ON THE CHANGES THROUGH WHICH THE NODULE ORGANISM (*PS. RADICICOLA*) PASSES UNDER CULTURAL CONDITIONS.

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(With Plates I–III and One text-figure.)

Since Hellriegel and Willfarth's discovery in 1888 of the peculiar nitrogen nutrition of leguminous plants by symbiosis with specific micro-organisms, and the further work on the isolation of the organisms and the mode of infection of the plant by Beijerinck and Prazmowski respectively, a considerable number of investigations have been made on the nodule organism and its relation to the host plant. The earlier studies included many observations on the morphology of the organism, but the realisation of the possibility of soil inoculation and its practical significance naturally directed attention largely to the development of cultural technique and no corresponding advances were made in our knowledge of the mechanism of the process of nitrogen fixation, or of the development of the organism itself. The work of Nobbe and Hiltner, Simon, Harrison and Barlow, and Kellerman has placed the preparation and use of virulent cultures on a definite basis. But as regards the morphological side we are still unable to reproduce, *in vitro*, the characteristic changes through which the organism is known to pass in symbiosis with the plant, nor has much light been thrown on the chemical processes in the nodule. Quite recently Burrill and Hansen (1) have pointed out that the life-cycle from the soil to the nodule and back to the soil is unknown and that we have as yet no clue as to the form in which the organism exists in the soil.

The early investigations of Beijerinck (2) led him to recognise the existence of three stages through which the organism passes prior to and during its period of existence with the host plant. The first of these is the swarmer stage in which form actual infection of the plant takes place. The cells are actively motile and of extremely small dimensions, 0·9μ long and 0·18μ broad, and on this account were regarded by

Beijerinck as representatives of the smallest living organisms. Subsequent to the infection of the plant, the swarmer gradually assumes a larger asymmetrical spindle-shaped rod form, about 4μ long and 1μ wide, which eventually develops into the characteristic branched individuals, the so-called bacteroids. Although Prazmowski (3) employed, in part at least, media of the same composition as those used by Beijerinck, the dimensions of the organisms encountered in pure culture differed. Prazmowski mentions, in addition to infinitely small cells, the occurrence of larger motile bacilli, 2μ–3μ long and $0{\cdot}2\mu$ wide, which are often to be found as chains of two, three, or four cells.

Gonnermann (4) employed plant-extract peptone gelatine, and claimed by the use of this medium to have isolated ten different nodule bacteria, seven of which passed as *Bacilli tuberigeni*, two as *Micrococci tuberigeni* and the other was identified as *Bacillus fluorescens non-liquefaciens*. In view of the peculiar conditions under which he obtained these organisms, and the absence of any of the precautions which are now considered to be necessary for the isolation of the nodule organism, it is difficult to attach any importance to his results. Mazé (5) regarded the organism as being markedly pleomorphic and mentions a fact worthy of note in connexion with our own results, namely, the tendency of the organism, when grown in an atmosphere of nitrogen, to assume a coccus form which, on subsequent cultivation under aerobic conditions and especially on potato, gave rise to bacilli again.

Although the predominant forms usually found in pure cultures of the nodule organism are those first described by Beijerinck, the occurrence of more complex cells such as bacteroids of the normal nodule has frequently been noted; in fact, definite attempts have been made to induce bacteroid formation on artificial media. Beijerinck (6) first mentioned their presence in pure cultures, and at a later date Stutzer (7) and Hiltner (8) carried out a considerable number of experiments with different media with the object of securing extensive bacteroid formation. While it was possible to obtain isolated cases of the formation of much branched cells in certain of these media, the experiments were only partially successful, the branched forms being relatively few in number and varying both in size and shape from those ordinarily found in the nodule. On the other hand, little attention has been paid to the reverse change of the organism, i.e. the "down-grade" transition from the more complex to the simpler forms. In the main, observations have been confined to the dissolution of the bacteroid forms obtained from living nodules, and Prazmowski (9) gives illustrations of the degeneration of

bacteroids into "oily" globules. Morck (10), although failing to realise the true nature of the bacteroid form, which he regarded as a product of the plant itself, recorded the presence in the bacteroid of a "micrococcus-like microbe."

Löhnis and Smith (11) studied the forms produced by *Ps. radicicola* and noted the presence of small globules and ovals, slime threads and cocci, regenerative bodies, and observed the granular decomposition of small rods and threads to form "spores."

THE PRESENT INVESTIGATION.

In the course of an investigation having for its object the production of active and virulent cultures of the organism, a number of nutrient media were prepared in the hope that a satisfactory substitute for the ash-maltose agar of Harrison and Barlow (12) might be obtained, since much appears to depend on the preparation of the plant ash used for this purpose. Cultures of the nodule organism were isolated from red clover, broad bean, lucerne and lupine plants and transferred weekly to a range of fresh media. It was noticed, however, during the microscopical examination of these cultures prior to transference, that certain of the tubes contained a number of coccoid forms scattered among the normal rod vegetation of the cultures. Especially was this the case in cultures of the red clover organism.

It was at first thought that these coccoid forms might represent a definite infection and our attention was consequently directed to their elimination by repeated sub-cultures. The Petri dish cultures gave rise to one type of colony only and, after transference of several of these to slant culture tubes, the growths were again replated and again tubed. This was repeated six times, and in none of the cultures was it possible to detect the coccoid forms upon examination after seven days. Finally the organism was tested for specificity by infection of clover seedlings. To this end, clover seeds were sterilised in mercuric chloride solution and, after being washed in sterile water, were transferred to sterile water agar in Petri dishes to germinate. After five days six sterile seedlings were transferred to Erlenmeyer flasks containing sterile mannite mineral salt agar and inoculated with a culture obtained in the above manner. At the end of one month the seedlings possessed from four to seven nodules each and thus satisfactorily demonstrated the conformity to type of the cultures employed. Furthermore, the cultures used for these infection experiments were again induced to give rise to the coccoid forms by the methods described below.

The observations recorded in this paper show that the life-cycle proceeds in the following five stages:

(1) *The pre-swarmer form (non-motile).* When a culture of the organism is placed in a neutral soil solution, it is converted after four or five days into the pre-swarmer form (see Fig. 1 1).

Fig. 1. 1. Pre-swarmer first stage. 2. Pre-swarmer second stage. 3. Swarmer. 4. Motile rod. 5. Highly vacuolated rods.

(2) *Second stage, larger non-motile coccus.* When the pre-swarmers are transferred to a suitable medium, such as mannite agar, they undergo a change. The original coccoid pre-swarmer increases in size until its diameter has been doubled, but still remains a non-motile coccus.

(3) *Swarmer stage, motile.* The cell then becomes ellipsoidal and develops high motility. This form is the well-known "swarmer" of Beijerinck.

(4) *Rod-form.* Proceeding in an "up-grade" direction, the swarmer becomes elongated and gives rise to a rod-form, which is still motile, but decreasingly so. So long as there is sufficient available carbohydrate in the medium, the organism remains in this form.

(5) *Stage of high vacuolation.* When however the organism is placed in a neutral soil extract (or the available carbohydrate becomes exhausted), it becomes highly vacuolated and the chromatin divides into a number of bands. Finally these bands become rounded off and escape from the rod as the coccoid pre-swarmer, 1.

Our experiments show that lack of available carbohydrates is conducive to pre-swarmer formation, while in the presence of available carbohydrates the pre-swarmers become swarmers and finally rods.

EXPERIMENTAL.

In general the media used for the cultivation of the nodule organism consisted of two solutions, soil extract and mineral salt solution, from which five stock agar media were prepared. The soil extract was made by steaming one kilo of garden soil with two litres of distilled water for a period of two hours, after which the liquid was filtered through a Berkefeld filter candle. The mineral salt solution was that suggested by Ashby(13) and consisted of:

Magnesium sulphate	0·2 grm.
Sodium chloride	0·2 ,,
Mono-potass. phosphate ...	0·2 ,,
Calcium sulphate	0·1 ,,
Calcium carbonate	0·2 ,,
Water (dist.)	1000 c.c.

The composition of the different agar media is set out below:

		Soil Extract Agar	Soil Extract Mannite Agar	Soil Extract Saccharose Agar	Mannite Agar	Saccharose Agar
Soil Extract (c.c.)	...	1000	1000	1000	—	
Mineral Salt Soln (c.c.)	...	—	—	—	1000	1000
Mannite (grms.)	...	—	10	—	10	—
Saccharose (grms.)	...	—	—	10	—	10
Agar (grms.)	...	15	15	15	15	15
Reaction	...	0°	−1·5°	−1·5°	−1·5°	−1·5°

During the course of repeated cultivations of the nodule organism on these media and especially in those cases when recourse was had to the use of soil extract media, the occurrence of coccoid bodies was noted. On the one hand, frequent transferences and repeated preparation

of plate cultures sufficed to demonstrate the purity of the cultures, which had thus given rise to divergent forms and, on the other, the final tests on the plant infection power were carried out on the lines already indicated. The identity of the organisms having thus been definitely established, it was decided to ascertain as far as possible the conditions that made for the occurrence of these coccoid or pre-swarmer forms.

In the main we have studied the conditions of aeration and food supply and the effect of various temperatures on the growth of the organism.

RELATIONS TO AIR SUPPLY.

Since the conditions in liquid media and in masses of actively growing bacteria might reasonably be expected to be only partially aerobic, a considerable number of observations have been made on the behaviour of the nodule organism when exposed to an anaerobic environment. Preliminary experiments included the use of the specific organism from red clover, broad bean, lucerne and lupin, the cultures being made on soil extract mannite agar, either in tubes or flasks. For convenience of operation the tubes were placed in a glass bottle with a supply of alkaline pyrogallol. After the necessary inoculation had been carried out, the flasks or bottles were evacuated by attachment to the pump, and normal pressure was then restored by allowing a slow current of air to pass through a vessel containing a deep layer of alkaline pyrogallol. This alternate evacuation and replacement by an oxygen-free atmosphere was repeated ten times and the cultures were then incubated. While the clover and lucerne organisms failed to grow under these conditions, those of broad bean and lupin after fourteen days covered the surface of the agar as a slightly mucilaginous mass. Further incubation under these conditions led to a decrease of mucilage and subsequent examination revealed the presence of the coccoid forms to which reference has already been made.

To test the effect of anaerobic conditions on the clover and lucerne organisms, a number of cultures were set up and submitted to preliminary incubation for seven days with free access of air. When growth of the organisms was sufficiently far advanced, the tubes were transferred to anaerobic conditions and observations were subsequently made at definite periods. Once a culture had been opened and examined it was discarded, the possible disturbance from intermittent aerobic conditions being thus avoided.

History of Original Cultures.

All the cultures were derived from strains isolated four months previously with the observance of the precautions set out by Harrison and Barlow (12). During this period they were repeatedly cultivated on soil extract mannite agar, and by means of transference once a week the organisms were maintained in the form of actively motile small rods. After certain periods under anaerobic conditions the following observations were recorded:

2 *Days*. *Bean and lucerne cultures*—thick, mucilaginous rod forms.
Clover—non-mucilaginous and increased vacuolation of cells.
Lupin—decrease in mucilage; increased vacuolation; rapid division to small rods in some cases.

3 *Days*. *Bean*—mostly rod form; very mucilaginous; some rods highly vacuolated.
Lucerne—small highly vacuolated rods.
Clover—small rods; increase in vacuolation.
Lupin—small and large vacuolated rods.

7 *Days*. All highly vacuolated. Plate II, fig. 3.

9 *Days*. Cocci appearing in clover culture. Plate II, fig. 4.

19 *Days*. Cocci appearing in all cultures.

One Month. Many cocci in all cultures.

After 19 days one set of tubes was removed from anaerobic conditions, sterile air was passed through the vessel and the cultures were then incubated.

2 *Days*. Number of cocci increased.

4 *Days*. Appearance of small rods.

12 *Days*. Mostly all rods; little or no vacuolation.

In continuation of these experiments the effect of other atmospheres on the growth of the organism has also been determined. While cultivation in oxygen leads to the production of good mucilaginous growth and of forms showing high differentiation of the cell contents (Plate II, fig. 1), confinement in an atmosphere of hydrogen results in a reduction of the mucilaginous character and an abundant formation of the pre-swarmer stage. Exposure to the action of free ammonia in normal atmosphere reacts unfavourably on growth of the organism and tends to the production of capsules. Coal gas is equally unsuitable—the cells become capsulated, while branched forms are frequent.

It is thus seen that any deviation from strictly aerobic conditions exercises a prejudicial effect on the growth of the organism, but the

extent to which these changes proceed appears to be determined by other conditions, such as, for example, those of nutrition. Under comparable aerobic conditions, cultivation on soil extract mannite agar and on soil extract saccharose agar induced a free formation of pre-swarmers, but this occurred to a much less degree on similar media without soil extract; soil extract agar without additional source of carbon gave rise solely to small rods.

THE PRODUCTION OF THE PRE-SWARMER STAGE FROM ROD STAGE.

In our original cultures grown on soil extract mannite agar and transferred weekly to fresh medium, the organism exists mainly as a short actively motile rod 3–4μ long and 1μ broad. Increase of age of culture is accompanied by decrease in motility and a marked production of mucilage. The cells no longer take the stain uniformly and densely, but exhibit a definite banding of their contents. [Plate II, figs 3, 5. Plate III, figs. 1, 2, 3, 5.] Soil extract media appear to be especially potent in rapidly inducing these effects.

Under restricted conditions of aeration the vacuolation increases until the chromatin substance has become segregated to small particles scattered along the cell. In branched forms the particles are seen at the angles and extremities of the arms as well as along the rod. Occasionally the cells appear to be regularly banded but at other times the change only gives rise to a reticulate effect. This condition has been previously observed by other workers, and Miss Dawson (14) asserted that these chromidial particles were simply due to vacuolation and showed no analogy with spore formation.

In a few instances the formation of chromatin patches and bands has followed a definite sequence of division, but usually there is a little evidence of a dominant phase. In one case, for example, the initial material consisted wholly of uniformly staining rods. At the end of one hour stained films from this culture showed a predominance of cells possessing an achromatic patch distant about one-third of the length of the cell from one end. A few minutes later this patch had extended as a band across the cell and half an hour later the larger chromatin particle had also divided, producing three equal granules in the cell. Two hours later each of these had again divided, thus producing six chromatin bands placed at regular intervals across the rod. As already indicated above no such definite subdivision is as a rule perceptible, and the chromatin cell contents break up into particles of equal size. In small rods the chromatin may be grouped as a central band or as

two polar bands, while still larger rods may possess as many as fourteen densely staining bands. Swollen globular forms have been found to exhibit a reticulate segregation with a number of chromatin granules scattered about the periphery of the cell (Plate III, fig. 6). Subsequent changes appear to proceed in either of two ways. At times the chromatin particles become definitely organised cocci within the rod (Plate II, fig. 3), but usually they remain as bands and only the terminal granules become coccoid. The cell wall may dissolve as a whole, leaving a string of "pre-swarmers" having the appearance of streptococci, or it may dissolve locally, similarly permitting of the emergence of a "pre-swarmer." Hence pre-swarmers are found to occur as masses, chains or singly. They are non-motile, and stain readily and densely with carbol fuchsin and gential violet, Loeffler's methylene blue, and with Heidenhain's haematoxylin. At the moment of liberation they do not appear sufficiently well-defined to suggest the possession of a cell wall, nor do they show the characteristic behaviour of spores towards the usual stains.

The original pre-swarmer is small (0·4μ diameter) and stains densely. Under favourable conditions it swells to about twice its original diameter; it then stains less densely, and finally becomes a swarmer of high motility. From this time, and with continued incubation, the usual transition through small rods, long rods, to ultimate pre-swarmer formation may be induced. The production of pre-swarmers is not limited to normal rods. We have observed branching bacteroids and large involution forms passing through the same changes and eventually giving rise to pre-swarmers.

With the object of ascertaining the relation of these changes to the normal process of development and disintegration in the nodule, a number of observations have been made on the contents of old bean nodules, immediately prior to dissolution. They were invariably black and pulpy within, but only those nodules were used whose outer walls were intact. Examination of the bacterial slime within the nodule showed most organisms to exist as the frequently observed, highly swollen, branched and vacuolated "ghost" form (Plate I, fig. 1). Dense masses of chromatin were found within the cells, sometimes as transverse bands and at others as a lining to the periphery. The chromatin masses break down into pieces of varying size and shape, some having the coccus form resembling pre-swarmers, while densely staining bacilli were also found.

In the old nodule of red clover, the "ghost" form is smaller than that of the bean, but much of the chromatin leaves the bacteroid in the pre-swarmer form.

There is thus some indication that the formation of pre-swarmers is a normal course and is not primarily determined, although it may be markedly influenced, by cultural conditions.

THE INFLUENCE OF SOIL CONDITIONS ON PRE-SWARMER FORMATION.

In view of the pronounced tendency to the formation of pre-swarmers in soil extract media, a number of observations have been made to ascertain whether this effect was a positive one, i.e. due to the presence of some definite substance or group of substances, or merely negative, as the result of a deficiency of nutrient materials. To this end, a number of young vigorous cultures of red clover, broad bean, lupin and lucerne organisms were taken, all of which showed the bacteria in the normal rod form. Soil extracts were prepared by digesting 30 parts of soil with 100 parts of distilled water, (*a*) in the cold, (*b*) by steaming for one half-hour and (*c*) by heating in the autoclave at 25 lbs. pressure for the same period. After this period of digestion, the respective extracts were filtered by passage through a Doulton filter candle, placed in separate portions of 100 c.c. in Erlenmeyer flasks and sterilised in the autoclave. Two controls were introduced, one with distilled water and the other being a flask with 30 grms. soil and 100 c.c. water autoclaved, but not filtered. In all cases quantities of about 10 c.c. of the respective liquids were transferred to strong agar slant cultures, the bacterial mass scraped off by means of a sterile platinum needle and the bacterial suspension then returned to the flask.

Examination after various periods gave the following results:

Medium	After 18 hours	After 5 days	After 19 days
Distilled water	Pre-swarmers appear in clover cultures	10% pre-swarmers in clover, bean and lupin. Few in lucerne	Number of organisms greatly reduced
Cold Soil Extract	,,	25% pre-swarmers in all cultures	,,
Extract of Steamed Soil	Pre-swarmers appear in clover and lupin cultures	50% pre-swarmers in all cultures	,,
Extract of Autoclaved Soil	Pre-swarmers appear in clover cultures	Clover practically all pre-swarmers. Bean, some pre-swarmers. Lucerne mostly small rods	Clover and lupin all pre-swarmers. Bean and lucerne about 50%
30 gms. Soil, 100 c.c. Water	Pre-swarmers appear in clover, bean and lucerne. Lupin showed rapid division of long rods	,,	,,

Transference of an active culture to soil extracts prepared in various ways, or to distilled water, thus rapidly leads to pre-swarmer formation, and this uniformity of result in itself suggests that this effect is due more to deficiency of food substances than to the presence of any substance formed by the action of high temperatures on the soil. This is rendered more probable by the following experiments.

(1) *The addition of cultures to non-sterile soil.* In this case mass inoculation of non-sterile soil was carried out, the bacterial suspension being of such a strength that the nodule organism predominated over the other soil organisms and could be readily picked out in film preparations.

After four days films were obtained which showed the nodule organisms in excess of the other soil forms. All the cells were producing pre-swarmers in large numbers.

(2) *The effect of a cold soil extract on the organism.* Since the soil extract prepared in series (1) was necessarily of a much lower concentration than the soil water, we attempted to prepare an extract of more normal strength. In the absence of facilities for the preparation of extracts by combined pressure and displacement methods, an extract made in the ordinary manner, by digesting 500 grms. soil with 400 c.c. of distilled water for 18 hours and filtered through a Doulton candle, was concentrated *in vacuo* at 40° under aseptic conditions to one-fifth of its volume, i.e. to the volume of water originally contained in the soil. A culture of the organism was then added to the concentrated extract and incubated at 30°. Observations made at regular intervals of 12 hours showed a gradual but definite increase of pre-swarmer forms from the seventh to the ninety-sixth hour, by which time they formed practically the whole of the population. After six days a quantity of 1·0 per cent. sterile saccharose solution was added to the culture, and resulted in the transformation of the organism from the pre-swarmer to the rod form. These ultimately gave rise to the pre-swarmers and were again transformed to rods on the addition of more saccharose. It is, therefore, evident that pre-swarmer formation may be attributed among other causes to lack of food material. We have, however, a certain number of indications not only that lack of air supply tends to make for pre-swarmer formation in otherwise suitable media (p. 151), but that free aeration exercises the reverse effect, i.e. it appears to promote swarmer formation in otherwise unsuitable solutions.

The possible effect of previous environment on the liability to pre-swarmer formation has been tested by growing strains of the nodule

organism, previously cultivated on a range of different media, in a soil extract known to favour the production of pre-swarmers. Cultures derived from soil extract agar, saccharose agar, mannite agar, and soil extract mannite agar, were each transferred to soil extract; after 20 days all cultures gave rise to extensive pre-swarmer formation.

The Relation of Temperature to Pre-Swarmer Formation.

Comparative observations have been made to ascertain how far pre-swarmer formation or, on the other hand, persistence of the rod form, is affected by temperature. For this purpose eight flasks, each containing 30 grms. of field soil and 100 c.c. of distilled water, were sterilised in the autoclave and, after inoculation, were incubated at different temperatures, viz. 37°, 30°, 25°, and at room temperature. After 20 days the cultures showed:

At 37°—large dense rods with few pre-swarmers.
„ 30°—smaller dense rods; number of pre-swarmer greater than at 37°.
„ 25°—pre-swarmers dominant.
„ room temperature—pre-swarmers dominant.

The Influence of Certain Soil Conditions on the Production of Pre-Swarmers.

In addition to the foregoing results which were obtained by the use of Rothamsted garden soil, some further information has been derived from an examination of the behaviour of the organism towards a number of other soil types. The general method adopted was to sterilise 30 grms. of the soil with 100 c.c. of distilled water in a 250 c.c. Erlenmeyer flask. Sterile distilled water (5 c.c.) was added to a tube culture of the organism, the growth brought into suspension with a platinum needle, and then transferred to the flask. The whole was incubated at 30° and observations made at intervals.

Without entering into a detailed account of the various results, it may suffice to state that the type of soil and particularly the presence or absence of available base appears to exercise a profound effect on the form of cell arising under otherwise identical conditions. Definitely acid soils lead to the production of involution forms and finally to the extinction of the organism. This, of course, is strictly in accordance with the known intolerance of the nodule organism of acid conditions.

Soils with an excess—either small or large—of calcium carbonate readily induce the formation of the pre-swarmer from normal rods, and this change can also be effected by the addition of calcium or magnesium carbonate to acid soils which would normally lead to the production of involution forms.

An interesting case was presented by a light sandy loam from Woking, the reaction of which was slightly alkaline to litmus. On the addition of a normal culture to this soil, the organisms persisted in the form of small and large densely staining rods, whilst no pre-swarmer forms were to be observed. In this case also the addition of calcium or magnesium carbonate resulted in the conversion of 95 per cent. of the cells into the pre-swarmer stage within the period of seven days. The great uniformity with which this change may be induced rather suggests the existence of the nodule organism in the pre-swarmer form in normal soils.

An extension of these observations, on the effect of soil conditions on the predominance of a particular form of the organism, led to an examination of a number of soils from the permanent barley plots on Hoos Field. The treatment of the different plots includes, as is well known, a range of mineral manures with and without the addition of nitrogen in the form of sodium nitrate and of ammonium sulphate, while one plot receives a dressing of farmyard manure annually in spring. The results of these experiments show in the majority of cases a definite tendency of the organism towards pre-swarmer formation after a period of 14 days, but in the case of the unmanured soil, peculiarly enough, the organism remained in the rod form.

Conversion of Pre-Swarmers into Swarmers.

In the following two series of tests we attempted to convert the pre-swarmer into the swarmer, in contradistinction to the earlier experiments, where attention was specially paid to the converse change. It was necessary, therefore, to induce the pre-swarmer stage before any of the test substances were supplied. This was done by previous cultivation in sterilised garden soil and distilled water containing 1·0 per cent. calcium carbonate, and when about 95 per cent. of the organisms were in the pre-swarmer stage, the compounds were added as sterile solutions.

Subsequent observations showed the following:

Compound added to control	Form of organism	
	After 1 day	After 7 days
Control (soil extract)	Pre-swarmers dominant	Pre-swarmers dominant
0·1% Sodium nitrate	,,	,,
,, Calcium nitrate	,,	,,
,, Ammonium sulphate ...	,,	,,
,, Asparagin	,,	,,
,, Peptone	,,	,,
,, Sodium chloride	,,	,,
,, Potassium sulphate ...	,,	,,
,, Calcium sulphate	,,	,,
,, Magnesium sulphate ...	,,	,,
,, Manganese sulphate ...	,,	,,
,, Ferric chloride*	,,	,,
,, Di-potassium phosphate ...	,,	Few swarmers, Pre-swarmers dominant
,, Mono-potassium phosphate	,,	Increase of swarmers, Pre-swarmers dominant
,, Calcium phosphate ...	Small rods (dense)	Small rods (dense)

* This substance allows of only a poor growth of the organism.

The addition of all the inorganic and organic sources of nitrogen and of the majority of the mineral salts is thus shown to be ineffective as a means of inducing the production of swarmers. The phosphates behave differently, however, and the effect of calcium phosphate, although at present inexplicable, might possibly constitute a secondary factor in the known response of leguminous crops to phosphatic manures.

The second series consisted in a similar comparison of the values of a number of higher alcohols and sugars, all of which were supplied in a concentration of 0·01 per cent. For convenience of comparison the compounds have been arranged in two groups according to their value for bringing about the desired change of the pre-swarmer to the swarmer. Furthermore, it should be borne in mind that three grades of action are possible. In the first, particularly suitable compounds rapidly induce the production of swarmers and later of normal rods, but with exhaustion of the carbon source there occurs a correspondingly early passage to the pre-swarmer stage. The second is that effected by less suitable compounds which, within a prescribed period, only slowly converts the pre-swarmers to swarmers and rods. The third grade gives purely negative results; it is seen when the compound is unsuitable for the growth of the organism. The results are given in the following table:

Compound tested	Form of organism: Initial stage	After 4 days	After 28 days
Ammonium tartrate	Pre-swarmers	Rods: swarmers dominant	Pre-swarmers dominant
Mannite	,,	Swarmers dominant	Small rods dominant
Maltose	,,	Swarmers and rods	Pre-swarmers
Lactose	,,	,,	,,
Dextrose ...	,,	Small rods	,,
Raffinose	,,	,,	Pre-swarmers dominant
Saccharose ...	,,	Dense rods	All pre-swarmers
Glycerine	,,	Pre-swarmers dominant	Small rods dominant
Laevulose	,,	,,	Swarmers dominant
Galactose	,,	,,	,,
Arabinose	,,	,,	Pre-swarmers dominant
Starch	,,	,,	,,
Inulin	,,	,,	,,
Dextrose	,,	,,	,,

The determining influence of carbohydrate supply on the form assumed by the organism is well illustrated by an experiment in which successive small doses of saccharose were supplied to a culture of the organism. In this manner it was possible to convert a pre-swarmer vegetation to one of dense rods and, from the alternate supply and exhaustion of the sugars, the numbers of either form could be well represented by an asymptotic curve.

It has already been shown that the addition of calcium carbonate to a soil containing the organism in the rod form, leads to a conversion to the pre-swarmer stage. This is likewise the case when excess of calcium carbonate is added to a saccharose medium containing the organism in the swarmer stage:

(1)	Soil flask	...	0·1% Saccharose	...	dense rods
(2)	,,	...	0·1% Saccharose 0·01% $CaCO_3$	... ...	dense rods
(3)	,,	...	0·1% Saccharose 0·1% $CaCO_3$	... ...	highly vacuolated rods producing pre-swarmers.
(4)	,,	...	0·1% Saccharose 0·5% $CaCO_3$	... ...	many free pre-swarmers: greater vacuolation of rods.

These experiments demonstrate that the following factors influence the formation of the pre-swarmer stage:

(1) Lack of available organic food material.

(2) The presence of excess of calcium carbonate.

SOIL CONDITIONS FAVOURABLE TO THE CONVERSION OF PRE-SWARMERS TO SWARMERS.

The conversion of pre-swarmers into swarmers is effected not only by the pure substances referred to in the above tables, but by other substances commonly present in the soil. It is facilitated by horse manure, straw and plant residues. Aqueous extracts were used in all cases and, as before, the organisms were initially in the pre-swarmer stage. The results are given in the following table:

	Form of organism		
Substance tested	After 2 days	After 7 days	After 28 days
Horse manure extract	All rods	All rods	Some pre-swarmers present
Straw extract	Rods dominant	Rods dominant	Some pre-swarmers present
Horse faeces extract	A few rods present	Pre-swarmers dominant	Pre-swarmers dominant
Bean root extract	Rods dominant	Rods dominant	Rods dominant
Clover root extract	,,	,,	,,
Lucerne root extract	,,	,,	,,
Vetch root extract	,,	Pre-swarmers dominant	Pre-swarmers dominant

Thus it appears that certain, if slight, differences exist in the nutritive value of the various extracts tested. Those prepared from the legume roots bring about a continued growth of the organism in the rod form; dung and straw extracts are less effective in this direction, whilst horse faeces extract is almost without action.

The up-grade and down-grade changes in the form of the nodule organism deserve attention on account of their bearing on field problems. Excess of calcium or magnesium carbonates in the soil makes for rapid pre-swarmer production and consequently provides the best starting point for a strong growth of cells capable of infecting the host plant. The effect of phosphates in converting pre-swarmers into swarmers might also be expected to facilitate infection. The importance of a proper supply of phosphates for the growth of leguminous crops is well recognised and may be due, in part, to some biological change to which it gives rise. On the other hand, there has been some difficulty, in the past, in reconciling theoretical conceptions of the necessary manurial treatment of leguminous crops with agricultural practice, since the application of farmyard manure has long been, and still is, believed to be of great value in the cultivation of legumes (E. J. Russell(15)). The results obtained in our experiments provide an explanation that

would quite well meet such cases. Finally, a considerable amount of experimental work has consisted in a comparison of the relative value of inoculating by means of pure cultures, as against that by means of soil. The general results obtained in this direction have almost invariably shown the superiority of the latter method. Although this is, no doubt, due in part to the lack of virulence in some of the cultures used, some of the effect might well be attributed to the form in which the organism was presented to the plant. Our investigations show that soil is instrumental in the formation of the pre-swarmer form, and this form might well be expected to make for an earlier or more effective infection of the plant. Investigations on the relative efficiency of the different forms in infecting the plant are still in progress.

SUMMARY.

It is shown in the preceding pages that under certain cultural conditions the nodule organism from the roots of red clover, broad bean, lucerne and lupin exhibits a tendency towards granular disintegration of the cell with the formation of small non-motile coccoid bodies, about 0·4μ diameter.

In the cultures ordinarily in use these coccoid bodies are not formed extensively, but cultivation on soil extract media rapidly leads to their production, until finally they constitute the predominant type in the culture.

A life-cycle consisting of five stages is described:

(1) *The pre-swarmer form (non-motile).* When a culture of the organism is placed in a neutral soil solution, it is converted after four or five days into the pre-swarmer form.

(2) *Second stage, larger non-motile coccus.* In presence of saccharose, certain other carbohydrates, and phosphates, etc., the pre-swarmers undergo a change. The original coccoid pre-swarmer increases in size until its diameter has been doubled, but still remains a non-motile coccus.

(3) *Swarmer stage, motile.* The cell then becomes ellipsoidal and develops high motility. This form is the well-known "swarmer" of Beijerinck.

(4) *Rod-form.* Proceeding in an "up-grade" direction, the swarmer becomes elongated and gives rise to a rod-form, which is still motile but decreasingly so. So long as there is sufficient available carbohydrate in the medium, the organism remains in this form.

(5) *Vacuolated stage.* When, however, the organism is placed in a neutral soil extract or the available carbohydrate becomes exhausted, it becomes highly vacuolated and the chromatin divides into a number of bands. Finally these bands become rounded off and escape from the rod as the coccoid pre-swarmer, 1 (see Fig. 1).

The formation of the coccoid bodies (pre-swarmers) may also be induced by the addition of calcium or magnesium carbonates to the medium or by placing the organisms under anaerobic conditions. Of a considerable number of compounds other than carbohydrates, calcium phosphate alone was capable of bringing about the change from pre-swarmers to rods.

The organism also appears to be affected greatly by the reaction of the soil. In the main, the normal rod rapidly changes into the pre-swarmer form in calcareous soils; acid soils cause the production of highly vacuolated cells and eventually kill the organism, while a slightly alkaline soil was found to be capable of supporting vigorous growth without altering the form of the cells.

The effect of various temperatures on the rapidity of pre-swarmer formation has been studied. Relatively high temperatures (30° and 37°) either prevent or postpone the entrance of down-grade changes.

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KEY TO PLATES.

PLATE I.

Fig. 1. Old nodule of the Broad Bean, showing ghost forms and free chromatin masses.

Fig. 2. Red Clover organism in pre-swarmer stage.

Fig. 3. Red Clover organism. Pre-swarmers transferred to fresh medium. Type of rod after 24 hrs.

Fig. 4. After 72 hrs. on fresh medium.

Fig. 5. „ 96 „ „

Fig. 6. „ 72 „ „ Showing all stages from Figs. 2 to 5.

PLATE II.

Fig. 1. Red Clover organism after seven days in atmosphere of oxygen; the position of the chromatin bands indicating rapid cell division.

Fig. 2. Red Clover organism after five days in atmosphere of nitrogen showing increased vacuolation of rods.

Fig. 3. Red Clover organism after seven days in atmosphere of nitrogen showing still higher vacuolation.

Fig. 4. Red Clover organism after nine days in atmosphere of nitrogen. The pre-swarmers have left the rods and are free in the medium.

Fig. 5. Red Clover organism after nine days in atmosphere of nitrogen. A typical culture showing highly vacuolated rods with segregated chromatin. The pre-swarmers can be seen within the rods; also free in the medium.

PLATE III.

Figs. 1 and 2. Lupin organism after seven days in soil water showing pre-swarmer production.

Fig. 3. Lupin organism after seven days in atmosphere of nitrogen.

Figs. 4 and 6. Lupin organism after nine days in soil water.

Fig. 5. Lupin organism involution form showing segregation of the chromatin and pre-swarmer production.

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