that the use of mature residue may actually serve to reduce the amount of available nitrogen in the soil. This locking up of the nitrogen is, of course, temporary.

A great many studies have been made to determine how much of the nitrogen of a green-manure crop is taken up by the succeeding crop and under what conditions the most effective green manuring can be accomplished. Availability studies of leguminous green manures indicate that 50 - 80 per cent of the nitrogen is returned in the first succeeding crop. The residual effect lasts over 2 or 3 years but in decreasing efficiency. The return is highest when the green manure consists of young plant material. According to Wagner, 1892, the nitrogen of green manure is much more available than that of stable manure but less so then sodium nitrate. The comparative figures are:

Nitrate of soda	100
Green manure	70
Stable manure	45

Mertz, 1918, found the nitrogen of leguminous green manure distinctly superior in availability to that of non-leguminous manure.

Tests of the availability of green manures are made by determining the rate of nitrification either chemically or indirectly by comparison of plant growth. Lyon and Bizzell, 1913a; and Lyon, Bizzell, and Wilson, 1920, have shown that the rate of nitrification is greater in soil on which a legumious crop has grown. This stimulation of the nitrifying bacteria is a fortunate fact which may in part account for the high value of leguminous green manuring. Other studies of nitrification have shown that the kind, degree of maturity, etc., of the green manure itself is influential in the rate of nitrification. There are many reports in the literature concerning the rate of nitrate formation from leguminous green manures (Thompson, 1917; Wilson and Wilson, 1925; Löhnis, 1926a; Whiting and Richmond, 1921 and 1927a and b; Martin, 1927; Lyon and Wilson, 1928, and others). According to Löhnis, 1926a, determination of the rate of nitrification of green manures is an accurate measure of the availability of their nitrogen. Such being

the case, the following reports are the more significant. Whiting, 1926, discussed some of the important points concerning the nitrification of plant tissue and emphasized particularly the fact that the rate of nitrification is largely dependent upon the water-soluble nitrogen content of the material. He was able to show that the green tops of such plants as sweet clover, alfalfa, red and alsike clover, and, to a lesser degree, the annual Leguminosae, nitrify more rapidly than non-leguminous plants. Lyon and Wilson, 1928, likewise found young green tops conducive to maximum accumulation of nitrates. Leukel, Barnette, and Hester, 1929, found variation in the rate of nitrification with age of the leguminous material decomposed. They observed progressive decrease in availability of *Crotalaria striata* tissue as it developed through young succulent stages, bloom, and full maturity. Lyon and Wilson, 1928, obtained some interesting results by comparison of the rates of nitrification of different plant tissues. Leguminosae are not always of first rank, as indicated by their list—vetch, rye, peas, oats, and buckwheat in the order given.

Nitrification of bacterial substance itself has been studied by Barthel and Bengtsson, 1928, who found great variability. *Rhizobium* after 2 months in soil yielded 34.1 per cent of its nitrogen, *Urobacillus* 60.7 per cent, and *Azotobacter* apparently none.

Effect of the Leguminosae upon succeeding crops. Green manuring, or the plowing under of a green crop to enrich the soil, is an old and well established practice. It had its origin in empirical farm practice, long before there was an agricultural science to tell the reason for the custom. In 1927 Pieters produced an entire book on the subject of green manuring. His presentation of the facts is so adequate that only a brief account need be given here. The chief influences of green manures may be summarized as follows:

1. Increase of the nitrogen supply of the soil
2. Increase of the available phosphorus and potassium content of the soil
3. Increase in the organic matter of the soil
4. Stimulation of bacterial activity in the soil
5. Improvement in the physical structure of the soil
6. Suppression of weeds
7. Change of reaction of the soil

The book by de Sornay, 1916, is of interest to those concerned with tropical leguminous plants. Nolte in 1923 presented a general discussion of green manuring problems. As stated by Lipman and Blair, 1928, green manures have the following functions in the soil:

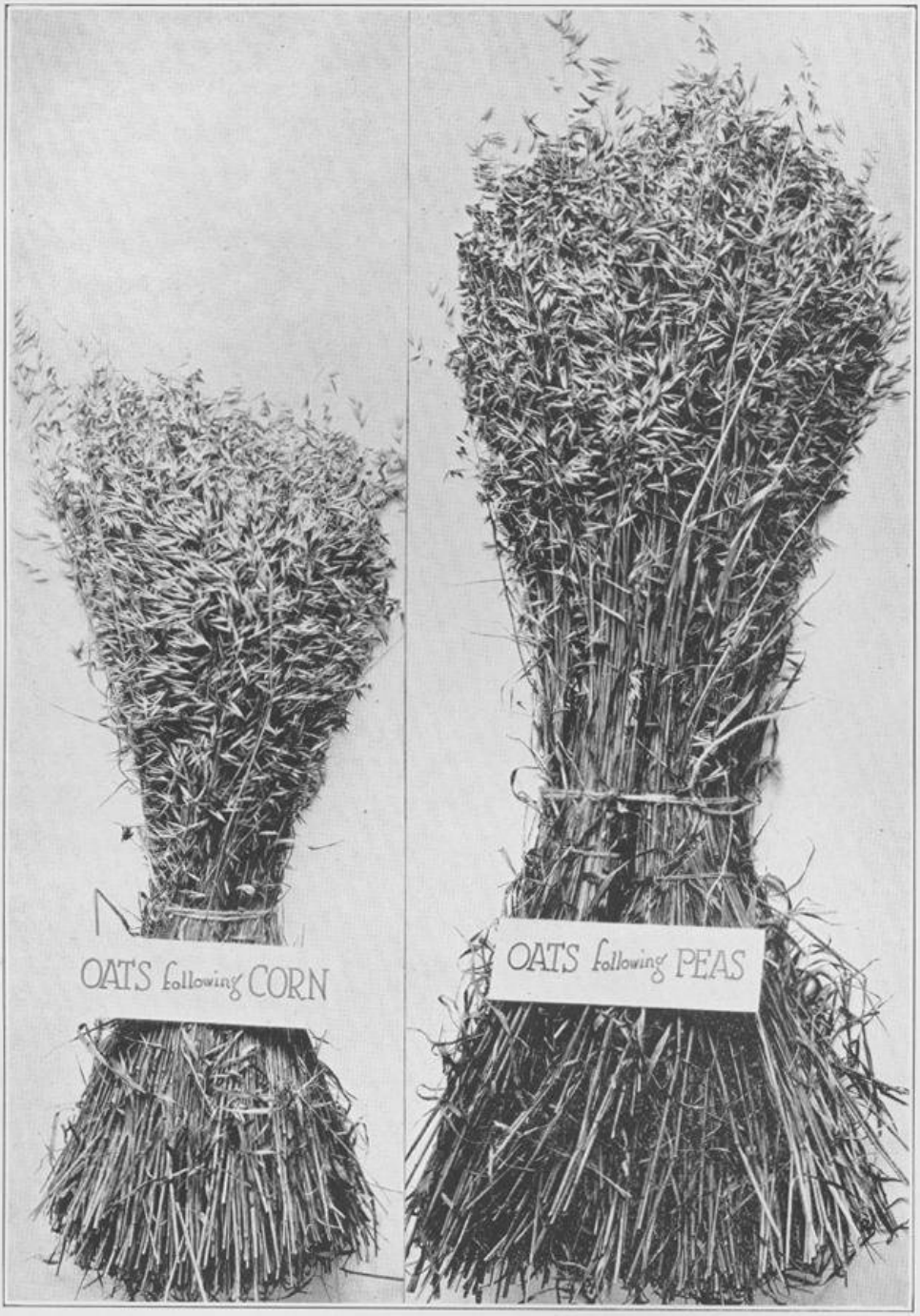
1. As cover crops they help to conserve plant food constituents
2. Protect the surface soil from erosion
3. Increase the store of soil nitrogen
4. Increase the store of organic matter
5. Improve soil texture
6. Influence the number, kinds, and activity of soil microörganisms

TABLE 22

Effect of various leguminous and non-leguminous plants on succeeding crop

Location	Investigator	Preceding crop	Yield of indicator crop per acre
Tennessee	Mooers, 1930	Sweet clover*	Corn 54.5 bushels, (av. of 5 yrs.)
Tennessee	Mooers, 1930	Orchard grass*	Corn 44.1 bushels, (av. of 5 yrs.)
Tennessee	Mooers, 1930	Lespedeza*	Corn 48.3 bushels, (av. of 5 yrs.)
Tennessee	Mooers, 1930	Corn	Corn 31.0 bushels, (av. of 5 yrs.)
New York	Lyon, 1925	Clover, red*	Oats 98.7 bushels
New York	Lyon, 1925	Timothy*	Oats 43.7 bushels
New York	Lyon, 1925	Alfalfa	Corn 80.3 bushels of ears
New York	Lyon, 1925	Timothy	Corn 54.1 bushels of ears
New York	Lyon, 1925	Alfalfa[1]	Oats 73.7 bushels of grain
New York	Lyon, 1925	Timothy[1]	Oats 62.8 bushels of grain
New York	Lyon, 1925	Alfalfa[2]	Wheat 39.6 bushels of grain
New York	Lyon, 1925	Timothy[2]	Wheat 36.0 bushels of grain
New York	Collison, 1931	Alfalfa	Barley 68.8 bushels of grain (av. of 3 yrs.)
New York	Collison, 1931	Alfalfa	Wheat 33.6 bushels of grain (av. of 3 yrs.)
New York	Collison, 1931	Timothy	Barley 25.75 bushels of grain (av. of 3 yrs.)
New York	Collison, 1931	Timothy	Wheat 25.6 bushels of grain (av. of 3 yrs.)
Indiana	Wiancko, Walker, Mulvey, 1928	Clover*	Corn 55.4 bushels of grain
Indiana	Wiancko, Walker, Mulvey, 1928	Timothy*	Corn 41.8 bushels of grain
Indiana	Wiancko, Walker, Mulvey, 1928	Soybeans*	Corn 48.8 bushels of grain
Ohio	Cutler and Hoyt, 1927	None	Corn 31.3 bushels of grain (av. 4 yrs.)
Ohio	Cutler and Hoyt, 1927	Clover, red**	Corn 42.3 bushels of grain (av. 4 yrs.)
Ohio	Cutler and Hoyt, 1927	Sweet clover (biennial)**	Corn 49.4 bushels of grain (av. 4 yrs.)
Ohio	Cutler and Hoyt, 1927	None	Oats 40.9 bushels of grain (av. 4 yrs.)
Ohio	Cutler and Hoyt, 1927	Clover, red**	Oats 43.5 bushels of grain (av. 4 yrs.)
Ohio	Cutler and Hoyt, 1927	Sweet clover (biennial)**	Oats 49.9 bushels of grain (av. 4 yrs.)

*Hay crop removed.
[1]Second year after the alfalfa and timothy had been turned under.
[2]Third year after the alfalfa and timothy had been turned under.
**Plowed under—green manure.


OATS following CORN
OATS following PEAS

The addition of nitrogen to the soil is by no means the only benefit to be derived from the Leguminosae. Of equal or greater importance may be the fundamental change which they induce in the soil particles—a setting free of nutrients for plant nutrition. As shown by Dvořak, 1912, immense quantities of CO_2 are set free by the microbial decomposition of the plant tissues. This CO_2, escaping to the atmosphere, may account at least in part for the great benefit to the succeeding crop. In the soil itself, the presence of CO_2 is beneficial to fertility because of its solvent action; Headden, 1927, for example, referred to the solution of feldspar as aided by the CO_2 discharged from the roots of Leguminosae or resulting from their decomposition in the soil. Various investigators have argued that the soil improving quality of the Leguminosae is due not entirely to the nitrogen they fix but also to their stimulating effect upon the numbers and activity of the soil microflora (Sears and Paden, 1929). Dvořak, 1912, and Fulmer, 1917, suggested such an indirect effect through stimulation of free nitrogen fixation. The carbonaceous portion of the plant tissue turned under yields a large amount of energy upon decomposition. A part of that energy is seized by free-living nitrogen-fixing bacteria, which then proceed to fix nitrogen.

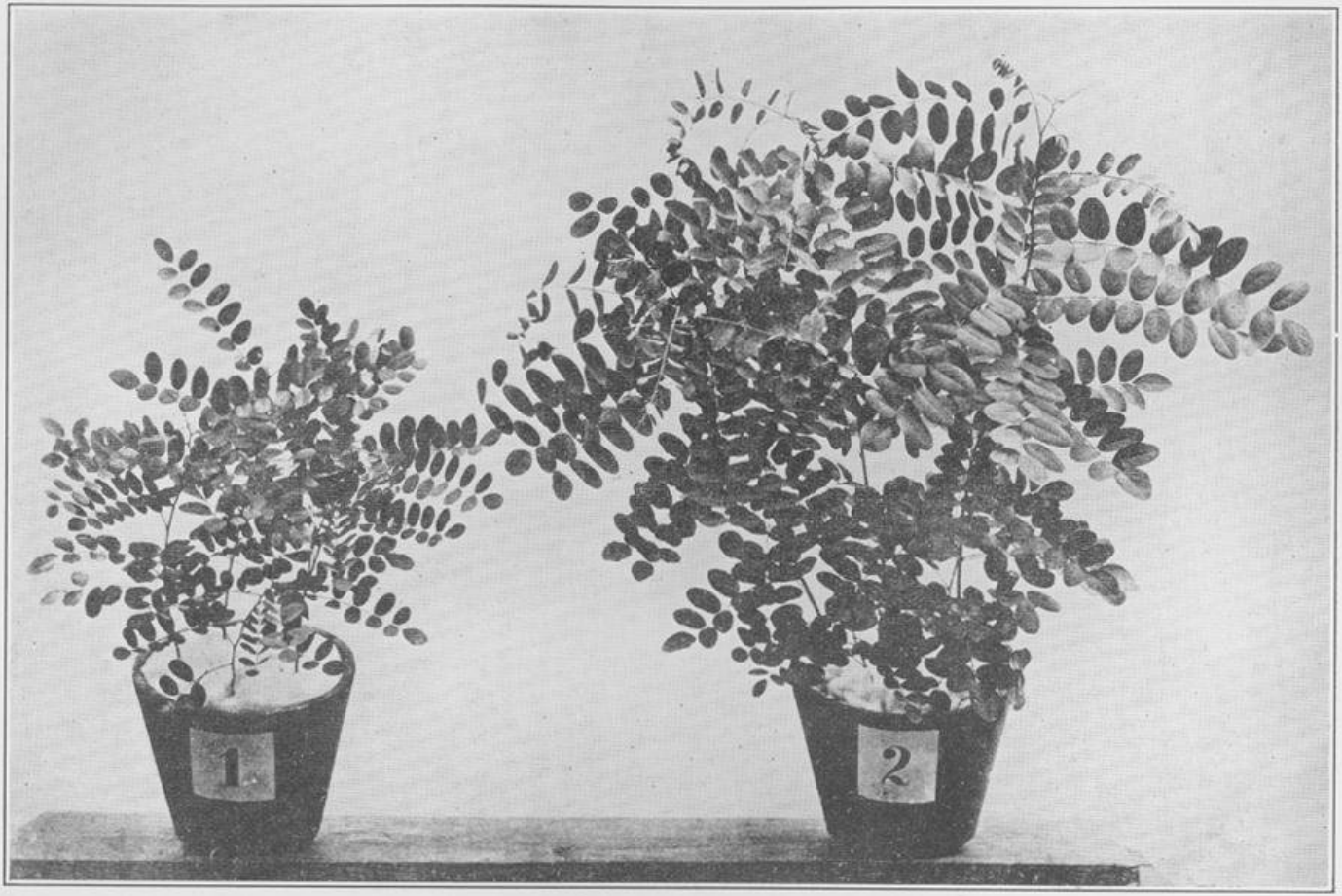
Lyon, 1930, concluded from a number of experiments that a leguminous crop, introduced at regular intervals in a rotation of crops, serves principally to keep the soil nitrogen *activated*. The effect appears to be temporary; hence the need for frequent renewal. Repeated growth of leguminous plants does not greatly enhance the effect. These beneficial offices of the green manures are reflected in the increased yields of succeeding crops. See Table 22 and Plate 40. This fact was noted early in the history of agriculture (see the specific statement of Varro, Chapter 3 of this monograph) and has been confirmed again and again until the present day. A few recent examples of increased yield in the succeeding or so-called indicator crop are cited in Table 22. They clearly show the remarkable benefit to be derived from leguminous green manuring.

A number of the recent papers have claimed that it is not necessary that the entire mass of the leguminous crop be returned as green manure. Löhnis, 1926b, remarked that removal of tops in the case of several Leguminosae made little difference in the after-effect. Mooers, 1930, in studying the remarkable soil-improving properties of sweet clover, found that the average yield of corn after sweet clover, with tops cut for hay, was 54.5 bushels per acre over a 5 year period. Lespedeza was somewhat less effective; even though the entire crop was turned under, the succeeding corn crop amounted to only 40.2 bushels per acre. In this connection the long-time experiment of Bailey, Williamson, and Duggar, 1930, deserves mention. They found that in their experimental plots the yield of corn has been maintained or increased over a period of 34 years, during which a leguminous crop has been sown in the corn at the last plowing. The control plot without the leguminous crop has shown a steady decrease and at present yields less than half the crop of the treated plot.

It would be unfair to close the discussion of green manuring without repeating the warning that removal of a large part of the leguminous crop may under some circumstances result in actual loss of soil nitrogen. The results of Swanson, 1917; and Swanson and Latshaw, 1919, have shown this to be so of alfalfa, continuously cut for hay. Wright, 1920; and Hall, 1917, have reported

somewhat similar findings. These are probably extreme cases involving removal of much plant tissue. Removal of tops once in the season, as implied in the reports of Löhnis, 1926b, and Mooers, 1930, apparently has little effect. It is then a matter of choice or of economic consideration whether it is best to use the entire crop for green manuring or to deduct one or two cuttings for hay, Nelson, 1929. Obviously if one intends to build up a poor soil by green manuring, the plowing under of the entire crop is to be recommended.

In a sense the wild leguminous plants may be thought of as natural agents for green manuring. This is a point of view which is not generally appreciated, especially as referring to the arboreous Leguminosae. Of these the black or yellow locust, *Robinia Pseudo-Acacia,* is perhaps the most important example. As early as 1890a Frank called attention to the nodules of the locust tree and a year later, Nobbe, Schmid, Hiltner, and Hotter, 1891, published the results of their study of the importance of rhizobia for the growth of *Robinia Pseudo-Acacia.* They obtained a great increase in dry matter and in total nitrogen from the use of the proper bacteria. The gain in dry matter amounted to 22-fold over the control and the gain in nitrogen to 105-fold. A later paper by Nobbe, Hiltner and Schmid, 1895, again emphasized the value of rhizobia for the growth of locust on soils poor in nitrogen. This point is clearly shown in Plate 41. The question of the nature of nodules of *Robinia* and their relation to nitrogen fixation is discussed in the paper of Nobbe and Hiltner, 1899c and again by Nobbe, Richter and Simon, 1908. They pointed out that the nodules of the locust when submerged in water are of very little value to the host plant. As might be expected, the addition of the proper bacteria to locust plants under greenhouse conditions resulted in marked stimulation of the higher plant. What is urgently needed, however, is further study of the value of nodule bacteria for the tree leguminous plants under field conditions. Ferguson, 1925, undertook such an investigation and it is hoped that more information will soon be forthcoming. There are several suggestive reports which point to the value of the locust for soil improvement. For example Mattoon, 1930, said that the black locust will grow rapidly on poor sandy soils and by means of its nodule bacteria enrich the nitrogen supply of these soils. Honey locust, on the other hand, does not form nodules. This fact was established as early as 1891 by Nobbe, Schmid, Hiltner, and Hotter. McIntyre and Jeffries, 1932, have given definite figures for nitrogen enrichment in the soils surrounding black locusts—in one experiment, 0.2469 per cent *vs.* 0.1575 per cent in an adjacent oak wood lot. They have also shown improved growth of catalpa trees grown in mixture or adjacent to black locusts, and they suggest that for this reason American foresters make greater use of black locust in mixed plantings..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CHAPTER 13

NATURAL AND ARTIFICIAL INOCULATION

"What really leads us forward is a few scientific discoveries and their application."

—PASTEUR

It is well known that the leguminous plant, when infected with appropriate rhizobia, is favorably influenced; its total growth is increased and the soil is enriched. The source of this material benefit is the nitrogen of the air, captured and made available to the plant by the rhizobia in its root nodules. Unfortunately, these microörganisms are not always present in the soil, and under such conditions nodules fail to form. The leguminous plant must then draw its nitrogen from the soil, like any other higher plant. These essential facts concerning the Leguminosae and their nitrogen nutrition had scarcely been reported before various methods were devised for introducing the nodule-forming bacteria into the soil, and thus insuring nodule formation. This process of adding bacteria is popularly known as "inoculation." The word "inoculation" is derived from the Latin "in" plus "oculare." "Oculus", the noun, means eye or bud; hence to inoculate literally means to "furnish with eyes". The term has been used in at least three ways: first, referring to a nodule-bearing plant, we say it is well *inoculated;* second, we *inoculate* seed or soil by the process of applying bacteria; and third, we call the cultures thus used, *inoculation.* To the authors, the first use of inoculation, *i.e.* in relation to nodule formation, appears inexact and should be abandoned in favor of *nodulation* of the plant. The use of inoculation in connection with seed or soil in reality implies a single process, that of bringing the nodule-forming bacteria into proximity with the young plant. Whether the bacteria are applied to the seed or directly to the soil is only a matter of technique. The third use of inoculation, as synonymous for culture, is unnecessary and may be misleading. In this book, therefore, inoculation is restricted to mean *the introduction of microörganisms into the soil or other substrate for the purpose of increasing crop production.* How best to accomplish this inoculation has been the subject of numerous investigations. At present two general methods are in use: (1) the soil transfer method, the scattering on a field of some quantity of bacteria-laden soil taken from a field where a previous leguminous crop of the same "cross-inoculation" group has borne nodules; (2) the pure or artificial culture method, the actual application of cultures of the desired bacteria to the seed or soil. The applicability of these two methods, their advantages and disadvantages, will be considered in this chapter.

SOIL-TRANSFER METHOD

The gradual distribution of the nodule bacteria has come about in ages past through the natural distribution of wild Leguminosae and in more recent times through years of growing leguminous crops. Some of the natural agencies involved in the dissemination of the bacteria are:

(1) The breaking down of root nodules and the consequent liberation of billions of rhizobia into the soil. These organisms may die, remain dormant for a time, or, under the proper conditions, multiply rapidly and eventually reach new plants.
(2) Flowing water or dust particles carried about by the wind.
(3) The seed to which bacteria adhere.
(4) Manure from barns where leguminous crops have been fed.
(5) Insects, worms, and other animals in the soil.

The practice of soil transfer as a method of inoculation was attempted within a year after Hellriegel, 1886a, made his famous report concerning the nitrogen nutrition of leguminous plants. The discussion of the Hellriegel experiments before the Agricultural Society in Celle greatly impressed August Salfeld, and on his return from this meeting he made the following remark: "Das ist dein einziger Rettungsanker, du musst es bei Bohnen, Erbsen, Peluschke und Wicklinse (*Ervum Monanthos*) mit der Boden-Impfung versuchen." Salfeld might well be called the father of soil-transfer inoculation, for his tests in 1887 (see Salfeld, 1888), at the Experiment Station at Bremen, represent the first field experiments on the distribution of soil to improve the growth of leguminous crops. It is true that the practice of transferring soil for the purpose of increasing the general productivity of land was carried out somewhat earlier. In 1896 Salfeld reported that in 1883, almost three years before the Hellriegel discovery, he himself had noted the value of a top dressing of the land with soil from another field. He observed that "Kuhlerde" spread on peaty soils (moorland) greatly benefited the succeeding crops. This benefit was the more apparent if the transfer soil was from a leguminous field where crops had been grown successfully.

According to the records, the transfer of soil from one field to another in order to improve the soil productivity is an old custom handed down from ancient times and was well established in Holland long before the time of Salfeld. In general, large applications of soil, several tons per acre of a soil rich in humus, were recommended; thus it served a triple purpose: the addition of plant food, the addition of bacteria, and the change in physical properties of the soil.

Salfeld, in his early experiments, directed attention to the kind of soil to be used, as well as to the methods of inoculation. The first experiment in 1887 was on a newly drained swamp to which transfer soil at the rate of about 3600 lbs. per acre was applied by hand. To insure the presence of the proper bacteria, the soil was secured from the upper layer of a field where horse beans had grown successfully for years. The treatment of the plots in this first field test was as follows:

(1) Without Chile saltpeter and without transfer soil.
(2) Plus Chile saltpeter.
(3) Plus transfer soil.
(4) Plus transfer soil and Chile saltpeter.

Four kinds of leguminous crops were planted: grey Prussian peas, field peas, garden peas, and vetch. The results showed decided benefit from the use of the soil inoculation. Because of the success of this experiment, Salfeld greatly expanded his tests the following year. A wide variety of leguminous plants was included, *e.g.*, peas, lentils, horse beans, and various clovers; and three kinds of transfer soil were used: (1) uncultivated loam (*Glaukonit*), (2) cultivated loam (*Kalenberger*) from a field where horse beans had been grown repeatedly, and (3) cultivated loamy marsh soil (*Wiererde*) from Holland, probably garden soil. As in previous tests, the soil was applied by hand at the rate of about 3600 lbs. per acre.

In April the plots were planted to peas, horse beans and peas, horse beans and lentils, and field peas. By June 13th a decided benefit was visible; the plants on the plots to which the cultivated soils were added, especially the loam (*Kalenberger*), had a dark green color, whereas those on the untreated plots were of a yellowish green. The beneficial effect obtained from the application of the two cultivated soils was most noticeable with the field peas, although a gain was noted with all of the crops tested. The figures below give the average gain on the treated plots of the horse bean and pea mixed crop, as well as of the horse bean and vetch mixture.

	Increase due to inoculation	
	Seed	Vines
	per cent	per cent
1. Beans and peas (*Kalenberger*)	67.0	87.7
Beans and peas (*Wiererde*)	90.3	117.0
2. Beans and vetch (*Wiererde*)	208.8	84.9

The roots of the plants from plots receiving cultivated soil were thickly studded with nodules; those from the untreated plots showed only an occasional nodule.

Salfeld argued that the remarkable increase in productivity brought about by the application of soil to this land could not be due to an improved physical or chemical condition, since the use of uncultivated loam (*Glaukonit*) soil failed to exert any effects, and in any case the actual fertilizing value of the added soils was extremely small. He, therefore, concluded that the benefits obtained must be due to the microörganisms contained in the soil.

One more experiment from the year 1889 deserves mention. Salfeld selected for this test burned-over land, a peaty swamp soil which for five years had been uncultivated and during this time had produced very little vegetation. Four different amounts of soil were spread over the surface of the experimental plots, about 900, 1800, 2700, and 3600 pounds per acre. In April the plots were planted to oats and in May to a mixture of red, white, and alsike clover, and grass. After the oats were cut, the young clover plants began to show the effect of inoculation.

On the plots without soil inoculation the plants made a fair growth, whereas those on the treated area showed a good growth, a dark green color, and produced roots with an abundance of nodules. So striking was this difference that Salfeld included in his report an illustration of the nodulated and unnodulated alsike clover. This drawing, from the original report of Salfeld, is reproduced as Plate 42; it is no doubt the first illustration of such plants from a field experiment, and shows in a striking manner the beneficial effect of inoculation. Salfeld found that larger applications of soil brought about greater yields. The difference in yield, however, between the plots receiving various amounts of transfer soil was far less than that noted between the treated and untreated plots. A description of these early experiments will be found in the reports of Salfeld, 1888, 1889, 1891, 1892, 1894a and b, 1895, 1899, and 1900; and Salfeld and Wolff, 1898. Attention is also called to Salfeld's book, *Die Boden-Impfung zu den Pflanzen mit Schmetterlingsblüten im landwirtschaftlichen Betriebe,* published by M. Heinsius, Bremen, 1896.

Reports concerning the effect of the nodule bacteria on the host plants and on the succeeding crops are almost innumerable; only a few of the early experiments will be mentioned here.

Von Feilitzen, in 1891, observed the beneficial effect of soil inoculation on peas grown on a raw peaty soil at Jönköping, Sweden. Similar results were obtained by Fruwirth in 1891. Like Salfeld, he secured best results from the use of large amounts of the transfer soil. On the contrary, Fleischer, 1893, obtained good results from very small amounts of soil, about 90 pounds per acre. He attempted to study the effect of adding rhizobia to old clover fields, but failed to secure any benefit. In 1893 Schmitter published a critical review of the early experiments relating to soil inoculation. The findings of Salfeld which had been reported in 1888 naturally had aroused great interest in agricultural circles, and with these in mind, Schmitter carried out a number of experiments to ascertain the practical value of soil inoculation on various soil types. He reviewed the work of Hansen, 1890; Fleischer, 1891; Fruwirth, 1891; and Arnstadt, 1891, calling attention to the differences obtained by these investigators. From his own work with lupines, he concluded that soil transfer is beneficial on moor soils recently cultivated, on sandy soils, and on burned-over soils. On the other hand, very little benefit results on old cultivated loam or clay soils. To the authors it seems probable that many of the old ideas of soil inoculation may be traced back to this report of Schmitter. Among other things he studied the relation between size and location of nodules and their effect on the host plants.

In the closing years of the 19th century there appeared many important contributions to the study of the Leguminosae and their unique association with bacteria. The papers of Atwater and Woods, 1889, 1890 and 1891a and b in America; of Lawes and Gilbert, 1890 and 1891; and Warington, 1892, in Great Britain; of Nobbe, 1890, and 1896a and b; Nobbe and Hiltner, 1893, 1896a, b, c, and d, 1898, 1899b and c, and 1900; Hiltner, 1900a; Jacobitz, 1901; and Wilfarth, 1893, in Germany, are examples of the work of the period. Dehérain and Demoussy in France, 1900a and b and 1901, also reported favorable results from the use of rhizobia-bearing soil. These are but a few of the investigators who have studied this problem. As might be expected, there is not complete agreement as to the

benefit obtainable; however, by far the majority have reported an increased growth following the use of soil inoculation. A review of this subject is given in a paper by Miller, 1896, who described some of the early work relating to inoculation.

In America the results of Duggar, 1897 and 1898; Goessmann, 1897; Shutt, 1898 and 1899; Otis 1898; Munson, 1898; and others emphasized the widespread need of inoculation. Duggar of Alabama, 1897, was one of the first in America to test the effect of inoculation. He carried out field studies with various leguminous plants, using two methods of distributing rhizobia; in one case, the soil containing the bacteria was transferred directly to the field, and in the other, the soil was mixed with the seed before planting. A general outline of his experiments and results is presented.

Four plots, one-fortieth of an acre each, lying side by side, were sown with inoculated and uninoculated seed of hairy vetch (*Vicia villosa*). The inoculation consisted of dipping the seed in water into which there had been stirred earth from a garden where common vetch (*Vicia sativa*) had grown thriftily for several years. A striking difference was noted between the treated and untreated plots. The plants of the treated plots were by far the most vigorous and were of a luxuriant green, whereas those of the untreated plots were brownish and small. Examination showed clusters of nodules on the roots of the inoculated plants and none on the roots of the uninoculated. The green and dry weights are shown:

Yield of hairy vetch per acre

	Green	Dry
	lbs.	lbs.
Uninoculated	900	232
Inoculated	9136	2540
Gain	8236	2308

Decided increases from inoculation were obtained with both Canada field peas and crimson clover. In one of his field tests, Duggar, 1898, harvested 4,057 lbs. of cured crimson clover hay per acre from the treated plot, in contrast to only 761 of cured hay from the untreated plot. Lane, 1900, of New Jersey; Otis, 1898, and Cottrell, Otis, and Haney, 1900, of Kansas, should be mentioned among the early workers in this field. A review of the early reports is given in the papers of Kellerman, 1912, and Golding and Hutchinson, 1914.

Amount of soil. How much soil should be broadcast per acre in order to secure well-nodulated plants? The answer cannot be given without knowledge of the various factors involved: the number of nodule bacteria in the soil, the method of distributing the soil, etc. Salfeld advised about two tons of soil per acre, although smaller amounts under favorable conditions proved nearly as satisfactory. Duggar, 1897, used 720 lbs. of soil per acre, broadcast and harrowed in with the seed. The soil for this purpose was taken from a field where the desired leguminous crop had grown successfully, or from an inoculation bed, a plot where the desired leguminous crops had been grown intensively for several years. In localities where soil with the proper bacteria is scarce, Duggar recommended dampening a small amount of soil and applying directly to the seed. In this con-

nection, the old method of Hellriegel and Wilfarth, 1888, should be recalled; they made use of a water extract prepared from soil which, from previous observations, was known to contain the desired bacteria in abundance.

Heinze, 1907, prescribed soil at the rate of ¾ to 1½ tons per acre. To secure the best results, the soil should be taken from below the surface layer of the old field, mixed, applied to the surface of the field to be treated, and the land harrowed.

Otis, 1900, reported good results with soybeans from the application of soil at the rate of 1200 lbs. per acre. Moore, 1902, stated that from 500 to 1500 lbs. of the rhizobia-bearing earth are required to produce a satisfactory growth of nodules. Pugsley, of Nebraska, 1913, obtained good results with alfalfa by mixing soil from an old alfalfa field with well-rotted manure and distributing this mixture with a manure spreader. Shoesmith, 1913, stated that 400 to 500 lbs. of transfer soil per acre may produce good nodulation, but to be sure of results, it is well to use larger amounts of soil: the more soil, the greater the certainty of having sufficient inoculation. Albrecht, 1919, suggested that 300 to 500 lbs. of soil be scattered over each acre and disked or harrowed in before seeding. According to the reports of the Kansas Agricultural Experiment Station, Throckmorton and Salmon, 1927, from 300 to 500 lbs. of soil per acre is needed to produce successful nodulation. This soil should be broadcast and the field harrowed immediately. Brown and Erdman of the Iowa Station, 1927, recommended that the transfer-soil be broadcast at the rate of 100 to 500 lbs. per acre and disked in immediately. From plot tests on various soil types, Alway and Nesom, 1927, observed that the growth of alfalfa is increased where large applications of transfer-soil are made. The amount of soil required to give the maximum effect could not be determined. In some cases they found as much as 2 tons of soil per acre advantageous. The Illinois Agricultural Experiment Station recommended 200 to 600 lbs. of soil per acre, Sears, 1928; and more recently 400 to 600 lbs. per acre, Burlison, Sears, and Hackleman, 1930. McKee, Schoth, and Stephens, 1931, likewise recommended 500 lbs. of soil per acre.

In summary, then, it is a common practice in America to broadcast from 100 to 500, or, in some cases, as much as 1,000 lbs. per acre, of soil taken from a nearby field where a good crop of the desired kind of leguminous plant has grown. The actual amount of soil transferred, within a wide range, is not of great importance, providing the proper bacteria are present. Of far greater moment is the question of distribution of the soil so as to bring the bacteria in close proximity to the seedlings to be infected. A number of ways of spreading the soil containing the bacteria have been reported. If the transfer soil is allowed to dry and is then passed through a sieve, it may be evenly distributed by means of a fertilizer drill. It is sometimes easier to distribute the soil with a lime spreader, or to mix the transfer-soil with manure, and to distribute the mixture by means of a manure-spreader. If the amount of transfer-soil is small, some of the methods described below will be found helpful.

Dust method. One of the oldest methods of inoculation consists of mixing the soil with the seed at the time of planting. For this purpose the soil should be collected from around the roots and nodules of the desired leguminous crop

and allowed to dry. This dry soil is then mixed with moistened seed before planting.

Soil-paste or muddy-water method. In the farm papers and bulletins of the agricultural experiment stations, many descriptions have appeared concerning the so-called "soil-paste or muddy water process." It is recommended that a small amount of screened soil, known to contain the desired rhizobia, be stirred with water until there is a heavy suspension or cream-like paste. A mixture of about one quart of water and one quart of soil is recommended for a bushel of seed. The muddy water is then poured over the seed and thoroughly mixed with it, so that every seed is coated. If the seeds are too wet, dry pulverized soil may be added. The seed should be planted as soon as dry. This method is described by Burlison, Sears, and Hackleman, 1930. It is important that the soil used for preparation of the muddy water be not of acid reaction. Richmond, 1926b, issued such a warning on finding that the rhizobia died within 7 days on seeds treated with muddy water from acid soil, whereas they survive a long time, some almost a year, upon seeds treated with muddy water from a neutral soil.

Glue or sugar method. This procedure is similar to the muddy water method except that the seeds are first coated with a glue or sugar solution. An early description of the glue method, attributed to the Illinois Farmers' Institute, will be found in Hoard's Dairyman, 1913. The directions call for a strong glue solution, 1 lb. of furniture glue to 3 gal. of water. Experience has shown that a lower concentration of glue, about 3 oz. of glue to 1 gallon of water, is sufficient. The seeds are moistened with the glue solution, then sprinkled with pulverized soil and mixed until each seed is coated with soil. A sugar solution can be used in place of the glue. For the sugar method, 1 to 4 tablespoonsful of sugar are dissolved in a quart of water and the seeds moistened with this sticky solution. The dry soil is then sprinkled over the seed as described for the glue procedure. A description of these methods was given by Sears, 1928a.

Because of the great saving in labor, it is natural that these modifications of the old method of broadcasting 500 to 1000 lbs. of soil per acre soon found favor among farmers. Magoon and Dana, 1918; Fellers, 1919; Arny and McGinnis, 1921, and other investigators have studied the value of some of these methods of inoculation. Arny and McGinnis noted that one bushel of seed dipped in a glue or sugar solution will take up about 6 to 12 pounds of soil; however, with few exceptions, the degree of nodulation thus secured is unsatisfactory. They obtained better results from the use of an equal weight of seed and soil without the glue. Somewhat similar results were reported by Ames and Casanova, 1928. They suggested that soybeans be treated with soil taken from a field where these plants had previously grown with an abundance of nodules. For this purpose, soil should be mixed with the seed at the rate of 0.5 pound of soil to each pound of seed.

The gradual method. For a very long time farmers have been practicing the so-called gradual method of inoculation. At least one might so interpret the advice of Cato, who charged the Roman farmers to plant leguminous crops "a second and a third time." Such practice should perhaps be called nature's method, for it involves the enrichment of bacteria in the field itself by successive planting of

the desired leguminous crop. For example, in preparation for alfalfa, a farmer may sow 1 or 2 pounds of sweet clover or alfalfa seed along with an ordinary grass mixture. A fair degree of nodulation from the natural seed or soil rhizobia will occur (unless soil acidity or some other unfavorable condition prevents). These nodules serve to build up the rhizobial flora of the field and insure adequate nodulation for alfalfa the following year.

It is really gradual inoculation that has built up in our agricultural lands a certain flora of resident rhizobia for the commonly grown leguminous plants. Thornton, 1929a, for example, cited the fact that alfalfa has been grown in the southeastern district of England since its introduction in the middle of the 17th century. The soil now contains a fair number of the rhizobia for alfalfa, and plants grown in the district develop plenty of nodules. Experiments by Thornton showed no benefit from additional inoculation in that district, although in other parts of England, where the natural soil is deficient in rhizobia, inoculation is highly successful.

The recent cultivation of a leguminous crop with nodules present is not, however, a sure guaranty of the presence of enough rhizobia to insure the best results. There are a number of agencies which inhibit the multiplication of rhizobia in soil; *e.g.,* the antagonistic microörganisms—bacteria and protozoa—, as well as adverse physical and chemical conditions. Wilson, 1926a and b, concluded that soil acidity is a very potent factor in determining the survival or disappearance of rhizobia from soil. He cited a case of soil apparently deficient in *Rhizobium leguminosarum,* although *Vicia sativa* had been successfully raised on it the previous year. On well-limed soil, however, the rhizobia may survive for years. This subject is further discussed in Chapter 11. It is mentioned here in support of the thesis that even cultivated lands on which leguminous plants are grown in the regular rotation may need supplementary inoculation.

Farmyard manure. Experience has shown that applications of manure made from alfalfa hay usually favor the development of well-nodulated alfalfa plants. The same relationship holds true for other common leguminous crops. According to the tests of Fred, 1918, however, cow manure produced from alfalfa hay which had been richly seeded with the proper bacteria just prior to feeding, failed to show any viable rhizobia. The bacteria apparently are killed during passage through the digestive tract. No doubt the dust and soil particles found on hay together with residues of bedding and feeding the animals are carried over to the manure and thus aid in the distribution of nodule bacteria.

THE ARTIFICIAL OR PURE CULTURE METHOD

For several years after the discovery of Hellriegel and Wilfarth, investigators were busy confirming and extending knowledge of the nature and function of the leguminous root nodules. In 1896a as a climax to their laboratory and field studies, Nobbe and Hiltner presented their first paper relating to the use of pure culture inoculation. These experiments with pure cultures began as early as 1890 but were not made public until February, 1896, in the Sächsische Landwirtschaftliche Zeitschrift. The cultures of bacteria were applied directly to the seed or to a small amount of soil, which was distributed over the field. In the fall of the

same year, 1896a and b, Nobbe presented another paper relating to the use of pure cultures and the effect of various factors on the nodule bacteria. Nobbe and Hiltner applied for a patent in England, June 12, 1895 (1896b), and in the United States, August 9, 1895 (1896d). Although the date 1895 may therefore be taken as the beginning of the commercialization of pure cultures, it should be recalled that at an earlier date Beijerinck, 1888, Nobbe, 1890, Prazmowski, 1891, and others obtained nodule formation from the use of pure cultures of the bacteria.

The Nitragin culture. The patented culture of Nobbe and Hiltner (British Patent No. 11,460 and United States Letters Patent No. 570,813) was placed on the market under the name "Nitragin," sold by Meister, Lucius, and Brüning, Höchst-on-the Main, Germany. According to Voelcker, 1896, the word *Nitragin* is derived from the Greek, ἄγειν, and the Latin *agere,* "to make active." Although suggestive of the English for nitrogen, the word "Nitragin" is merely the trade name for all of the preparations put out under the Nobbe and Hiltner process. Because of the opinion of Nobbe and Hiltner that each leguminous plant demands its own variety of rhizobia, 17 kinds of Nitragin were prepared.

Nitragin at first consisted of a pure culture of the desired strain of rhizobia grown in 8 to 10 ounce glass bottles containing only a small amount of the solid medium, about 3 cm. deep. The original substrate consisted of sugar, asparagin, gelatin and a water extract of leguminous plants. After sterilization, when the jelly had solidified, the surface was planted with the bacteria, and the culture was allowed to grow. As soon as the bacteria produced sufficient growth, the bottles were sealed, labeled, and shipped to the trade. The 17 different varieties of Nitragin, representing as many leguminous crops, were designated by differently colored labels. Each bottle was sufficient for about half an acre and sold for 60 to 75 cents. The directions cautioned against exposure to a strong light and temperature above 98° F.

The inventors suggested two methods for the use of Nitragin. The first method consisted of the direct application of the bacteria to the seed. In the second method, the bacteria were mixed directly with soil and this inoculated soil was then spread over the field and worked in to a depth of about three inches. This second method was favored by Nobbe and Hiltner.

The value of their discovery was soon the object of much discussion and much study. The farmers and experiment station workers of Germany and other countries became keenly interested in this new bacterial product and tried it out in numerous carefully planned experiments. These early tests included plot and field experiments on various types of soil and with almost all cultivated varieties of leguminous plants. The reasons for this great interest were manifold. As stated by the originators, some of the advantages of artificial culture are: (1) early infection takes place; the bacteria are brought into direct contact with the seedling and soon penetrate into the root hairs; (2) the cost of this laboratory culture is less than that of transfer soil; (3) the danger of carrying harmful fungous diseases is eliminated; (4) the labor involved is lessened.

One of the first tests of the value of Nitragin was made by Kühn, 1896, of the Agricultural Institute at Halle. He failed to observe any beneficial effects, but admitted that the bacteria already present in the soil may have rendered his

test useless. Without submitting any results, Kühn suggested that the bacteria, when cultivated in an artificial medium, gradually become weaker and eventually lose their power to benefit the host plant. Actual tests, however, carried on by Nobbe and Hiltner, 1893, and Hiltner, 1897, failed to support Kühn's theory. They found that cultivation of a year or more on a gelatin medium, prepared according to Beijerinck's formula from pea plant extract, asparagin, sugar, and gelatin, results in enhanced growth of the bacteria, apparently without loss of the power of the bacteria to aid the host plant. Additional support for the views of Nobbe and Hiltner will be found in the paper by Beijerinck, 1890, who found that rhizobia may be cultivated on artificial media for a long time without injury. Although the conditions in a tightly corked bottle are not ideal for the longevity of rhizobia, Nobbe, 1896, showed that these organisms in Nitragin are not seriously injured when kept for three months in sealed vessels.

Baessler, 1896, at the Köslin station, obtained an increased yield from the use of Nitragin with peas and serradella. This gain was noted in yield of both vines and seed. Loges and Glaser, 1896, at Pommritz studied the effect of inoculation on the yield of peas, beans, and vetch grown on a light sandy soil. Inoculation was carried out by allowing the seed to stand 24 hours in water containing a pure culture of the bacteria. All the crops were benefited by the inoculation, the percentage gain being 124 for field beans, 46.7 for peas, and 400 for vetch. They made the interesting observation that the vetch with nodules is much more resistant to the attack of beetles than the vetch without nodules. Von Spillner, 1896, found no effect from the use of Nitragin for serradella and lupines, but thought that dry weather interfered with the test.

Dietrich, 1897, using Nitragin, obtained an increase in the growth of blue lupines on sandy soil. In this case, the inoculation was not applied until 12 days after the seed was sown. Maercker, 1898, found no effect on peas, sweetpeas, or lupines. Dickson and Malpeaux, 1897, noted an increase in growth both in pot and field experiments with clover, lupine, and vetch treated with Nitragin. The best results were with Nitragin applied directly to the soil instead of to the seed.

Lauck, 1899, advised the use of fresh cultures of Nitragin, and in order to keep the organisms in an active state, he recommended a medium rich in carbohydrate, *e.g.,* potatoes. Nobbe and Hiltner, 1899b, answered this suggestion of Lauck and called attention to the fact that Nitragin is grown on a medium rich in carbohydrate, *i.e.,* sugar.

In England, Miss Dawson, 1901, found that Nitragin is of no value for peas except when they are grown upon gravel soil. In America, the tests of Nitragin met with even less success than in Europe. Goessman, 1897, of Massachusetts, reported Nitragin a complete failure, whereas Shutt, 1898 and 1899, found it an effective and economical agent to establish the growth of clover and horse beans. Shutt later, 1900 and 1905, reserved judgment on the value of Nitragin, saying that for clover at least no inoculation was necessary in Canada. By 1910 Shutt clearly recognized the desirability of inoculation for alfalfa, since it increases both yield and nutritive value. Duggar, 1897 and 1898, in Alabama; Munson, 1898 and 1899, in Maine; Halsted, 1900, in New Jersey; and others obtained erratic results from the use of Nitragin. Brooks and Thomson of Massa-

chusetts, 1899, also reported poor results with Nitragin. The majority of investigators recommended the transfer of soil as a much more certain and effective method of distributing the nodule bacteria.

For a more detailed discussion of the early tests of Nitragin, the reader is referred to Frank's papers of 1898 and 1899. A paragraph from his report follows:

> Ich vermute, dass die Ursache der häufigen Nichtwirkung desselben darin liegt, dass die Bakterien durch die Kultur auf Gelatine an ihrer Lebenskraft soweit sie die physiologische Wirkung auf den Pflanzen-organismus betrifft, einbüssen was bei ihrem Leben im Naturboden nicht der Fall ist, und dass eine andere Herstellungsweise des Nitragins diesen Übelstand überwinden lassen dürfte.

Of the 12 stations in Germany where Nitragin was tested, only 4 reported an increase in growth from the use of the culture. As compared with the soil transfer method of inoculation, Nitragin was found decidedly inferior. According to Frank, this failure of Nitragin is due probably to a loss in the efficiency of the bacteria. Nobbe and Hiltner, 1899b, replied to this criticism of Frank, and called attention to the need for a better understanding of the limitations of bacterial cultures. They refused to agree with Frank and others that failure of Nitragin is due to a loss of ability of the bacteria to grow within the roots, but they recognized that long cultivation of rhizobia in a medium prepared from plant extract plus gelatin results in certain changes in the physiological efficiency of the bacteria. To remedy this condition, Nobbe and Hiltner proposed to replace the old gelatin medium with an agar medium containing an extract of the plant, preferably of the exact species of plant to be inoculated. In this way they thought it possible to prevent deterioration of the culture and also to insure the adaptation of the bacteria to certain species of plants. According to this suggestion, only bacteria grown on pea extract should be used to inoculate pea seed. Among other things, they emphasized the necessity of using fresh cultures. Nobbe and Hiltner, 1899b, stated that if the cultures are contaminated with fungi or other bacteria, they are no longer of value; in fact, they claimed that these foreign organisms soon kill off the nodule-forming bacteria. Concerning the number of bacteria and the efficiency of the culture, they reported that the quality or activity of the culture is the all-important point; the total number of bacterial cells is less important.

When Nitragin was first put on the market, Nobbe and Hiltner recommended that the gelatin of the culture be dissolved in pure water, but later, 1899b, they modified this suggestion. The addition of pure water, they thought, might cause death of the bacteria, and hence they suggested that the water be replaced with milk, or, better, a water extract of the seedlings of the same variety of the plant as the one to be treated.

As reported by Nobbe and Hiltner, 1899b, the use of milk as a medium in which to suspend the bacteria originated with F. Spiegel of Spremberg, 1899, who made use of milk to overcome the harmful effect of the bacteria naturally present in the dilution-water. Voorhees and Lipman, 1907, also called attention to the danger of using contaminated water in preparing the bacterial suspension for inoculation. It is, however, questionable whether the few foreign organisms likely to be thus added would make a significant difference.

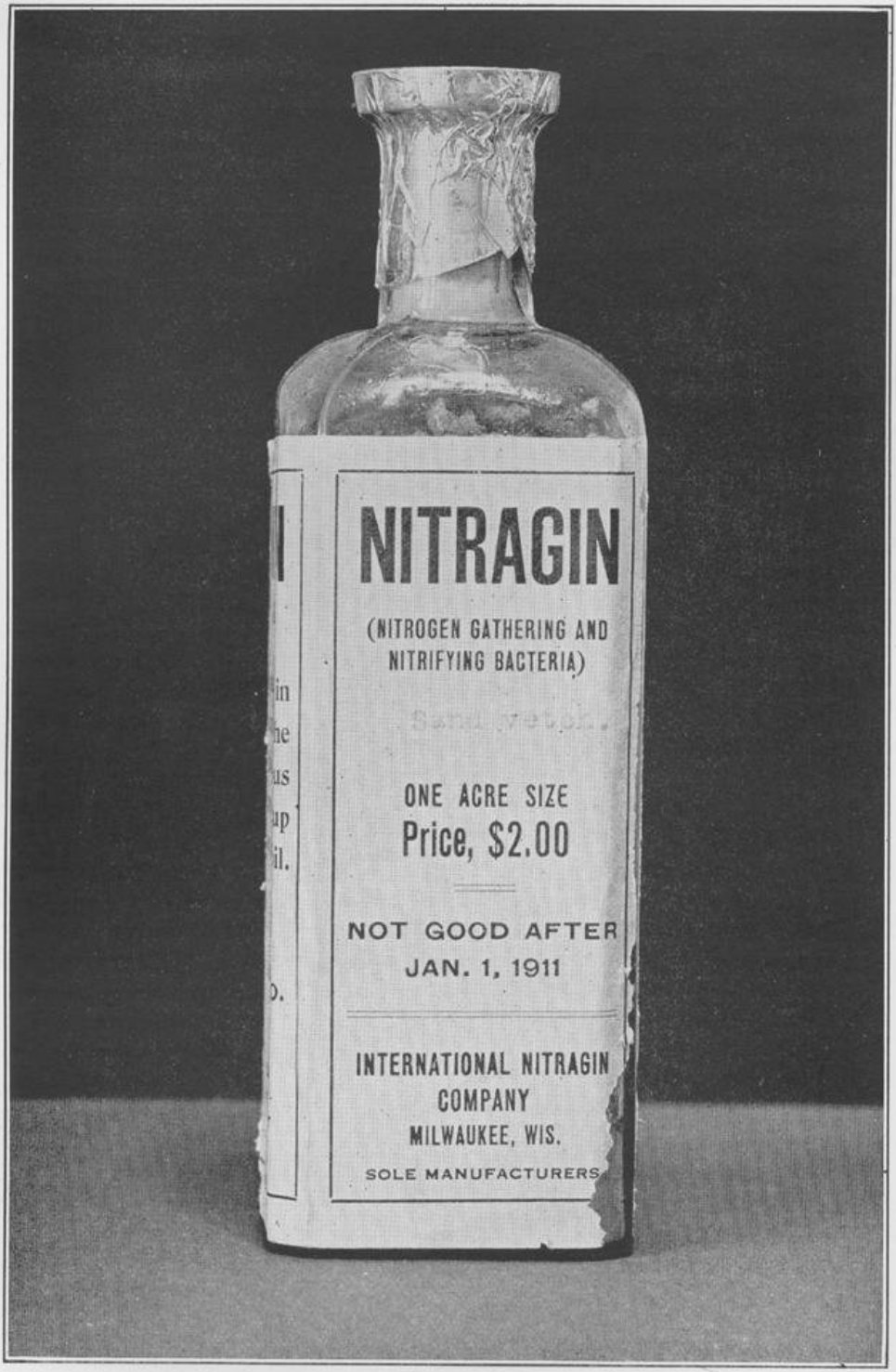
Nobbe and Hiltner, 1899b, at first questioned the value of milk and stated they would withhold their approval until they had carried out experiments. Hiltner and Störmer, 1903a, and Hiltner, 1904b, did advise the use of skim-milk but for another reason. They thought that it reduced to a minimum the injurious substances produced by germinating seeds, and also helped to prevent the harmful effect of water through plasmoptysis of the bacterial cells. Quite the opposite view is taken by Simon, 1908-09, who concluded from his studies that the use of milk or of peptone and sugar in the dilution of the culture to be used on the seed is of questionable value. These substances, he argued, may favor the growth of harmful bacteria and thus injure the nodule organism. Many modifications of the original Nobbe and Hiltner method of inoculation have appeared. In their various papers Nobbe and Hiltner, 1899b, Hiltner and Störmer, 1903a and Hiltner, 1904b, have suggested: (1) the mixing of moist soil plus bacteria with a little dry soil; (2) the mixing of the bacterial culture with finely cut leguminous hay and then the distributing of the culture so prepared over the field instead of on the seed. Nine years later, 1908, Coates of the United States was granted Letters Patent No. 899,155, in which the chief claim relates to the use of meal as an absorbing agent prepared by the grinding of clover plants, peas, beans, etc. This meal is sterilized and then moistened with the pure culture of the desired bacteria.

At one time, 1904, a small package of peptone and glucose was supplied with the Nitragin culture with directions to dissolve in a specified amount of water and use this solution for the diluting of the bacterial culture.

In general, the results of the tests of Nitragin, as carried out in Germany and other countries, were unsatisfactory and indicated that, while the artificial culture may benefit leguminous plants, the gain is not equal to that obtained from the use of natural soil. In the year 1899, the manufacture of Nitragin, the original pure culture on gelatin, was discontinued at Höchst-on-the-Main.

Fortunately, this failure of Nitragin to meet the expectations of its originators did not discourage Nobbe and Hiltner. They believed it possible to make and distribute an effective pure culture of rhizobia, and with this hope they continued their investigations. The use of pure cultures, they reasoned, is sound theoretically, provided that the proper bacteria are present and are sufficiently active to produce effective nodules. In 1904b, Hiltner described the preparation of the new Nitragin then sent out in a liquid form. Several years later, Nitragin in jelly form was sold in the United States. See Plate 43.

In the light of our present knowledge of the nodule organisms, it is not at all surprising that Nitragin in its original form, or in the modified form of the liquid culture, failed to meet the high expectations of the farmers. Hiltner and Störmer, 1903a, stated that they could not sanction the extravagant claims of the commercial company. The writers cannot but feel that the exaggerated and sometimes totally unwarranted claims of some of the advertisements, as well as the lack of information concerning the proper method of handling did much to discredit the use of cultures. No doubt the opinions of scientists from other countries also contributed to the skeptical attitude of the public regarding pure-culture inoculation. For example, the French investigator Mazé, 1899, maintained that inoculation does not result in an increased growth of leguminous plants; he based his conclusions on two assumptions: first, if the soil is in the proper state for


NITRAGIN
(NITROGEN GATHERING AND NITRIFYING BACTERIA)
ONE ACRE SIZE
Price, $2.00
NOT GOOD AFTER JAN. 1, 1911
INTERNATIONAL NITRAGIN COMPANY
MILWAUKEE, WIS.
SOLE MANUFACTURERS

the growth of the nodule bacteria, the few rhizobia already present will develop, making inoculation unnecessary; second, if the soil reaction is unfavorable to the plant or the general conditions for growth are adverse, then inoculation is useless, for the bacteria cannot function. The fallacy of such a line of reasoning is shown clearly by the results of a number of investigators. Some of the reasons for failure and their remedies are given in this chapter.

Hartleb culture. During the first decade of the twentieth century, a number of commercial cultures for use with leguminous crops appeared. Hartleb in 1901b was granted United States Letters Patent No. 674,765 which relates to a method of inoculating seeds with microörganisms. According to his scheme, the seeds are first washed with pure water and then covered with a water suspension of the bacteria in the form of bacteroids (a method for producing bacteroids in the absence of the host is given in United States Letters Patent No. 674,764, also by Hartleb, 1901a) and allowed to stand until they begin to swell. It is claimed by Hartleb that the seeds swell and the seed coat loosens and thereby an increased surface is offered for the microörganisms. Seeds so treated germinate quickly after planting and allow early nodule formation. Hiltner, 1902, and Hiltner and Störmer, 1903a, criticized the Hartleb procedure on the ground that soaking of the seeds in water results in a reduced germination. The use of bacteroids, they felt, is not sound, at least not until there is evidence that this swollen form is more resistant than the rod form.

Nitro-culture. Among the critics of the Nitragin culture was Moore, 1902; he claimed the failure of Nitragin was due in part to the method of cultivating the organisms on a rich nitrogenous medium. Moore granted that these bacteria grow luxuriantly on such a substrate, but he maintained that they gradually deteriorate and eventually lose their power of fixing nitrogen. He and his associates reported that cultures grown in the presence of nitrates actually lose their power to form nodules, while those on a nitrogen-free medium produce an abundance of nodules.

To obviate this defect in principle, Moore, 1902, proposed the cultivation of the nodule bacteria on a nitrogen-free medium (see p. 38) in order to maintain the nitrogen-fixing power of the rhizobia. His method of inoculating the seed or soil with the appropriate strain of *Rhizobium* is outlined in the U.S.D.A. Yearbook for 1902. In 1904, he took out United States Letters Patent No. 755,519, which deals with the preparation of cultures of the root nodule bacteria. He stated that in a medium containing some available form of combined nitrogen, *e.g.,* proteins, nitrates, or ammonium salts, the bacteria do not develop their full nitrogen-gathering power but rapidly deteriorate. At first the cultures from the United States Department of Agriculture were sent out in dry form. Each preparation was made up of three packages: Package No. 1—nutrient salts, magnesium sulphate and potassium phosphate, and sugar; Package No. 2—bacteria. Absorbent cotton was saturated with a culture of the desired organism grown in a medium relatively low in combined nitrogen. This moist absorbent cotton was air-dried in the absence of contaminating organisms and was then wrapped in tin or zinc foil; Package No. 3—a quantity of ammonium phosphate.

The directions for the use of these cultures recommended dissolving the salts of Package No. 1 in one gallon of pure water and then adding the contents of No. 2. This solution was to be set in a warm place for 24 hours to allow the bacteria to multiply. At the end of 24 hours, the ammonium phosphate of Package No. 3 was to be added, and once more the solution was to be allowed to incubate for 24 hours. If the culture developed properly, the solution acquired a cloudy appearance. Moore, 1905, recommended that the seed be moistened with this solution, but not soaked, and as soon as possible spread out to dry, or that the culture be used to moisten dry earth and this inoculated soil be spread over the ground. The investigators of the United States Department of Agriculture soon realized that the cotton culture of Moore was not satisfactory, and in 1905 Kellerman and Robinson replaced the old form of cotton culture with a pure culture in liquid medium and sent it out in hermetically sealed glass tubes. At first the packages of salts were included and the propagation of the culture by the farmer was recommended. Later, however, this part of the process was abandoned and, instead, the United States Government supplied a large liquid culture which was ready for immediate use.

The Moore process, later carried out by the National Nitro-Culture Company, of West Chester, Pennsylvania, began to advertise and sell for $2.00 enough of the culture to inoculate the seed for one acre. The supporters of the Moore process claimed that the problem of distributing active cultures of the nodule bacteria was solved. The enthusiasm was great and the response enormous. The period of experimentation and failure, like that in Germany during 1896 to 1899, was now under way in the United States. Roughly, the years of this boom period for cultures were from 1902 to 1907. The use of pure culture inoculation spread almost like wildfire, and it seemed to the farmers that the solution of the age-old problem of insuring adequate nitrogen supply to their land was at hand. For a picture of the public mind of this period, the reader is referred to a popular article by Grosvenor, *The Century Magazine,* October, 1904. "Vaccinate your land" or "Use the vest pocket fertilizer" are examples of the wild claims published at this time. That the investigators did not realize the nature of the nodule bacteria is quite apparent from another quotation taken from Grosvenor (p. 823): "The bacteria burned themselves out and disappeared without producing a single nodule on the plant."

Again the farmers were doomed to disappointment. Numerous tests of this widely advertised culture, by Harding and Prucha, 1905; Voelcker, 1905; Starnes, 1905; Butz, 1906; Remy, 1907; Harding and Wilson, 1908; and others proved it to be even less efficient than the original Nitragin. Sent out in dry form, the bacteria on the absorbent cotton soon perished or became so reduced in numbers that the culture was worthless. Why the same mistake of greatly over-estimating the value of cultures, as had been made in Germany several years before, should have been repeated seems difficult to explain. The desirability of careful scientific study and careful control of all bacterial cultures seems to have been overlooked.

As pointed out by Harding and Prucha, 1905, the two chief claims for the Nitro-culture of Moore were neither new nor original, for the drying of cultures on cotton is a well-known process in the yeast industry, and the use of a

medium low in nitrogen for the nodule bacteria was emphasized by Beijerinck as early as 1888; Prazmowski, 1891; Laurent, 1891; and others. A general review of this period is given in the report of Voorhees and Lipman, 1907. Those interested in the application of science to agriculture were forced to admit that the safe and sure way to secure nodulation of leguminous plants was the old and laborious method of soil transfer. Fortunately the scientists would not admit defeat, and there appeared during the years 1905 to 1915 a number of important contributions relating to the general nature and behavior of the nodule bacteria. Among these reports, the papers from the Dresden Experiment Station are of interest. There, under the direction of Simon, a great deal of work was carried out, especially in relation to the isolation of the nodule organisms, their cultivation and distribution. These papers by Simon and his associates were published in 1907, 1908-09, 1911, 1912, 1913, 1914, 1915, 1918, and 1925.

The Azotogen culture. Simon, 1907, maintained that the bacteria do not reach their maximum activity in synthetic media, and particularly gelatin, but are most active when cultivated in sterilized field soil plus lime. With this in mind he devised the moist soil culture, Azotogen. It is essentially a pure culture of a selected strain of rhizobia, developed in sterilized soil. Its superiority over Nitragin is, according to Simon, 1925, due to the fact that the bacteria for Azotogen are allowed to multiply directly in the soil, whereas for Nitragin the bacteria are propagated in an artificial medium and are then transferred to soil. In 1913 Simon prepared Azotogen not as a pure culture of rhizobia but as one containing in addition to the nodule bacteria, a small number of other nitrogen-fixing organisms. This partnership of rhizobia with representatives of the *mesentericus* group, *e.g., B. danicus* and *B. Malabarensis,* as described by Löhnis, 1905, and Löhnis and Suzuki, 1911, was perhaps suggested by the reports of such an association within the nodule.

According to Kühn, 1911, the preparation of Nitragin in the form of a soil culture was patented as early at 1906, or two years before Azotogen was placed on the market. Simon in 1925 described the early development of the Azotogen and Nitragin soil cultures. Reports of the value of the root nodule bacteria applied either by the soil transfer method or by means of artificial cultures, and also a comparison of principal cultures then on the market, *e.g.,* Nitragin, Azotogen, Nitro-Culture, and Nitro-bacterine of Bottomley, will be found in the papers of Gerlach and Vogel, 1908; Grabner, 1909; Grandeau, 1909; Kühn, 1911; Löhnis and Suzuki, 1911; von Feilitzen, 1896, 1909, 1910, 1911, 1912, 1919; von Feilitzen and Nyström, 1914; Schindler, 1911; Teisler, 1912; Makrinoff, 1913 and 1924a; Rhodin, 1914; and many others. Grandeau, 1909, from the results of bacteriological analysis of Nitro-bacterine, reported the presence of clostridial and coccus forms and the entire absence of root nodule bacteria. Plant tests of Nitro-bacterine also failed to show any nodules. Grabner, 1909, on the contrary, found Nitro-bacterine more effective than Nitragin in the production of nodules. Feilitzen, 1910, carried out plot tests with blue lupines on virgin soil. He compared transferred soil, Nitro-bacterine, and Nitragin, and found that he secured the best results with transferred soil. Nitragin caused a slight increase in growth, but Nitro-bacterine proved worthless.

Farmogerm culture. In 1905, Earp-Thomas applied for a patent relating to the growth and distribution of root nodule bacteria and in 1906 secured such a patent (United States Letters Patent No. 816,850). According to the patentee, "The process essentially consists of growing the bacteria in corked and sealed vessels by a nitrogen-free medium for a given period until the organisms reach a stage of exalted virulence." Since that time a number of other patents have been granted him; Nos. 865,965 in 1907; 1,099,121 in 1914; 1,137,388 in 1915; 1,252,332 in 1918; 1,309,723 in 1919 contain further claims and modifications of his process. As in the patent of George T. Moore, United States Letters Patent No. 755,519, 1904, much emphasis is placed on the fact that in the Earp-Thomas culture the organisms are propagated in a nitrogen-free or, as stated in Patent No. 816,850, *substantially nitrogen-free medium,* consisting of water 2210 cc., wood ashes 14 gm., agar 26 gm., and maltose 35 gm.

The claims of these patents might lead one to suppose that the idea of selecting "virulent" organisms originated with Earp-Thomas; however, a mere glance at the literature reveals the fact that Nobbe and Hiltner, 1896c and d; Moore, 1904; and Kellerman and Robinson, 1906; as well as a number of others, had long since realized the value of selecting highly active cultures.

Some idea of the device suggested by Earp-Thomas for permitting the passage of air to cultures, may be gained from a description of his container. In United States Letters Patent No. 1,137,388, of April, 1915, he claimed "A container comprising a closed receptacle, a conduit tube connecting the atmosphere within the receptacle with the atmosphere external to the receptacle and having its inner end reduced and curved circularly and ending and open to the interior of the receptacle at a point removed from the walls of the receptacle, and a plug of fibrous material in the conduit tube."

In 1914 Earp-Thomas proposed the use of sterilized soil for the propagation and distribution of the nodule cultures and also suggested the value of adding other forms of soil bacteria. A later invention, 1918, suggested the use of humus material, inoculated with the desired bacteria, and coated with agar or some protective material. It was claimed that the coating of the particles to which the bacteria are adhering prevents contamination and protects these bacteria from the harmful action of such agents as fertilizers, acids, etc. The granules of peat coated with agar and bacteria may also be mixed with lime, and thus it is possible to inoculate and lime the soil in one operation. According to Manns and Goheen, 1916, Earp-Thomas, 1921 and 1922, and others, humus alone or properly reinforced with nutrient salts offers a very favorable medium for nodule bacteria.

Composite culture. The desire to have one culture for several crops has led to the preparation of the so-called "dual purpose" or "universal culture," Bredemann, 1912, and Vogel, 1917. In some such preparations, rhizobia of only two or three cross-inoculation groups are included, while in others the makers have gone to the extreme of mixing representatives of all of the more common cross-inoculation groups. Although this method may bring about fair nodule formation, the results are variable. The writers feel that composite cultures offer few advantages and certainly many disadvantages. Perhaps the only justification for this mixing of strains is in the case of the small-size garden culture. Because of the limited amount of seed to be sown, it may be desirable to have one culture

containing the organisms for such crops as beans and peas and thus to reduce the cost of inoculation.

Crushed nodule culture. Remy, 1902, attributed the partial failure of Nitragin to an inherent weakness of the culture itself and not to external factors. In order to secure the best results from inoculation, he suggested the use of fresh crushed nodules instead of the pure laboratory cultures. The results of his experimental work clearly demonstrated the superiority of the crushed nodule method of inoculation. Since the report of Remy, Makrinoff, 1913, and others have used cultures prepared directly from nodules, but have found no indication that this type of culture is better than that prepared in accordance with sound bacteriological methods. For example, Ferguson, 1906, at the Virginia Agricultural Experiment Station, sent out cultures for leguminous crops prepared by seeding a suitable medium with a fresh, washed, and crushed nodule taken from the roots of the particular variety of plant to be treated. Undoubtedly such a procedure offers a simple means of preparing cultures; however, there can be no guaranty that the organisms are in a high state of activity and are in any sense superior to the pure cultures kept under regular laboratory conditions. It is needless to say that the Remy method has not found wide favor. Hiltner, 1902, answered Remy's criticism of cultures grown in the laboratory and showed that the nodule organisms do not lose their efficiency as soon as they are removed from the host, but may be grown for years without degeneration if kept on a suitable medium.

Christensen, 1914 and 1916, compared the value of pure culture and crushed nodules for the inoculation of alfalfa. He obtained the best results from the use of the pure culture, although the differences were not great. Because of the perishable nature of the crushed nodule preparations, he maintained that such a method is useless except under local conditions.

Alinit and mixed cultures. From time to time there appear on the market bacterial preparations for use with non-leguminous plants or in some cases for use with both the Leguminosae and Gramineae. Generally it is claimed by the manufacturer that these cultures intensify the beneficial bacterial processes of the soil, thus bringing about a gain in the available nitrogen of the soil and consequently an increase in the growth of the higher plants.

Caron as early as 1895 reported an increase in yield of non-leguminous plants from the use of pure cultures of an aerobic bacterium. The process of cultivating this organism known as *Bacillus ellenbachensis alpha* was patented by Caron in 1901. The culture was widely exploited under the trade name of "Alinit," and extravagant claims were made concerning its favorable effect on higher plants. Needless to say, the results of careful field and pot tests failed completely to support the claims of the inventor and distributor.

Patents for mixed cultures of bacteria which were supposed to greatly favor plant growth were taken out by Bottomley in 1911c and 1914. In 1924 Earp-Thomas was granted a patent relating to the culture of beneficial soil bacteria, rhizobia, azotobacter, sulphur bacteria, and other forms in a medium composed of green-sand marl mixed with organic food and moisture-retaining material. The names of some of these bacterial preparations follow: Nitro-bacterine, Phosphogerm, Sulphogerm, All-Crop-Farmogerm, Nitrobacter Soil Vaccine, Soilgro,

Soil Vita, Vitamite, Terra Vim, and Growmore. The effect of such cultures has been carefully studied by Leonard, 1925a; Lochhead, 1926b and 1931b; Erdman and Brown, 1928, and Zucker, 1928.

The results of such studies have been most discouraging, and in the light of these investigations it must be admitted that up to the present time there is no definite proof of the value of such cultures for non-leguminous crops.

Dry cultures. In view of the complexity and difficulty of preparing an efficient and uniform culture of the nodule bacteria, even under the best of circumstances, it would seem unwise to supply cultures in a dry state known to prevent or destroy bacterial life. To attempt to do so will no doubt further complicate a process which is already involved and far from clear. The desire, however, for something easy to distribute and to use, is perhaps the explanation of the popularity of the so-called dry cultures now in the market. Regardless of the past history of cotton cultures and of the known physiology of rhizobia, there have appeared in recent years a number of these cultures, prepared by absorbing the bacteria on the surface of the dry organic and inorganic compounds. Some of these preparations will be described.

L. R. Coates, 1908, was granted United States Letters Patent No. 899,155 in which he suggested the use of sterilized meal prepared from clover plants, peas, beans, or cotton seed instead of the cotton as used by the George T. Moore Patent, No. 755,519, as an absorbent material to make up the liquid cultures of bacteria. Two years later, 1910, Coates was granted United States Letters Patent No. 947,796, which relates to the use of sterilized ground raw bone or some analogous substance as an absorbing agent for the pure or mixed cultures of the bacteria. A somewhat similar method of absorbing the culture of bacteria is also described by C. F. Diller, United States Letters Patent, No. 1,354,808, in 1920, in which he suggested the use of such pulverized inorganic solid substances as silica, calcium carbonate, calcium phosphate, etc. The bacterial culture is simply allowed to dry on this unsterilized material and then sent out in dry form.

During the last three or four years there have appeared on the market in the United States a number of dry commercial cultures which consist in the main of bacteria adsorbed upon some finely divided substance such as dry sand, soil, peat, carbon black, etc., to be applied without water. These cultures are highly recommended by their manufacturers, who claim for them long life and ease of application. As might be expected, the reports from the use of these cultures are not encouraging. Comparative tests of the dry and moist cultures carried out by the United States Department of Agriculture showed that the agar or moist-soil cultures are far more efficient than the dry cultures. Somewhat similar results are reported by Albrecht, 1930b, who found that for alfalfa the dry cultures are by no means equal to the agar culture or soil inoculation. Although the use of dry cultures possesses a certain appeal to the farmer in the way of ease and convenience of application, the writers feel that their use is not to be recommended.

It would be unfair to close this discussion without calling attention to some of the possibilities of the dry cultures. A modification of the Rogers, 1914, method for rhizobia, or the procedure now followed in his laboratory for the distribution of cultures of the propionic-acid-forming bacteria, seems worthy of careful study.

Special methods used in the production of commercial cultures. In the previous paragraphs we have seen that the nodule bacteria for the artificial culture method of inoculation have been grown in liquid, solid, and soil media. They have been offered to the market in a great variety of containers, tubes, bottles, tin boxes, etc. Many have carried special patented devices for aeration, which according to the inventors, prolong the life of the culture and insure its reaching the farmer in good condition. The patent ventilator of the Earp-Thomas invention in 1915 and a somewhat similar device by Hollowood, 1921, United States Letters Patent No. 1,389,659 are examples. The special cork stopper, pierced by a toothpick and covered with plaster of Paris, as designed for the Nodogen culture, might also be mentioned.

Enough has been said in Chapter 4 to indicate the essential conditions for cultivation of the rhizobia. For methods of growing mass cultures of the bacteria, the reader is referred to F. J. Matchette, United States Letters Patent No. 1,618,461 in 1927 and to C. Vigreux, United States Letters Patent No. 1,623,896 also in 1927. Likewise the papers of Harrison and Barlow, 1907; Harrison, 1915; Magoon and Dana, 1918; Fred, Whiting, and Hastings, 1926; Shutt, 1928; and others will be helpful for general information on the preparation of artificial cultures. A rapid and particularly effective method of inoculation by means of an atomizer has been described by Fred, Whiting, and Hastings, 1926, and Shutt, 1928. The atomizer apparatus has been in use at Wisconsin for over five years and has proved rapid and dependable for sufficient and uniform inoculation, and when properly handled, for comparative freedom from contamination.

In order to reduce the cost, it has been suggested that the farmer purchase only a small culture of the desired species of rhizobia and then propagate this culture until the desired amount is obtained. Such a recommendation is sound, provided that the farmer is equipped and understands the conditions necessary for the culture of bacteria. To one untrained in this field, such a procedure is of doubtful value and may result in the growth of foreign bacteria instead of rhizobia.

COMPARISON OF THE SOIL TRANSFER AND ARTIFICIAL CULTURE METHODS

In the early years of the 20th century, farmers in general had little regard for artificial cultures. Moreover, their distrust was not without reason, for many of the culture preparations then on the market were of very doubtful value. Gradually, however, a better understanding of the requirements of the rhizobia in respect to media and maintenance of vigor has resulted in material improvement in the quality of cultures now produced. These improvements in cultural methods and in distribution of cultures have helped to overcome many of the failures which characterized the early experiments.

In order to get a fair picture of the relative value of pure culture and soil transfer methods of inoculation, a brief review is given of some of the more recent studies. There has been no attempt to make this review a complete discussion

of the subject of soil *versus* artificial culture inoculation. The early literature has been presented in the first part of this chapter and therefore need not be repeated. Unfortunately, many of the recent contributions to the subject are meager, the experiments limited and far from clean cut. It has not in all cases been recognized that a valid comparison of pure culture and soil transfer methods must take into account several factors: (1) The inoculating materials must be fair representatives of their kind. The pure culture must contain an abundance of viable rhizobia of an efficient strain for the host plant in question. The transfer soil must be carefully selected from a field which has successfully raised the desired leguminous crop for a number of years. (2) Both pure culture and soil inocula should be prepared and used in such a way as not to injure their chances of success. In other words, the experiment must be so planned that other possible limiting factors shall not operate to influence the success or failure of either type of inoculant. (3) At least for the larger applications of soil, *e.g.,* 1 to 10 tons of rich soil per acre, allowance must be made for the beneficial effect necessarily following from supplement to the total plant food. On poor soil particularly, this "top-dressing" effect may be very appreciable.

Christensen, 1914 and 1916, carried out an extensive comparison of soil inoculation with pure culture inoculation on various soil types. Briefly, his results showed that for alfalfa, pure culture inoculation at its best is in every way the equal of the soil-transfer inoculation. On the other hand, various artificial cultures are not alike in their power to benefit the plant. Some cultures are worthless. In general, he found that the pure culture method offers the advantages that infection takes place earlier, and that the cost of this method is less than that of transfer soil. From the results of pot tests with yellow lupine, Feilitzen and Nyström, 1914, observed the inoculation with soil, Nitragin, or Azotogen were all beneficial, whereas Farmogerm was negative. Lipman and Blair, 1916b, concluded from their tests with soybeans that the quality of commercial cultures as well as that of soil may vary widely in relation to the effectiveness of nodule production. A general discussion of the efficiency of the pure culture method, as compared with the soil-transfer method on acid soils, was reported by Alway and Nesom, 1927. They pointed out that bacteriologists in general support the use of artificial cultures, whereas agronomists consider the soil-transfer method preferable. They found that on limed fields the artificial culture method of inoculation for alfalfa is fully as effective as a heavy application of soil from a field where sweet clover or alfalfa has been well established; but that when the soil is acid, the soil-transfer method of inoculation proves far better than the pure culture method.

There are certain arguments which may be advanced in favor of the pure culture method. In the first place, the physiological efficiency of the bacteria is now recognized to be of prime importance. It is not enough that nodules shall be formed; they must benefit the host plant, as shown by increased plant growth and increased total nitrogen of the tissues. The soil-transfer method is a reliable way to secure nodules, but there is no assurance that they will be such as will benefit the plant. The pure culture, on the other hand, may be a chosen strain, which has proved beneficial in previous tests.

In the second place, artificial cultures are commonly applied directly to the seeds, and consequently there is probability of an *early infection.* This early infection of the seedlings is especially valuable for the quick-growing annual leguminous crops; *i.e.,* beans, cowpeas, peas, soybeans, vetches, etc. For others, like alfalfa, it is also desirable that the seedlings be vigorous and well nourished in the early stages when they are endangered by competition with weeds.

In the third place, the cost of transporting soil for application of 500 to 1000 lbs. per acre for any considerable acreage is almost prohibitive. Furthermore, it is often difficult at any price to secure enough soil for successful inoculation. For 100 acres, for example, 50,000 or 100,000 lbs. of soil would be required.

The difficulty in securing sufficient soil is especially acute when a crop is being introduced into a new district. Long haulage with consequent increase in expense is then necessary. It is conceivable that this factor would be a serious limitation in many places.

In the fourth place, the transfer of soil is always open to the danger of transmitting harmful insects, weed seeds, and the germs of bacterial and fungous diseases of plants. Obviously, the pure or artificial culture method overcomes the danger of bringing in disease. On the other hand, it would be unfair not to call attention to the danger of spreading disease, especially fungous diseases, during the process of mixing seed and culture. The water in which the bacteria are suspended, plus the process of mixing, may serve to distribute the spores of disease-producing organisms already present on the seed. Leonard, 1923c and 1924, noted an instance of this in the case of *Phaseolus vulgaris,* inoculated by the wet process. Water alone produced the same effect, indicating that the effect was due to mechanical spreading presumably of *Bacterium flaccumfaciens.*

From a summary of the *pros* and *cons* of these two methods, it appears to the writers that the proper use of pure cultures offers the best way to secure beneficial nodulation. It is realized that many scientists will not agree with such a statement. Without question, the results of the early investigators indicated the superiority of the soil transfer method. Such a claim was well founded at that time, but it fails to take into account the results of more recent investigations, namely, the discovery of the great difference in physiological efficiency of the rhizobia and the improved methods of preparing cultures. The successful nodulation of a leguminous crop depends on at least three conditions: *presence of the proper organism; the proper nutrition of the plant;* and *the development of nodules which are active and efficient in furnishing the plant with nitrogen.* The use of artificial cultures of bacteria appears to offer the best way to meet these conditions.

FACTORS THAT INFLUENCE ARTIFICIAL CULTURES

A vast number of studies has been made concerning the factors which may assist or interfere with the effectiveness of artificial cultures. Attention is called to the papers of Hiltner and Störmer, 1903a; Galloway, 1906; Kellerman and Beckwith, 1906a; Kellerman and Robinson, 1906; Prucha and Harding 1906; Chester, 1907; Simon, 1907; Kellerman and Robinson, 1908; Kellerman, 1910a; Lipman, 1910b; Harrison, 1915; Manns and Goheen, 1916; Ockerblad, 1918; Temple, 1916; Fellers, 1918a; Snyder, 1925a; Lochhead, 1926a, 1927b and c;

Fred, Whiting, and Hastings, 1926; Vandecaveye, 1927b; Shutt, 1928. The paper of Fred *et al.*, 1926, gives a summary of the more recent work in relation to the effect of various agencies on the viability of these organisms.

Relation between number of bacteria and effect of inoculation. Nobbe and Hiltner, 1899b, and Hiltner, 1900a, were the first to study the relation between the numbers of bacteria in the inoculum, the number and size of nodules produced, and the relationship between degree of nodulation and degree of benefit to the host. In their first report, 1899b, they detected no appreciable advantage in nodulation upon increasing the number of bacteria to 25 times the usual application, and they made the following highly significant statement: "Die Quantität der zur Impfung verwandten Bakterien ist also weit unwesentlicher, als die Qualität, d.h. die Virulenz derselben." This may be taken as a very early appreciation of the fact of strain variation and of the importance of choosing active strains for inoculation purposes. The following year, Hiltner, 1900a, experimented with even greater extremes in size of inoculum; using three dilutions with a total variation of 10,000 between lowest and highest, he was yet unable to induce great differences in number, shape, and size of nodules. In discussing results, he postulated that there exists a fine equilibrium between the plant and the bacteria, which operates to prevent any marked difference in the degree of nodulation.

Quite different results were reported by Süchting, 1904; Kellerman and Robinson, 1906; Noyes and Cromer, 1918; Fellers, 1919; Perkins, 1925a; Wilson, 1926a and b, and 1929a; Erdman and Wilkins, 1928; and Thornton, 1929b. These investigators report that, within limits, the number of nodules is increased when the number of bacteria is increased. Perkins observed an increase in nodules when the number of cells per seed increased up to 50, but with still greater numbers, there was no gain. Somewhat similar results were obtained by Wilson. Thornton, on the contrary, noted with alfalfa in a field experiment an increase in number of nodules when the number of bacteria per seed was raised from 2,500 to 20,000 organisms. In a pot experiment with runner beans, the use of more than 1,280,000,000 organisms per pot (6 seeds per pot) was still effective in increasing the number of nodules, though the increase was not in direct proportion to the increase in number of cells. The results of Thornton, however, do not disprove that there is a limit to nodulation imposed by the plant itself.

For the benefit of those who may wish to calculate the number of bacteria per seed, an average count of the numbers of seeds per pound of the more important leguminous plants is given below. The figures are taken from Fellers, 1918a; Munn, 1924; and U. S. Department of Agriculture, 1928.

Total Number of Seeds Per Pound

Alfalfa	226,800	Winter vetch	16,330
Sweet clover	258,550	Soybean	2,500
Red clover	272,160	Field bean	1,500
Alsike clover	680,400	Cowpea	3,570
White clover	680,400	Field pea	2,210
Crimson clover	149,690	Peanut	1,200
Lespedeza	370,000	Velvet bean	1,500
Spring vetch	8,620	Beggar weed	420,000

Mineral fertilizers. The action of mineral fertilizers as affecting the behavior of the nodule bacteria in soil is discussed in Chapter 11. At this time, only the effect of mineral fertilizers on the vitality of bacteria on the seed will be considered. The practice of seed inoculation and the planting of the seed along with fertilizers is a well established practice in farming. Not infrequently the inoculated seed is brought in direct contact with the mineral fertilizer, and consequently the bacteria may be affected. Löhnis and Leonard, 1926, reported that cyanamide of calcium, hydrated lime, or sulphur are very harmful to the nodule bacteria on seeds. On the other hand, quite the opposite is true when inoculated seed and fertilizers are sown at the same time but from different compartments of the grain drill. Helz and Whiting, 1928, carried out both laboratory and field tests of the effect of fertilizers on the formation of nodules. In the main, they found that fertilizer applications large enough to lower germination of the seed also injure nodulation. However, applications of phosphorus and potassium fertilizers in the amounts commonly applied actually bring about an increase in number of nodules. From the several reports, the conclusion seems warranted that the vitality of inoculum on leguminous seed is in all probability not seriously influenced by the addition of mineral fertilizers, unless in excessive amounts or in immediate contact.

Milk. As early as 1899, Spiegel, and in 1903a Hiltner and Störmer recommended that cultures of nodule bacteria when received on the farm be mixed with milk and not water before being applied to the seed. If skim-milk is not available, a suspension of the culture may be prepared in an extract of germinating seed or in a solution of peptone and sugar. According to Giltner and Langworthy, 1916, milk is especially to be recommended for its protective action on the rhizobia. Since 1899 the skim-milk method has been widely used by the farmers of Denmark, Great Britain, and Canada. Christensen, 1914, in Denmark recommended that the culture of bacteria be washed off the surface of the agar with milk and this suspension then applied to the seed. Somewhat similar directions are given by the Canadian bacteriologists at Guelph, by Lochhead, 1926a and 1927b and c, and again by the English investigators, Thornton and Gangulee, 1926, and Thornton, 1929a. According to Lochhead, 1926a, sweetened skim-milk may be used to advantage in the inoculation of seed. He emphasized that the sweetened skim-milk forms a residue which aids in holding the bacteria on the seeds, and also that when the seeds are sown, this residue furnishes an available food for the bacteria. On the other hand, Harper and Murphy, 1928, from the results of only a limited number of tests, reported that a milk suspension of soybean bacteria fails to give any better result than a water suspension.

Milk plus phosphate. Thornton and Gangulee, 1926, called attention to the fact that in the soil these organisms pass through a series of changes in their life cycle. At certain stages they are motile and move about in the soil, while at other stages they are incapable of vital movement. It is in the motile stage that they attack the root hairs and thus force their way into the plant. The significance of this observation and its relation to successful nodulation is apparent. The chance for infection is greatest when the bacteria are capable of movement, and with this in mind the English investigators evolved the phosphate and milk inoculation pro-

cedure. The bacteria are suspended in milk containing calcium phosphate and are so applied to the seed. It is claimed that in addition to the valuable effect of the milk film, the phosphate stimulates development of motile forms and consequently greater spread of the rhizobia. The net result is a decided increase in nodule formation.

The beneficial effect of phosphates on nodulation is well known. For a discussion of the subject, see the reports of Marchal, 1901; Laurent, 1901; Löhnis, 1902; Wohltmann and Bergené, 1902; Dehérain and Demoussy, 1902; Flamand, 1904; Eichinger, 1912; Prucha, 1915; Truesdell, 1917; Wilson, 1917; Hutcheson and Wolfe, 1922; Alicante, 1926; and others. The use of phosphates with artificial cultures to increase nodulation, however, was first suggested by Thornton and Gangulee, 1926, and Thornton, 1929a.

In the United States, Albrecht, of Missouri, has carried out a number of experiments arranged to measure the effect of calcium, phosphorus, and potassium treatments on the formation of nodules on soybeans. These studies (Scanlan, 1928, Albrecht and Davis, 1929a and b) show that the beneficial effects of calcium-bearing substances, such as lime, acid phosphate and other calcium salts, are essentially the result of calcium stimulation. They found that calcium carbonate on an acid soil already well supplied with rhizobia results in a decided improvement in nodulation.

In New Zealand Reid, 1930, has found that superphosphate and sulphate of ammonia, intimately mixed with inoculated seed, kills the nodule bacteria. Superphosphate plus lime, rock phosphate or basic slag, however, are beneficial to both nodulation and plant growth.

Recently Albrecht and Davis, 1929b, have reported on the physiological rôle of calcium in favoring nodule production of soybeans. Their results show that calcium exerts an effect on the higher plant and possibly on the nodule organism as well. Cross sections of stems of calcium-starved and calcium-bearing plants showed a difference in the general structure of the cell walls. These investigators think that possibly the calcium present in transfer soil plays a decided part in the effectiveness of this method of inoculation.

Experiments carried out by Karraker, 1927, with alfalfa, showed an increase in nodulation from applications of salts of calcium.

Dyes. The lack of adaptation of red clover and alfalfa seed grown in certain foreign localities to certain farming sections of the United States led to the enactment by Congress of an amendment to the Seed Importation Act of August 24, 1912, whereby it became a requirement that according to their origin one or ten per cent of each lot of seed should be colored for purposes of identification. Some of the dyes which have been recommended[1] for this purpose are malachite green, crystal violet, and rhodamine B. For practical seed staining, water solutions are advised, although dyes mixed in molecular proportion with oleic acid and dissolved in alcohol are preferable when seed in the sack is to be stained.

The effect of these dyes on nodule bacteria of alfalfa and red clover in sterile nutrient solutions, on seed and in soil has been determined and the general conclusion reached that if alfalfa and red clover seed are stained in the manner and

[1]Leonard, L. T. Personal communication, 1930.

degree prescribed and the inoculation applied promptly before sowing, there should be no perceptible damage to the nodule bacteria either on the seed or in the soil.

Disinfectants. The use of various chemical compounds either in the powdered or liquid form to combat plant disease is a well-established practice. Since the report of Hiltner, *et al.,* 1903a and b, there have appeared a number of papers, emphasizing the value of these various substances for seed or soil disinfection. The majority of these reports deal with the general question of soil sickness. In relation to seed inoculation, the use of these seed disinfectants offers a problem. As first pointed out by the German investigators and also Robinson, 1910, it is impracticable to treat the seed with a strong poisonous agent and immediately thereafter to add a pure culture of bacteria or *vice versa.* Obviously such treatment will injure or destroy the bacteria. In recent years the effect of disinfectants on nodule production has been studied also by Simon, 1907; Heinze, 1907; Müller and Stapp, 1925; Schirmer, 1926; and Sayre, 1928. Müller and Stapp carried out an extensive study of the agents commonly used for soil and seed disinfection, *e.g.,* copper sulphate, copper carbonate, formaldehyde, mercuric chloride, Uspulun, Germisan, carbon bisulphide, calcium sulphide, etc.; they found that in pure culture a concentration of 1 to 10,000 of Uspulun or Germisan greatly retards the growth of the nodule bacteria. On the other hand, their tests in pots with the bacteria present in the soil instead of on the seed, failed to show any decided injurious effect on nodule production. In certain cases, these disinfecting agents actually exerted a stimulating effect on nodule production. Hiltner and Störmer, 1903b, observed that CS_2 applied for purposes of soil sterilization causes almost complete destruction of the rhizobia present. Quite the opposite effect was reported by Stapp, 1929.

Schirmer, 1926, investigated the effect of the new compounds, Uspulun, Germisan, Tillantin, etc., on seed inoculation. He found that these agents do not injure nodulation provided that the seed is treated first with the poisonous substance and some time later with the bacteria. On the other hand, Sayre, 1928, stated that the organic mercury compounds will readily destroy the nodule bacteria on the seeds.

One might suppose from the above reports on the extreme sensitivity of the rhizobia to disinfectants that it would be possible for experimental purposes to prepare rhizobia-free soil by disinfection. Such a procedure would seem feasible for green house experiments where it is desired to avoid the usual excessive heat sterilization or for field experiments where the natural rhizobial flora is a confusing factor. The attempt has been made by Leonard and Newcomb, 1925, who treated a field soil with SO_2 and formaldehyde in 1 per cent concentration. The result was only superficial sterilization, nodules being found 2 to 3 inches below the surface. For green house work, however, the method offers possibilities.

Germination of seeds. The question has been raised by Hiltner and Störmer, 1903a, as to whether or not the process of germination has an injurious effect upon the nodule-forming bacteria with which the seeds are treated. They pointed out the difficulty of securing nodules on such large-seeded plants as lupine and soybean, and concluded from the results of certain experiments that the excretion of substances from the seed coats during germination may injure the rhizobia. As

early as 1888 Beijerinck; and Haas and Fred, 1919, pointed out that the germination of the seed of Leguminosae may bring about conditions favorable for the growth of rhizobia. In this connection mention should be made of the effect of age on the reaction of seed. Wilson, 1929b, found that in general the older the seeds the more acid the reaction.

WHAT CONSTITUTES A SATISFACTORY CULTURE OF RHIZOBIA

Just 29 years ago Hiltner and Störmer, 1903a, published the results of their exhaustive and painstaking studies concerning the essential points to consider in the preparation of pure cultures of the nodule bacteria. Briefly these factors are:

1. Purity. The culture should be true to name and free from foreign organisms.
2. Physiological state. To retain the physiological efficiency of the bacteria they should be cultivated on a suitable medium. A gelatin medium is unsatisfactory.
3. Virulence. The organisms must be able to enter the plant and multiply, but must not be so virulent that they act as parasites on the host.
4. Nitrogen-fixing power. The culture of rhizobia should be selected on the basis of its ability to aid the host plant.

Since 1903 the use of artificial cultures has developed rapidly. Unfortunately those in charge of the preparation of these cultures have not always made use of the scientific facts as laid down by the early workers in the field. The proper application of the scientific discoveries would no doubt have prevented much of the disappointment and loss from the use of poor cultures.

To the authors, the value of a culture seems to depend in a large measure on at least five factors.

1. Upon the organism. It is essential that the culture of bacteria contain the proper organism to form nodules on the desired host plant. In general, it is desirable to have only the one type of organism in the culture.
2. Upon efficiency of the strain. Select an active strain of the desired organism. Because of the variation between strains in relation to effectiveness, every effort should be made to select strains of the bacteria which are known to be of decided benefit to the host plant, and to keep these tested cultures under conditions which will maintain the organisms in an active state.
3. Upon the number of bacteria. Just how many bacteria are required in order to produce the maximum number of effective nodules is not known. In general an abundance of the bacteria, probably several thousand per seed, produces the best results.
4. Upon age of culture. Old cultures may be low in total number of viable cells and those which are alive are presumably in a weakened condi-

tion. Naturally a biological product such as a culture of bacteria decreases rapidly in number of vegetative cells with an increase in age. To secure the best result use only young active cultures.

5. Upon culture medium. The substrate on which the organisms are grown exerts a profound influence on the number and efficiency of the cells. The authors prefer a medium low in available nitrogen and with a neutralizing agent, such as calcium carbonate, present.

Above all, since the rhizobia do not form spores, it must be recognized that such a biological product as a culture of rhizobia must be handled with great care if it is to retain full activity. Failure to keep this point in mind no doubt explains many of the negative results obtained from the use of artificial cultures. In other words, the first requisite to success is, of course, the culture itself. It must be inherently capable of producing effective nodules on the type of leguminous plant specified, and it must reach the farmer in a sufficiently active state to do so.

WHEN TO INOCULATE AND WHAT TO EXPECT FROM INOCULATION

From the vast number of experiments concerning inoculation numerous examples might be cited in which the presence or the absence of the proper bacteria literally determined the success or failure of the crop. Such striking differences, however, are the exception and not the rule. But they have been given much publicity with the result that the farmer is frequently blinded to the more modest but much more common small increases in yield. A gain of 10 per cent, which is scarcely noticeable by observation of the crop, will far more than pay the cost of the cultures. Other benefits, such as improved quality of the crop and increased soil fertility, can only be revealed by careful scientific studies. Here also relatively small increases will justify the use of inoculation.

The idea seems to prevail among scientists of almost every country that the benefits to be derived from inoculation are inversely proportional to the number of years the leguminous crops have been cultivated. Strange as it may seem, however, the actual facts do not agree with this statement. Many studies are now available which show this (Wilson, 1926; Wilson and Leland, 1929; and Whiting, Fred, and Stevens, 1925). The following data, obtained in Wisconsin, also indicate that inoculation of seed to be sown on fields which have previously borne the same crop may be very beneficial.

Effect of inoculation on the yield of Alaska peas

Soil		Number of years since pea crop	Gain due to inoculation
Class	Acidity*		
			per cent
Clay loam	very slight	10	no gain
Fine sandy loam	slight	4	4.13
Fine sandy loam	strong	3	9.87
Fine sandy loam	strong	5	34.50
Fine sandy loam	strong	8	51.49

*Truog test

The value derived from the artificial inoculation is to a considerable measure dependent upon climatic conditions, cropping systems, and fertilizations. If the crop is planted in a soil plentifully supplied with nitrates, either because of the climatic conditions or cropping and fertilizer systems, little benefit may be obtained from inoculation. Under other climatic conditions which result in a dearth of nitrates in the soil, inoculation of the seed may be extremely beneficial or even essential. The farmer can determine the value of this practice only by trial on his own fields and under his own conditions.

Some idea of the frequency of benefit traceable to the use of artificial cultures for the inoculation of leguminous seed is seen from the results of Edwards and Barlow, 1908; Harrison, 1915; and Lochhead, 1927a. Of 28,000 cultures distributed in Canada between 1905 and 1915, Harrison found that with alfalfa 82.7 per cent gave favorable results and with red clover 76.0 per cent. Also Lochhead, 1929, in Canada, found with alfalfa 84.6, sweet clover 75.6, red and alsike 80.6, and field pea 61.3 per cent favorable. More recently Lochhead, 1931a, gave the following figures for field tests actually carried out by farmers. Total number of reports, 1,352; total number showing benefit from rhizobia, 1,054; per cent showing benefit, 78. Barthel and Bjälfve, 1930, in Sweden carried out 113 field tests of inoculation with various leguminous plants and obtained 91 per cent of positive results. He found a decided gain in the yield of alfalfa from the use of the nodule bacteria, whereas clover, peas, and vetches responded to this treatment to a lesser degree.

The following recommendations give a general idea of when to use cultures:

a—Inoculate the leguminous seed to be sown on all fields where the same crop, or a member of the same cross-inoculation group, has not recently been grown.

b—Inoculate all leguminous seed which is to be sown on markedly acid soil.

c—Inoculate all leguminous seed unless recent field tests show that the soil is abundantly supplied with effective rhizobia. This last suggestion for the use of cultures is supported by the results of numerous recent experiments.

In conclusion reliable artificial cultures for the inoculation of leguminous plants have proved of inestimable value to the agricultural economy of our country. Without them it would have been impossible or at least infinitely more difficult to achieve the present extensive cultivation of alfalfa, soybeans, and other leguminous crops. It may be fairly said that the scientific studies leading to the present development of these cultures represent the greatest achievement in applied soil bacteriology and that further studies of the rhizobia will not only extend our knowledge of the general physiology of bacteria but also will lead to improved and more profitable agriculture.

APPENDIX

DIRECTIONS FOR THE USE OF ARTIFICIAL CULTURES

The manufacturers of artificial cultures usually send out with their products specific directions for inoculation. It will be well to cite here two typical sets of instructions, illustrating the milk and water methods of inoculation. The Rothamsted method for alfalfa, as stated by Russell, 1926, is as follows:

1. Keep the cultures in the dark until used.
2. In the cultures the bacteria appear as a whitish slime on the inclined surface of the jelly medium.
3. Transfer the contents of the tube into fresh skim milk, using about ¼ pint of milk and 1 gram per quart of calcium phosphate (CaH_4 $(PO_4)_2$. $2H_2O$) per tube of culture.[1] Turn out the contents of the tubes, using a clean stick, and *thoroughly mix the bacterial slime* with the milk, picking out the lumps of jelly medium. Rinse the tubes out into the milk.
4. The seed should be piled on a clean surface and the milk poured on to it and well mixed with the seed so that *every seed* is moistened with milk. It usually takes about a quart of milk to every 24 lb. of seed. If there is not enough milk, add a little clean water till all the seed is moistened.
5. The seed should be sown as soon as possible after inoculation. If the seeds are too wet and stick together, mix them with a little dry earth or sand till they are dry enough to drill.
6. The seed should be drilled and not broadcast, as it must not rest on the surface of the ground, because light kills the bacteria. For this reason, also, the inoculated seed must not be exposed to the sun before drilling.

A slight modification of this method appeared in the paper by Thornton, 1929a. The principal changes consist in the use of 1 tube of culture to 7 lbs. of seed and of ¼ pint of skim-milk containing 0.1 per cent of calcium di-acid phosphate to 7 lbs. of seeds. Skim-milk instead of whole milk is recommended with the remark that it "greatly shortens the time of drying."

The directions for use of cultures as recommended by the University of Wisconsin will serve as an illustration of the water method of inoculation. The cultures are supplied in large- and small-sized bottles. The quantity of seed for which one bottle is intended is specified on the label; it varies according to the variety of

[1]The required amount of calcium phosphate is sent out with the cultures. This should be dissolved in the milk before the bacteria are added.

leguminous seed concerned. The general directions for application of the inoculum are as follows:

1. Fill bottle ½ full with cool water. Close, shake and pour cloudy liquid into a clean cup. Repeat twice.
2. Make the cloudy liquid up to 1 pint.
3. Sprinkle over seed. Mix thoroughly. Dry in the shade and sow the same day.

NOTE: This culture will be replaced free of charge if not used within 30 days.
Do not open until ready to use. Keep in a cool place.

Very recently there has appeared on the market a special machine to be used in treating large amounts of seed with cultures of bacteria. According to the manufacturer this so-called Hydro-Electric Inoculating machine will treat as much as 60 bushels of seed per hour.

AGENCIES FOR DISTRIBUTION OF ARTIFICIAL CULTURES

State and agricultural colleges. At the present time inoculation by the artificial culture method is extensively practiced in the United States and other countries. The growing demand for cultures, encouraged as it is by agricultural agents, the agricultural press, and recommendations by various agricultural colleges, necessitates an extensive system of production and distribution of cultures of the rhizobia. At irregular intervals, usually every two or five years, the various experiment stations and the United States Department of Agriculture prepare bulletins or circulars giving a brief popular account of the various methods of applying nodule bacteria for distribution to the farmers of the several states. The reader is referred to the publications of Albrecht, 1930a; Brown and Erdman, 1927; Conn, 1929; Hastings and Fred, 1925; Löhnis and Leonard, 1926; Sears, 1928a; and others.

It goes without saying that the cultures offered to the farmer should be of the highest possible grade and of reasonable price. The exaggerated claims and "high pressure salesmanship" sometimes used to induce farmers to buy cultures should be discouraged. Rather should there be, as there now is in most cases, a general appreciation among farmers that inoculation is profitable, both for the immediate crop and for long-time maintenance of soil fertility.

There are now in the United States and Canada some 20 state institutions, mostly Agricultural Experiment Stations, and numerous commercial companies which are supplying cultures for inoculation. Naturally there is great variety among their products—in form of culture sent out, condition of culture as it reaches the farmer, amount recommended for a given amount of seed, and probably effectiveness of the bacteria themselves. Too often the limitations of bacterial cultures are not considered, and, as a result, the bacteria may perish before they reach the seed. The cost, the nature of the medium, and also the approximate


NEW YORK STATE
College of Agriculture
LABORATORY OF
PLANT PHYSIOLOGY
ITHACA, N. Y.
Legume Bacteria
FOR
ALFALF
ALFALFA
or
SWEET CLOVER
FOR 60 LBS. SEED
DIRECTIONS
UNIVERSITY OF WISCONSIN
AGRICULTURAL BACTERIOLOGY
MADISON, WISCONSIN
Nitrogen Gathering
Bacteria
UNIVERSITY OF IDAHO
MOSCOW
DEPARTMENT OF
BACTERIOLOGY
O. A. C.
Legume Inoculating
Bacteria
TWO ACRES
LEGUME BACTERIA
SOYBEANS
MAR 20 1924
GEORGIA
DEPARTMENT OF
AGRICULTURE
This bottle contains
bacteria for the inoculation of one acre of
VETCHES OR
AUSTRIAN WINTER PEAS
and should be used
within 30 days from
date.
Lot No.
(1)
BACTERIAL CULTURE
DIRECTIONS
State College of Washington
Pullman, Washington

number of cultures sent out in 1926 are given in Table 23. The figures of this table show, in a general way, the variations in size, price, and composition of these cultures. Eight of the fifteen state institutions in the United States send out annually more than 10,000 cultures each. For a more complete description of these cultures, the reader is referred to the reports of Zucker, 1929, and others. In Plate 44 are shown some of the cultures supplied by the Agricultural Experiment Stations.

The total distribution of cultures for four states, Missouri, Wisconsin, Idaho, and Washington are shown in Tables 24 and 25. Table 24 gives the figures for the distribution according to crops in the states of Missouri and Wisconsin for the year 1928. In Missouri the soybean is by far the leading crop, while in Wisconsin alfalfa is the most important. Other leguminous crops, in these regions at least, are far less extensively grown or less generally inoculated. For the clovers particularly the possibilities for benefit from inoculation are not generally appreciated.

Commercial companies. A list of some of the companies selling cultures in the United States, Canada, and certain foreign countries, and the general nature

TABLE 23

Showing the number, kind, and price of cultures sent out by different states in the United States and Canada

UNITED STATES[1]

No.	State	Address	Nature of medium	Price	Cultures supplied 1926	
1	Georgia	Atlanta	Agar	$0.30 acre	1,296	
2	Idaho	Moscow	Agar	0.25 acre	10,116	
3	Maryland	College Park	Agar	0.30 bushel	11,634	
4	Massachusetts	Amherst	Agar	0.25 bushel	695	
5	Michigan	East Lansing	Agar	0.25 bushel	46,689	
6	Missouri	Columbia	Agar	0.25 bushel	41,248[3]	
7	New York	Ithaca	Soil	0.25 acre	37,000	
8	North Carolina*	Raleigh	Liquid	0.50 acre	1,500	
9	Oregon	Corvallis	Agar	0.50 (2 acres)	8,000	
10	Pennsylvania	Agricultural College	Agar	0.25 acre	500	
11	South Carolina*	Columbia	Agar	0.50 acre	250	
12	Virginia*	Richmond	Agar	0.50 acre	15,582	
13	Washington	Pullman	Agar	0.25 acre	12,312	
14	Wisconsin	Madison	Agar	0.25 acre	69,058	
15	Wyoming	Laramie	Agar	0.25 acre	300	
					Total	256,180

CANADA[2]

1	Alberta	Fort Vermilion	Agar	----------	3,500	
2	Dominion Government	Ottawa	Agar	----------	6,113	
3	Manitoba	Winnipeg	Agar	----------	7,333	
4	Ontario	Guelph	Agar	----------	13,556	
5	Saskatchewan	Saskatoon	Agar	----------	6,000	
					Total	36,502
GRAND TOTAL						292,682

*Cultures sold by the State Department of Agriculture.

[1]In some cases these figures are for 20 pounds of seed instead of 60.

[2]Each culture is enough for 1 bushel of seed.

[3]1926-27 fiscal year.

TABLE 24

The distribution of cultures for leguminous crops in Missouri and Wisconsin

1918–1929

Fiscal year ending June 30	Missouri		Wisconsin
	Cultures shipped*	Individuals served	Cultures shipped**
1918	------	------	800
1919	2,667	800	16,688
1920	2,932	900	28,496
1921	5,309	1,665	31,471
1922	11,161	2,950	48,145
1923	20,010	4,145	50,602
1924	37,595	7,173	106,016
1925	36,163	6,970	116,660
1926	58,441	9,621	94,072
1927	41,248	6,096	69,058
1928	50,517	6,088	75,605
1929	41,407	5,331	72,510

Number of cultures for the various leguminous crops during 1928

	Missouri	Wisconsin
Soybeans	38,522	14,793
Sweet clover	4,353	34,177[1]
Alfalfa	3,906	------
Peas	------	16,862
Red and alsike clover	2,103	9,642
Cowpeas	1,537	------
Miscellaneous	96	131

*Calculated for 1 bu. of seed.

**Bottles: 1 bottle enough for 20 lbs. of clover or alfalfa, 30 lbs. of soybeans and 60 lbs. of peas.

[1]Includes alfalfa and sweet clover.

TABLE 25

The distribution of cultures for leguminous crops in Idaho and Washington

1915–1928

Year	Idaho		Washington	
	Acres*	Individuals served	Acres*	Individuals served
1915	3,139	----	----	----
1916	6,536	----	----	----
1917	21,265	----	9,300	----
1918	14,755	----	11,693	----
1919	----	----	11,463	----
1920	12,811	676	10,044	----
1921	7,755	466	8,325	----
1922	7,704	501	6,191	795
1923	9,848	709	10,949	1,386
1924	12,255	----	11,933	1,596
1925	(approx.) 12,000	----	13,037	2,087
1926	" 13,000	----	12,312	1,519
1927	--------	----	9,473	1,267
1928	--------	----	7,205	850

*These figures were taken from Annual Reports and may in some cases represent bottles instead of acres.

TABLE 26

Commercial cultures for sale in the United States in 1929

Trade name	Composition	Manufacturer	Address
-------	Processed field soil	C. T. Ashley Co.	Nicholasville, Ky.
Compo-Humus*	Peat in bulk	Agr. Humus Corp.	Lake Wales, Fla.
Dry Inoc	Charcoal	Franklin Laboratories, Inc.	Columbus, Ohio.
Edwards Legume Bacteria	Agar	Edward's Laboratory	Lansing, Mich.
Farmogerm	Agar	Earp-Thomas Farmogerm Co.	Bloomfield, N. J.
Azotogerm*	Peat (mixed culture)	Earp-Thomas Farmogerm Co.	Bloomfield, N. J.
Humogerm	Peat	Earp-Thomas Farmogerm Co.	Bloomfield, N. J.
Phosphogerm*	Peat in bulk	Earp-Thomas Farmogerm Co.	Bloomfield, N. J.
G.L.F. Legume Culture	Sand	Coop. G.L.F. Seed Service	Syracuse, N. Y.
Hazelmere Bacteria	Soil	Hazelmere Bacteria Co.	Bowling Green, Ind.
Humite*	Peat in bulk	Plant and Land Food Co.	Haines City, Fla.
McQueen's Dust Kote Inoculator	Soil	The McQueen Bacteria Co.	Milwaukee, Wis.
K Brand Inoculation	Soil	The McQueen Bacteria Co.	Milwaukee, Wis.
------	Liquid	National Canners Laboratory	Pittsburgh, Pa.
NitrA-germ	Soil	The NitrA-germ Co.	Savannah, Ga.
Nitragin	Peat	The Nitragin Co.	Milwaukee, Wis.
Certigerm	Peat	The Nitragin Co.	Milwaukee, Wis.
Nod-o-gen	Agar	A. Dickinson Co.	Chicago, Ill.
A. A. Inoculation	Agar	A. Dickinson Co.	Chicago, Ill.
Acme Inoculation	Agar	A. Dickinson Co.	Chicago, Ill.
Bell Brand Inoculation	Agar	A. Dickinson Co.	Chicago, Ill.
Funk Farms Inoculation	Agar	A. Dickinson Co.	Chicago, Ill.
Old Gold Inoculation	Agar	A. Dickinson Co.	Chicago, Ill.
Top Notch Culture	Agar	A. Dickinson Co.	Chicago, Ill.
Nodule-Bacter	Agar	Standard Inoc. Co.	Troy, Pa.
Pennewell's Pure Culture	Agar	The Penn Laboratories	Redlands, California
Pioneer Compost	Ground Rock	Pioneer Compost Co.	San Francisco, California
Plantgro*	Soil (mixed culture)	Julius J. Dolgos	Cleveland, Ohio
Scott's Bacteria	Sand	O. M. Scott and Sons Co.	Marysville, Ohio
Shur Inoc	Soil	M. B. Wilson	London, Ohio
Stimugerm	Agar	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
Certigerm	Peat	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
Ga. Legume Inoculator	Peat	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
Hoffman's Inoculant	Peat	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
Stimugerm	Peat	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
V. S. S. Inoc. Culture	Peat	Stimuplant Laboratories, Inc.	Long Island City, N. Y.
Superyield Culture	Agar	Strasburger and Siegel	Baltimore, Md.
U-need-R Inoculator	Manure mixture	Bressler Bacteria Co.	Cohocton, Ohio
Urbana Culture	Agar	Urbana Laboratories	Urbana, Ill.
Vitamite*	Manure mixture	A. R. Gregory	San Francisco, California

*All crop materials.

TABLE 27

Cultures for sale in foreign countries

	Trade name	Manufacturer	Address
1	Pure culture of nodule bacteria Hiltner's inoculation for legumes	Bayrische Landesanstalt f. Pflanzenbau und Pflanzenschutz	Munich, Germany
2	Nitragin Kühn's inoculation for legumes	Agrikulturwerke	Berlin-Grunewald 2, Germany
3	Azotogen Simon's inoculation for legumes	Azotogen-Institut, Dr. Teisler and Dr. Eckholdt	Dresden-A 1. Germany
4	Baljväxt-Bakterier Inoculation for legumes	Prof. C. Barthel	Stockholm, Sweden
5	Inoculation for legumes	Experiment Station	Lyngby, Copenhagen, Denmark
6	Nitrobion I Nitrogen bacterial fertilizer for legumes	Chem. Fabrik. Janke	Schiffbeck, Hamburg, Germany
7	Reformin Bacterial inoculation for legumes	Wirtschaftsverein for seed inoculation	Wien, I, Wallnerstrasse 8, Austria
8	Agrobakter Inoculation for legumes	Firma Cap, Bakteriologische Düngemittel	Praha, Nusle, Hvlickova 401, Czechoslovakia
9	Leguminosebakterien	Prof. A. I. Virtanen	Valion Laboratorio, Helsinki, Finland
10	Cultures for lucerne	W. D. Reid	Mycological Laboratory Plant Research Station, Palmerston North, New Zealand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of their products are shown in Tables 26 and 27.[3] These cultures are called by an assortment of names and are prepared in an almost endless variety of shapes and sizes of containers. Of far greater moment, however, is the variation in the nature of the culture media. Instead of making use of plant extract as a substrate, as in the original Nitragin, the manufacturers of cultures now usually employ a jelly medium often low in nitrogen, or a sterilized soil or peat.

Some of these commercial products have been on the market for a number of years; the *Nitragin* culture, for instance, for over 35 years. During this time these cultures have undergone various modifications and improvements. The best of the cultures represent the efforts of trained bacteriologists, coupled with the results of years of field experiments. Plates 45 and 46 illustrate some of the commercial cultures now on the market. It is not possible to give anything like accurate figures for the total number of cultures sold by commercial companies. In many cases, the companies prefer to keep their processes, as well as their total sales figures, secret.

In the year 1915, Harrison estimated that a sum total of 50,000 cultures was used annually in the United States. Löhnis and Leonard, 1926, stated that "at present several hundred thousand cultures of bacteria for the inoculation of legumes are distributed annually by the United States Department of Agriculture, the state experiment stations, and commercial concerns." In relation to the total acreage planted to leguminous crops, this amounts to about 2 per cent, according to their estimate. One of the largest commercial companies selling cultures reported the distribution of cultures according to cross-inoculation groups for the year July 1, 1928 to June 30, 1929, as follows:

Kind of Culture	*Bushels*
Alfalfa and sweet clover	141,054
Clover, red, alsike, etc.	30,358
Peas, canning	102,604
Peas and vetch	32,704
Soybeans	69,522
Cowpeas, Lima beans, peanuts	9,712
Beans	2,505
Dalea	2,400
Miscellaneous	3,339
Total	394,198

This one company sold enough cultures for approximately 400,000 bushels of seed. The grand total of cultures supplied annually by the various manufacturing institutions is naturally not available. But it appears to the authors that by conservative estimate the figure 400,000 perhaps represents one-third or one-fourth of the total. There would then be in the neighborhood of 1,500,000 cultures per year distributed in the United States alone.

The use of these cultures is widely accepted by the farmers of certain sec-

[3]The authors are indebted to Mr. L. T. Leonard, of the Bureau of Chemistry and Soils, United States Department of Agriculture, for supplying the names and addresses for Table 26 and Table 27.

tions of the United States. According to the estimate of the United States Department of Agriculture (Knight, 1930) the value of the commercial cultures distributed annually amounts to about $1,000,000.

INSPECTION OF ARTIFICIAL CULTURES

Cultures for the inoculation of leguminous crops are essentially biological products and, as such, are subject to the natural laws of variation and to the limitations of life itself. Too frequently, companies try to prepare cultures without due regard for the difficulties involved. Consequently, there are on the market cultures of dubious quality, and it behooves the purchaser to buy the best possible cultures if he would have success with his inoculation. But how is he to recognize the good cultures? His best course is to buy only from reliable producers, who have the facilities and recognize the need of scientific control of all steps in the process. Fortunately the testing of cultures is making much headway in recent years and is tending to improve and uphold the quality of cultures now on the market. The testing is not a simple matter, as may be appreciated from the difficulties encountered in the isolation and study of the rhizobia and in the greenhouse technique necessary for growing test plants. The necessity of growing the plants in strict absence of extraneous microörganisms is a serious stumbling block, unless the tests are made by someone well trained in bacteriology. They also require expensive apparatus and much time. For a discussion of this subject, see Klein and Kisser, 1924 and also Hopkins, Wilson, and Fred, 1931. It is not surprising that many commercial companies are unable to adequately test their own cultures. Fortunately, there are a number of places where such tests are now carried out. The Federal Government, as well as certain state agencies, is charged with the duty of testing cultures, and of issuing licenses to the makers of satisfactory cultures. At the present time, the states of Kansas, Maryland, New Jersey, New York, and Wisconsin have laws which require that companies offering artificial cultures for sale must first take out a permit, and if at any time the product is not of proper quality, permission to sell within the said state is cancelled. Although not required by law, there are a number of states where tests of cultures are made. Paragraphs from the laws of New Jersey, Wisconsin, and Kansas follow.

New Jersey. In 1919 the New Jersey State Legislature passed a law regulating the sale of inoculants for leguminous plants.

> The term legume inoculant or legume inoculants when used in connection with the law shall be construed to mean any material which contains the nitrogen-fixing organism, *Bacillus radicicola,* and which is sold, offered or exposed for sale for the purpose of conveying organisms of *Bacillus radicicola.* This law states that all preparations sold for this purpose shall bear a label giving the following information:
>
> 1. The brand name.
> 2. The kind or kinds of legume crops for which the contents may be used.

3. The month and year in which the inoculant was prepared.
4. Probable date beyond which the inoculant would be of doubtful value.
5. Quantity of material in terms of amount of seed or acreage for which the contents may be used.
6. Name and address of manufacturer.

It is required, furthermore, that all persons or companies wishing to sell legume inoculants shall file with the Experiment Station annually a statement of the brand or brands that they wish to sell and the name and address of the manufacturers.

An authorized inspector secures by purchase samples from the open market. These samples are forwarded and analyzed at the Experiment Station.

The penalty for violation of the law is a fine of twenty-five ($25.00) to one hundred dollars ($100.00), for each offense.

In addition to this regular control by means of official analysis, the law provides that any citizen of the State of New Jersey may obtain free of charge a report on the examination and analysis of any sample or samples of legume inoculants.

Wisconsin. The Legislature of 1921 amended the Fertilizer Law to provide for tests of cultures of bacteria:

Before any person or firm shall sell or offer for sale any pure or mixed culture of micro-organism or other material to be used for promoting directly or indirectly, the growth of higher plants, he or they shall file with the commissioner of agriculture a statement under oath specifying the composition of the substance and the kinds of micro-organisms contained therein which promote the growth of higher plants, and shall secure a permit from the commissioner of agriculture.

Kansas: The Report of the Kansas State Board of Agriculture (see Mohler, 1927) concerning the new fertilizer law, reads as follows:

For commercial fertilizers for which any claim of inoculation is made the following information should be given in the application for registration:

(1) The purpose of organisms present, with their names.
(2) The name of the crop or crops for which the material is intended.
(3) The guaranteed minimum number of bacteria per cc. or gram capable of performing the function for which the brand is sold, at any time previous to the date of expiration of the guaranteed value, shown on label.

Numerous methods for testing cultures have appeared in the papers of Harding and Prucha, 1905; Lipman, 1910b; Kellerman, 1913; Garman and Didlake, 1914; Temple, 1916; Emerson, 1918; Fellers, 1918a; Noyes and Cromer, 1918. The reports of the official tests of cultures at New Jersey are given in the papers

of Perkins, 1922, 1923; Perkins and Fudge, 1924; Fudge, 1925; Fudge and Stahl, 1926; Stahl, 1927, 1928. 1929; Stahl and Porges, 1930; Porges, 1931a; and those for Wisconsin in the papers of Strowd and Stevens, 1923, 1924; Griem, 1925, 1926, 1927 and 1928; and for New York, Hofer and Conn, 1931.

The method of testing cultures, as used at Wisconsin and at the United States Department of Agriculture, is illustrative of the technique and difficulties involved. In the main the inspection of cultures involves two steps—the determination of the number and kinds of microörganisms present in the supposed culture of rhizobia, and secondly the test for nodule production on the host plant claimed.

METHODS OF TESTING CULTURES OF ROOT NODULE BACTERIA

In order to secure representative samples in the condition in which a farmer might buy them, an official inspector should purchase cultures on the open market. As soon as the samples are received at the laboratory, they should be dated and given a serial number.

For the actual tests of nodule production, the plants are conveniently grown in half gallon earthen jars, filled with sand or soil of low nitrogen content. In order to destroy the rhizobia the jars are sterilized for 12 hours at 120° C. For the small, seeded leguminous plants, glass bottles may be used instead of the jars. In that case it is well to sterilize the empty bottles, and when cool, to fill them with sterilized sand. The bottles are then plugged with cotton stoppers and again sterilized for ½ to 1 hour. Long sterilization of the bottles is thus avoided, and breakage of the poor glass is very much reduced.

Before seed is planted, the sand in the jars or bottles should be fortified with a sterilized modified Crone's solution of the following composition:

KCl	10.0 gm.
$CaSO_4.2H_2O$	2.5 gm.
$MgSO_4.7H_2O$	2.5 gm.
$Ca_3(PO_4)_2$	2.5 gm.
$FePO_4$	2.5 gm.

This mixture of salts is ground in a mortar and 1.5 gm. taken for 1000 cc. of water. It may also be made the basis of an agar medium for the culture of small leguminous plants in large test tubes. The agar gel should be comparatively soft, as given by a 0.7 per cent agar concentration.

The seeds may be rendered free from bacteria by a variety of methods; see Robinson, 1910; Pinoy and Magrou, 1912; Wilson, 1915; Duggar and Davis, 1919; Walker and Erdman, 1926; Hopkins, Wilson, and Fred, 1931. The writers have found the following methods satisfactory. Place the seed in a sterile petri dish or container of similar shape and cover with 1 to 1000 mercuric chloride solution. In order to secure intimate contact of the $HgCl_2$ solution and seed, the container with seed is placed in a vacuum desiccator and evacuated for about 5 minutes. The seeds are then washed at least twice under vacuum with sterile water and covered with a modified Dakin's[4] solution. This is prepared by diluting

[4]Dakin, H. D., and Dunham, E. K. A handbook on antiseptics. The MacMillan Co., New York, 129 pp., 1917.

a sodium hypochlorite solution with a saturated solution of boric acid until the resulting mixture contains 0.5 per cent of free chlorine as determined by the method of Dakin and Dunham, 1917. After 15 minutes exposure (longer with certain seeds), the seeds are washed until the odor of chlorine is no longer detected in the washings; usually 5 to 8 washings are required.

A somewhat simpler method is that recommended by Wilson, 1915. Prepare a solution of calcium hypochlorite ($CaOCl_2$). The chlorinated lime should be fresh (100 gm. of $CaOCl_2$ in 1400 cc. of water) and the seed should be held in the solution for 2.5 hours. The time may be increased or decreased according to the variety of seed. The $CaOCl_2$ solution is then removed and the seed is washed at least four times in sterile distilled water. As shown by Neuberger, 1914, leguminous seeds may be immersed in water at 65° C. for 15 minutes or even longer, without serious injury. This temperature and time are sufficient to kill any rhizobia which may be on the seed but will not, of course, kill all other bacteria.

To approximate farm conditions, the commercial cultures should be diluted as recommended by the manufacturer. For example, according to the directions, alfalfa cultures for 60 pounds of seed are diluted to 600 cc., and 1.0 cc. of the bacterial suspension is used to inoculate 0.1 pound of seed. After the seed and inoculum are thoroughly mixed, the seed is ready for planting.

Each sample is planted in duplicate in half-gallon jars of sand, otherwise in triplicate in glass bottles in sand or in large test tubes of soft agar as desired. All cultures should be kept in a green house, at a temperature of from 60°—65° F., and watered with sterile water or Crone's solution at appropriate intervals. Nodules will develop in a few weeks, if the tests are carried out in proper season (adequate lighting). The size and placing of nodules should be noted, as they bear some relation to the "effectiveness" of the rhizobia in question. The type of nodulation has been emphasized in the consideration of strain variation, Chapter 10. The nodulation of the host plant and the benefit occurring therefrom is the crucial test of the quality of a culture. It is suggested that with every test of unknown samples, a control set of plants inoculated with a culture of known efficiency be included. These will form a standard for comparison of increase in weight of plant tissue and in amount of nitrogen in the test plants.

Total number of bacteria according to plate counts. To gain some idea of the total number of viable cells in the samples of culture, it is well to pour dilution plates according to regular laboratory technique. These plates will also give a general impression of the purity of the culture. Dilutions of 1:1 million, 1:10 million, or 1:100 million, depending upon the type of culture, are plated in triplicate with fresh Congo red-yeast water-mannitol agar.[5]

[5]The formula of the yeast water-mannitol agar is repeated here for the convenience of the reader.

Mannitol	10.00 gm.
Di-potassium phosphate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.1 gm.
Calcium sulphate	0.05 gm.
Calcium carbonate	3.00 gm.
Agar	15.00 gm.
Yeast water (1)	100.00 cc.
Water, distilled	900.00 cc.

(1) The authors prefer a starch-free yeast water. Mix 100 gm. of the pressed yeast (about 60 per cent moisture) with 1000.0 cc. of water, and sterilize for 2 hours at 15 pounds steam pressure. Set aside in the laboratory until the solution is clear. Siphon off as needed for media.

The rapidity with which colonies develop depends to a large extent on the variety of rhizobia in the culture. Usually, well-developed colonies are obtained from alfalfa, sweet clover, red clover, Wood's clover, peas, and beans in from 5 to 10 days. From cowpea and soybean cultures, the growth is slower, sometimes requiring 10 to 20 days for the colonies to reach the maximum size.

Purity tests. As soon as the dilution of the culture is prepared, loopfuls should be transferred to tubes of litmus milk, potato slopes, brom-thymol-blue yeast water-mannitol agar and Congo red-yeast water-mannitol agar. These tests are for the purpose of ascertaining the degree of contamination. The confirmatory purity tests are made from typical colonies on the plates. The colonies are first streaked on Congo red agar slopes, and after growth they are inoculated into tubes of litmus milk, potato, and brom-thymol-blue agar. These sub-cultures are incubated at 28° C. and examined after two and four weeks. If the plate contains several types of organisms, a superficial attempt is made to differentiate between the root nodule organisms of the leguminous plants and the contaminating forms. At least ten well isolated colonies should be taken from such plates and studied. From the sub-cultures on the various media, information is obtained concerning the purity of the culture, and also concerning the cultural characteristics of the organisms.

The results of these tests may be recorded on cards in some such forms as given below:

No._____

The Report of Tests of Cultures of Nodule Bacteria

Trade name_____For_____
Manufactured by_____
Sold by _____
Invoiced _____Sampled_____
Size _____Container_____
Condition _____Date of Expiration_____
Type of medium _____
Report _____
Total No. bacteria in each container_____
Approximate percentage of rhizobia-like colonies_____
Approximate percentage of fungi_____

Original transfer from cultures:*

Milk test_____Potato test_____
Congo red medium_____

*If the original culture is contaminated, it is necessary to use transfers from plate isolations.

Isolations from plates:

Milk test_____Potato test_____
Congo red medium_____
Microscopic examination _____

Nodule production:

Sand in jars_____ Number_____ Shape_____ Size_____
Sand in stoppered glass bottles_____
Agar tubes _____
Date planted_____Date examined_____
Laboratory test by_____Greenhouse test by_____

Remarks:

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SUPPLEMENT TO

ROOT NODULE BACTERIA AND LEGUMINOUS PLANTS

BY

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AUTHORS' NOTE

Since the publication of *Root Nodule Bacteria and Leguminous Plants* in 1932, interest in this field has increased, and many important investigations dealing with the characteristics of the leguminous plants and of the bacteria associated with them have been reported. During the last five years there has been a distinct shift in the research approach, and greater emphasis has been placed on the physiology of the plant-bacterial association and on the mechanism of nitrogen fixation. The old problem of excretion of nitrogenous compounds from the leguminous plants has again been investigated to explain the benefits of intercropping of leguminous and nonleguminous plants. In addition, the cross-inoculation groupings, particularly of the cowpea, lupine, and soybean groups, have called for further work.

Because of the great economic importance of the leguminous problem and the scientific interest in it, it has been deemed advisable to publish the available references to the recent work in this field. No attempt is being made at this time to interpret the results of the investigations, but simply to list the papers published from 1932 to 1938. A few important papers which were overlooked in the authors' report in 1932 are also listed separately.

For valuable aid in compiling this bibliography the authors wish to thank various staff members of the department, particularly Drs. P. W. Wilson, W. B. Sarles, and W. Umbreit.

In response to suggestions from various sources the index is here supplemented by a list of the scientific names of all the plants cited in the original monograph and by an author index. The authors are greatly indebted to Dr. O. N. Allen and Ethel K. Allen for the preparation of these two indices.

ERRATA IN *ROOT NODULE BACTERIA*, 1932

Page

xxi Par. 2, line 7. *For* Buckout *read* Buckhout.

8 Par. 1, line 5. *For* Ville, 1885 *read* Ville, 1855.

16 Par. 1, line 2. *For* 1926 *read* 1925.

22 Lines 1 and 7. *For* Buckout *read* Buckhout.

29 Par. 4, line 1. *For* Zimmerman *read* Zimmermann.

50 No. 18. For *Aztobacter* read *Azotobacter.*

53 Par. 3, line 4. *For* Kny, 1879a *read* Kny. 1879.

59 Column 2. For *Medicago sativum* read *Medicago sativa.*

63 Par. 4, line 4. *For* 1924a and b *read* 1923a and b.

72 Par. 1, line 27. *For* cephalothrichous *read* cephalotrichous.

76 Column 1. *For* Bottomley, 1910a *read* Bottomley, 1910.

78 Par. 1, line 4. *For* 1910a *read* 1910.

83 Par. 1, line 11. *For* to be writers *read* to the writers.

91 Par. 5, line 2. *For* product *read* produce.

95 Par. 4, line 7. *For* tyrosinose-producing *read* tyrosinase-producing.

115 Line 1. *For* Bottomley, 1910a *read* Bottomley, 1910.

131 Line 1. For *Phaseolus Mungo* read *Phaseolus mungo.*

144 No. 5. For *Rh. japonicum* (Kirchner) comb. nov. read *Rh. japonicum* (Kirchner) Buchanan.

No. 6. For *Rh. lupini* (Schroeter) comb. nov. read *Rh. lupini* (Schroeter) Eckhardt, Baldwin and Fred.

149 Par. 2, line 7. *For* 1879a *read* 1879.

156 Par. 2, line 2. *For* conclusion *read* conclusions.

181 Par. 1, line 10. *For* Bréal, 1899b *read* Bréal, 1889b.

188 Par. 3, line 3. *For* Fermi and Buscalioni *read* Fermi and Buscaglioni.

190 Par. 3, line 5. For *tuberuclorum* read *tuberculorum.*

194 Par. 1, line 5. *For* following *read* previous.

Par. 1, line 5. *For* Reynolds, 1907 *read* Reynolds, 1905.

221 Par. 1, line 4. *For* Luekel *read* Leukel.

224 Par. 2, line 4. *For* legumious *read* leguminous.

235 Par. 2, line 12. *For* 1928a *read* 1928.

238 Line 12. *For* Nobbe, 1896 *read* Nobbe 1896a.

253 Par. 1, line 3. *For* Hiltner *et al* read Hiltner and Störmer.

Par. 3, line 6. *For* Newcomb *read* Newcomer.

255 Par. 3, line 5. *For* Wilson, 1926 *read* Wilson, 1926a.
258 Par. 2, line 12. *For* 1928a *read* 1928.
271 Line 47. *For* Pédalogie *read* Pédologie.
272 Line 1. *For* Okhawara *read* Ohkawara.
Line 3. *For* Pédalogie *read* Pédologie.
273 Replace lines 26 and 27 in Barthel, 1919, reference by "Cultures de bactéries sur terre stérilisée. Meddel. K. Vetensk. Akad. Nobel-Inst. **5**: No. 20. 13 pp., 1919.
281 Line 28. *For* Davis *read* Davies.
Line 30. *For* 280–301 *read* 280–292.
288 Line 44. *For* 1808 *read* 1908.
295 Line 30. *For* 307–328 *read* 305–328.
305 Line 43. *For* 1913a *read* 1913.

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INDEX TO SCIENTIFIC PLANT NAMES

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The following index embraces all scientific plant names of non-leguminous as well as of leguminous species, which appear in the text. Mention has not been made of those scientific names that appear opposite photographs, since these are included in the explanatory legends under List of Plates, pages xiii-xix.

No attempt has been made to include scientific microbial names because of (a) their inclusion in the original general index and (b) the prevalence of their common names, such as pea, bacteria, clover cultures, etc., throughout the text.

Synonyms of the plant species that have been used in the text appear in parentheses under the more generally accepted plant name. In those instances where the generic name of the synonym is identical with that of the accepted name, the synonym has not been repeated in the alphabetical sequence. Synonyms with different generic names have been listed alphabetically, followed by a reference to the accepted plant name in parentheses. For example: *Genista scoparia, Sarothamnus scoparius,* and *Spartium scoparium* are followed by a reference to the more generally accepted synonym, i.e., see *Cytisus scoparius;* while under *Cytisus scoparius* the synonyms are parenthetically recorded.

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The following remarks may serve to explain the index. A superior numeral one ([1]) following the date of a paper, as in Buchanan, R. E., and Fulmer, E. I., 1928[1], denotes a footnote reference which does *not* appear in the original bibliography. A footnote citation of a paper which *does* appear in the bibliography, is designated by the same symbol following the specific page number.