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Further Observations on the Nature and Functions of the Nodules of Leguminous Plants

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IV. *Further Observations on the Nature and Functions of the Nodules of Leguminous Plants.*

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Communicated by Professor H. Marshall Ward, *F.R.S.*

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[Plates 7, 8.]

In a paper communicated to the Royal Society in December, 1898,* an account was given by the writer of various experiments bearing on the nature of Leguminous nodules and the organisms concerned in their formation. The work, there described, had reference almost exclusively to the nodules borne by *Pisum sativum* and *Vicia hirsuta*, but it is now proposed to record some further results obtained from a more comparative study of nodules from other genera of the Leguminosæ, and, in addition, to discuss, in some detail, subsequent experiments upon the nodule organisms, both in connection with their behaviour on artificial media, and the effect of their action upon the host plants, and, in conclusion, to consider these experiments in relation to the intimate connection, which they suggest as existing, between the host plants and their parasitic organisms on the one hand, and the very varied and complicated physical and biological factors of the environment on the other hand.

As regards anatomical characters, the facts to hand are still not sufficiently comprehensive to admit of any general conclusions being drawn as to the relation of the nodules borne by the genera of the different sub-orders of the Leguminosæ; it will, therefore, be convenient to consider each genus separately, irrespective of its systematic position in the order.

In the study of the nodules of *Phaseolus* (*P. multiflorus* and *P. oleraceus*) it is noticeable that the formation of nodules in this genus appears to require a greater length of time than in the majority of our common leguminous crops. For instance, in ordinary garden soil, *Phaseolus* plants have been grown for at least three weeks without a trace of nodules appearing upon the roots, whilst on peas, vetches, clovers, &c., they are as a rule easily detected after about ten days.

At the end of a month very small nodules were found in *Phaseolus*, and it was

* 'Phil. Trans.,' B, vol. 192, pp. 1–28.

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especially noticed that in every experiment these were confined almost entirely to very slender lateral roots, whilst the main roots were quite free from them. Here again we have a marked difference from the peas and vetches. In these plants it is the rule to find the first formed nodules upon the main roots, whilst the younger ones are scattered over the lateral roots, and of these the largest are usually situated upon the proximal part of the root.

Sections of large nodules of *Phaseolus* show that the tissues contain a remarkably large quantity of starch, both in the cells containing bacteroids, and also in a definite starch layer outside these cells. Situated from one to three cells below the surface of the nodule is a layer containing large crystals of calcium oxalate (Plate 7, fig. 1 *a*). In sections of portions of roots devoid of nodules, crystals of exactly the same type occurred in abundance in the layer of the cortex next to the endodermis.

In 1891 LAURENT* described and figured filaments within the bacteroid-cells of very young nodules of *Phaseolus*. FRANK, in 'Die Pilzsymbiose der Leguminosen,' regards this type as one in which filaments are normally absent. He figures one instance of such a filament crossing a cell of the piliferous layer, by which infection of the root had taken place, but he concludes that this is an exceptional case.

For some time I failed to find any trace of filaments in the nodules of this genus, but early in 1899 in some very minute specimens they were seen, not only within the cells of the bacteroid tissue, but also crossing the cortical layers (fig. 2 *a*, *b*). The characters of the filaments were strictly comparable to those described in the filaments invariably found in the nodules of *Pisum* or *Vicia*. In *Phaseolus* they were never found, however, in nodules larger than a pin's head, and only once was an "infection tube" seen within a root-hair (fig. 2 *c*), whilst appearances such as FRANK represents in his fig. 15 were not uncommon. Indeed, on the roots of *Phaseolus*, hairs are comparatively few in number.

Such results as these suggest that in this genus the germs are capable of entering the host, either by a root-hair, or directly across the cells of the piliferous layer: also that within the root they can continue their growth for a while with or without the formation of a filamentous zooglœa-like structure. It is evident that when formed these filaments are but transitory, since they do not seem to occur in any but the very smallest nodules. In this type we may, perhaps, have a representative of an intermediate stage in the adaptation of the parasite—*i.e.*, the aggressive symbiont—to its host, since both methods of growth seem possible, the filamentous zooglœa form apparently being the rarer of the two. On the other hand, in such plants as *Lupinus* (in the nodules of which filaments have not yet been observed) this latter mode of growth has either been entirely dispensed with, or has not as yet been developed by the organism.

In the genus *Acacia* the species chosen for study were *A. heterophylla* and *A. cornigera*.

* Laurent, 'Ann. Inst. Pasteur,' 1891.

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As in the nodules of *Phaseolus*, no filaments were found within the bacteroid containing cells of fully formed nodules upon *Acacia* roots; but, again like *Phaseolus*, fragments of filaments occurred in sections of very young nodules. The method of infection in this genus has not been followed in detail, but it is at least possible that here again we have an intermediate type in the development of the parasite in question (see fig. 3).

Inconspicuous filaments such as have been described in *Acacia* nodules were also observed in those borne upon the roots of *Carmichaelia australis*.

In *Coronilla glauca* the filaments are very numerous in the meristematic region of the nodules, but they disappear entirely in the older cells.

In the nodules of *Acacia* and *Robinia* a crystal-containing layer was observed similar to that described in *Phaseolus*. In the nodules of *Lupinus*, *Flemingia semialata*, *Edwardsia sp.*, *Adenocarpus decorticans* and *Psoralea sp.* no filaments have been observed.

During this comparative study of the nodules borne by various genera of different sub-orders and tribes, special interest was derived from some work upon the species *Desmodium gyrans*. The study of the nodules of this plant was suggested by experiments which were being conducted by Mr. R. I. Lynch in the University Botanic Gardens, Cambridge. For some time he had found it impossible to cultivate this plant with any success in ordinary Cambridge soil, nor had nodules ever been observed upon the roots. In October, 1898, in view of what was known regarding the organisms of the nodules of Leguminosæ, the attempt was made to grow *Desmodium* in its native soil from Calcutta, with the result that healthy plants bearing flowers, fruits, and seeds were secured, and in addition numerous nodules appeared on the roots.

Whether this is due to the existence of the specific organism especially adapted to *Desmodium* which is entirely absent from the soil of this district, or whether the unhealthiness of the plants themselves, arising from the unsuitability of the soil, had prevented the organism from penetrating the roots, is an open question. From further experiments, the results of which Mr. Lynch has kindly communicated to me, and from the fact that, as will be shown later, there are no characters peculiar to the organisms obtained from these tubercles which distinguish them from those obtained from every other genus, I am disposed to think that failure in growing these plants was due to the presence of some injurious factor—possibly organisms—in the Cambridge soil, rather than the entire absence from it of a specific organism capable of producing tubercles upon *Desmodium* roots. I may add that I have confirmed the above results.

A detailed examination was then made of the nodules borne by this genus—*Desmodium*—and, as is described on p. 55, pure cultures were made of the organism concerned in their formation, since this was observed to be of an unusually large size, and therefore especially well adapted for detailed microscopic study.

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Organisms of a similar large size were also found in the tubercles of *Flemingia*, *Psoralea*, and *Coronilla*.

In section, the nodules of *Desmodium* resemble those of *Lupinus* and *Phaseolus*. In the majority of cases no trace of filaments was seen, but as in *Phaseolus*, *Acacia*, and others, in some of the very young specimens fragments of filaments occurred in some cells along with the masses of bacteroids (fig. 5 *a* and *b*). The bacteroids fill all the infected cells; they are long straight rods with swollen ends, and mingled with these are others having the usual X and Y forms (fig. 6). In the outer cortical region is a layer of cells containing large crystals of calcium oxalate, as found in *Phaseolus*, *Acacia*, and *Robinia* (fig. 4 *b*). In *Desmodium*, however, this layer was not found in any of the roots available for examination. Possibly this may be due to the age of the roots, the cortex of which is thrown off by the formation of a cork layer very near to the vascular cylinder. The number of plants at my disposal was too small to allow of the sacrifice of seedling roots for the further study of this point.

In sections of the nodules of *Desmodium* a very remarkable feature was noted for the first time. In material hardened in absolute alcohol, bright apple-green bodies were seen in the cells of the bacteroid tissue. These became particularly obvious, in either hardened or fresh material, after digestion for four hours in artificial gastric juice at 34° C. Further sections of younger nodules in the fresh condition showed these bodies occurring in almost every cell of the bacteroid tissue, and in the cells immediately surrounding this region. As a rule there is only one in each cell, sometimes, however, two or more are present. When treated with 5 per cent. potash in the fresh condition they disappear suddenly and completely; 1 per cent. potash dissolves them more gradually. They are partially decolorised by 1 per cent. sulphuric acid. After treatment with absolute alcohol, or after digestion in gastric juice, they become differentiated into a colourless groundwork, with one or more dense masses lying within it, to which the green colour is confined (see fig. 7 *a*—*c*).

Thus far the exact nature of this body and the cause of its green colour have not been determined. Some preparations suggest that this green body is the cell nucleus; others, however, show a colourless nucleus-like body in the cells in addition to the coloured body. Its rapid solution in potash is evidence against a nuclear nature. A similar though less marked green colour was, however, found in the nucleus of cells of the nodules of *Robinia pseudacacia*, and here too the colour became more intense after digestion, but the body itself was not further differentiated (see fig. 8 *a*, *b*).

This method of clearing sections in gastric juice proved useful in many instances. Sections of tubercles, such as those of *Pisum*, *Vicia*, *Robinia*, which contain abundant filaments, after such treatment show clearly the true nature of these filaments. In the root-hair also the rodlets could be seen embedded in the "infection tube."

Amongst the nodules obtained from one *Desmodium* plant, which died suddenly, some were found with the tissues attacked by a septate fungus resembling *Rhizoctonia* (fig. 9). This point is of interest as suggesting the possible means by which the

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nodules which are not absorbed by the host may undergo decay at the close of the vegetative period.

Of the remaining nodules some were empty shells, others were filled with a watery mass of organisms, all of which were short straight rods, probably the broken-down portions of the longer rods found in still healthy nodules. (Refer to p. 57.)

In addition to these normal nodules a second kind was present, wholly or partially filled with Nematodes. To these the death of this plant was probably due.

Upon *Cassia* roots the formation of nodules has not, as yet, been observed. The roots of this genus are remarkable for the jet-black colour of the older portions, whilst the young root-tips which protrude through the mould are of a pale greenish-yellow colour, and form a very striking contrast to the older portions if the plants be turned out from their pots without disturbing the soil round the roots.

Without attempting to draw any general conclusions from the above account of the anatomy of the tubercles found on scattered genera of the *Leguminosæ*, I propose to turn next to the consideration of some further points in the biology of the "*bacteroids*" contained in the nodules. It has been shown that the filaments consist of numbers of straight rodlets embedded in a colourless matrix, from which, at intervals along the filaments, the rodlets are liberated, and subsequently become modified in form within the cells of the nodule.* Descriptions have been given of the cultivation of these rodlets in drops of different artificial media, and of the observation of their multiplication by repeated division into more or less equal halves. To these results can now be added the details of the formation of bacteroids from the straight rodlets, as well as some account of the general behaviour of these organisms when studied according to the ordinary methods of modern bacteriology.

In the account given on p. 53 of the morphology of *Desmodium* nodules, reference was made to the unusually large size of the organisms contained within them. They were some three or four times as long as those found in the nodules formed upon the roots of the commonly cultivated British *Leguminosæ*.† A mean of several measurements gave their size as from 3 to 7μ long by 1·3μ broad.

The majority of them were straight rods with swollen ends, but mingled with these were others having the X and Y form. Their large size at once suggested this organism as being probably especially well adapted for study in drop cultures. Accordingly I cultivated it from the nodules in the manner described in my former paper concerning *Pisum*, &c., by a series of separations on tube and plate cultures. In pure cultures on nutrient gelatine large numbers of X and Y forms occurred, and from these microscopic preparations were made. The organisms stain very readily with iodine, Gram's solution, methylene blue, carbol fuchsin, &c. With methylene blue or

* See *e.g.* figs. 9 and 10, 'Phil. Trans.,' B, vol. 192, Plate 1.

† As already mentioned, a comparative study of nodules from several extra-British genera has shown that large organisms of this type are by no means peculiar to *Desmodium;* for example, they occur also in *Acacia*, *Flemingia*, *Carmichaelia*, *Coronilla*, *Psoralea*.

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carbol fuchsin very deeply stainable bodies are seen in all the rods. They occur at the ends, and also one or more along the course of the rod. In the branched forms they are seen at the extremities of the arms, at the angles, and sometimes along the course of the main rod. These are doubtless the bodies which have been described as spores by SCHNEIDER,* and as cocci by FRANK† (see fig. 6).

The possibility of either of these having been discovered by these observers cannot be denied, but my preparations, both of this special type and of those from other genera suggest rather that the phenomenon is due to vacuolation of the contents.

Throughout my work on the bacteriological characters of the nodule organisms, I have had under observation a triple series of cultures, which for convenience in the following descriptions may be referred to as cultures of organisms A, B, and C. They are as follows :—

A. Organisms from sub-cultures of commercial "nitragin" for *Pisum sativum.*
B. Organisms cultivated directly from the nodules of *Pisum sativum.*
C. Organisms cultivated from the nodules of *Desmodium gyrans.*

A preliminary examination of "nitragin" was made by separation on a series of gelatine plates to test the purity of the commercial product. In four days numerous colonies were visible on all the plates, and no difference could be detected between them. No trace of any other form appeared after one month's cultivation.

The "nitragin" supplied to me, therefore, may be regarded as a bacteriologically pure culture.

The general characters of each of the three cultivated organisms are alike, though small differences are noticeable in aggregate cultures. They all grow readily on gelatine, or agar, containing a decoction of pea stems and leaves, asparagine, and a small percentage of sugar, and giving a very faintly acid reaction. If as much as 10 per cent. of sugar be present, growth is very slow indeed, in some cases no sign of growth appearing until seven or eight days after infection. The organisms are distinctly aërobic. On a gelatine medium they grow most rapidly at 15°—18° C., on an agar medium at 30°—35° C.

As already described, the leguminous organisms generally occur in colonies as minute rodlets about 1 to 3μ long by ·9μ broad. These measurements refer to the forms found in the common British *Papilionaceæ*, and exclude some especially large forms such as those of *Desmodium gyrans.*‡

In hanging drops of water, or of water containing inorganic salts, I have observed no growth at all. Nor did growth take place in a medium containing inorganic salts and nitrogen in the form of organic salts.§

* Schneider 'Beiträge zur Kenntniss der Rhizobien.' Ber. d. Deut. Bot. Ges., 1894.
† Frank, 'Die Pilzsymbiose der Leguminosen.' Berlin, 1890.
‡ See p. 55.
§ Uschinsky's medium, viz. :—

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Multiplication by division was observed in gelatine drop cultures of all three of the forms taken as types. The daughter cells produced are approximately equal, but there does not appear to be absolute regularity in the sequence of further divisions. Most frequently both halves divide again in a regular manner; sometimes, however, one only divides, whilst the other undergoes no further change or takes on the "bacteroid" form. Again, both daughter rods resulting from a division may become thus transformed, though rarely simultaneously, into bacteroids.

For the purpose of a close comparison of "nitragin" with organisms direct from the nodule grown on gelatine, a double series of tubes (gelatine, 10 per cent.; asparagine, ·25 per cent.; saccharose, 1 per cent.; pea extract) were infected with cultures A and B* respectively, and kept under exactly the same conditions at ordinary temperatures (15°—20° C.). From these, at intervals of 24 hours, preparations were made, and stained with carbol fuchsin.

Plate 7, fig. 10, *a* and *b*, shows that the organisms of both origins were throughout quite similar in microscopic characters, though a marked change in size had occurred in the organisms A. After 24 hours' growth on gelatine they had enlarged to nearly twice their former size, and the deeply stained bodies, already referred to in *Desmodium* organisms, were clearly visible. The maximum size was reached after, at the most, 48 hours' cultivation on gelatine, when branched forms also occurred in small numbers. From this time the size gradually diminished again, and after five days the mean length of the rodlets was the same as that of the original "nitragin" organisms. No X and Y forms were seen in the last preparations of either type. The diminution in size, as well as the disappearance of branched forms, is probably due to the breaking up of the larger individuals into smaller portions, since all intermediate sizes occur. Such a breaking up of the rods was indeed frequently observed in drop cultures when multiplication by division had quickly ceased, evidently owing to some unfavourable condition of the environment—very possibly an excessive accumulation of the products of metabolism. In the living nodules, no doubt, the removal of these products by the Leguminous plant is a powerful factor in ensuring the continuance of the activity of the organisms: and it is possible that if some other agent could be introduced into these artificial cultures to perform the functions which in nature are undertaken by the host plant, a more extended period of bacteroid formation might be secured.

In drop cultures of *Desmodium* organisms, after 8—10 days, the colonies, as a rule, consisted of very large numbers of quite small rodlets with a minority of long rods, and others of intermediate sizes, which supports the view that these rods tend to

Distilled water, 500 cub. centims.	NaCl, 2·5 grammes.
Amm. lactate, 5·0 grammes.	K_3PO_4, 2·5 ,,
Na. aspartate, 2·5 ,,	$CaCl_2$, ·01 gramme.
Na_2SO_4, 2·5 grammes.	$MgSO_4$, ·01 ,,

* See p. 56.

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break up into smaller and smaller individuals; and, indeed, this stage in their life history was recorded in 1894 by GONNERMANN,* who, when criticising FRANK'S theory of the origin of the Y's from a network of mycoplasm, says that in drop cultures these forms break up into mobile bacilli, and that their compound nature is made visible by staining. He concludes that the nodule organisms pass the winter as spores in the soil.

For the purpose of drop cultures I have found it most convenient to dilute the gelatine medium to 5 or $2\frac{1}{2}$ per cent. In such cultures the formation of very satisfactory colonies was observed in all three type forms (see p. 56); but, as would be expected, the larger one (C, p. 56) proved much more convenient for continuous study, and it was in this that the formation of bacteroids was followed in all its stages. At a temperature of 15°—20° C., in hanging drops of nutrient gelatine, the rods were seen to divide, and in from $1\frac{1}{2}$ to 3 days circular, discoid, faintly granular colonies were formed. If water did not condense to any excessive extent upon the surface of the drop, the colonies continued to enlarge, and in 12 to 14 days became domed and clearly visible to the naked eye as tiny whitish drops. From such colonies as these gelatine tubes were infected, and streak cultures grown, from which in turn seeds were inoculated. Frequently, owing to the condensation of a considerable quantity of water upon the gelatine drop, the colonies loosened after a few days, and eventually broke up. This gave excellent opportunities for observing the behaviour of individual rods in a state of vigorous growth—and it was in such cultures as these that the most satisfactory observations upon bacteroid formation were made.

To give some idea of the rate of growth it will be well to follow the formation of a typical colony such as is shown in fig 11.

The hanging drop was made at 3 P.M., when two rods (fig. 11 *a*) were fixed, lying side by side in the middle of the field. By 10 A.M. on the next day a group of 8 rods (*c*) had formed which grew to a colony measuring $11 \times 4\mu$ by 3 P.M. (*d*). The next morning at 9 o'clock the colony had increased to $15 \times 7\mu$ (*e*), and on the fourth day at 10 A.M. it measured $20 \times 15\mu$. On the fifth day at 10 A.M. it had become a circular colony of 28μ diameter (*f*). From this time it disintegrated gradually, when it was obvious that a large number of the individuals had already become transformed into X and Y shapes—the latter form largely predominating (*g*). Many of the rods were more or less curved, others were still quite straight. Several such individuals were, one after the other, fixed in the middle of the field, and carefully watched, with the result that repeated confirmations of earlier observations made under less convincing conditions were obtained, viz., that X and Y forms arise as the result of a distinct *branching* of the straight rods. In

* Gonnermann, 'Landw. Jahrb.,' vol. 23, 1894, heft 4; see also Smith, 'American Naturalist,' October, 1895.

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14 days this drop contained a few large rods, with large numbers of smaller ones and all intermediate stages. The branched forms had also disappeared.

When branching is about to take place, the rod, as a rule, becomes at first curved, and then from the point of greatest curvature on the convex side a lateral branch grows out giving the resultant Y form. In other cases both arms—here usually very short ones—appear to form simultaneously, suggesting a kind of dichotomous branching of the already swollen head of the rod. (See Plate 8, fig. 12 *a–f*.)

The earlier observations, above referred to, had been made upon individuals which after one, two, or at most four divisions, ceased to multiply; but one or more of these daughter rods assumed the V or Y shape by branching. It was obvious that these cultures were suddenly killed, possibly by some rapid rise or fall in the temperature, so that it was very satisfactory to have a further confirmation of these results in cultures which were obviously under conditions favourable to normal growth, since while some rods branched, others continued to multiply by division in the usual manner. The time required for the growth of a lateral branch averages about $1\frac{1}{2}$ to 2 hours. These observations have been made under obj. $\frac{1}{6}$, Zeiss D and E, and Zeiss Hom. Imm. $\frac{1}{12}$.

It will be seen that the above observations support the theory formulated in 1888 by Beyerinck.* This author drew his conclusions from observations made upon numerous individuals assumed to represent different stages in the process of bacteroid formation, but he did not trace the successive stages on one living rodlet, as I have done. He writes as follows:—"Die Stäbchen sind nämlich einseitig und zwar etwas neben der Mitte gebuckelt, in der Weise, dass wenn sich diese Anschwellung weiter erhebt—was factisch bisweilen geschieht—die eigenthümliche zweiarmige Gestalt der gewöhnlichen Bacteroiden erreicht wird."

It may be remembered that in my former paper it was stated that no case of X and Y formation had been observed on solid media, which it was thought was possibly due to an accumulation of the products of metabolism. From the above experiments it will now be seen that since that was written these forms have been grown in considerable numbers on solid media, indeed from the first they occurred on gelatine in colonies grown from organisms from *Desmodium*. In cultures of other forms their numbers have been, however, relatively small.

Before leaving the discussion of drop cultures, mention should be made of the occurrence of a motile stage in some cases. After the cultures are a week or more old, it sometimes happens that the rods will become slowly motile—they move about, but never travel very far from one part of the field to another; indeed, in many cases, one end of the rod seems to be stationary whilst the other end oscillates to and fro, in a manner reminding one of the movement of an *Oscillatoria* filament. This motile stage only continues a short time, at the most 12 hours.

When the drop contains rods of very varying length, it is noticeable, that when

* Beyerinck, 'Bot. Zeitung,' 1888, p. 760.

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motile, the smaller ones move much more rapidly and more jerkily than the larger. They seem also to retain their motion for a longer time, and may be seen darting in and out amongst the longer individuals after they have come to rest. The presence of cilia has not been demonstrated. These minute motile rodlets are presumably the *Schwärmer* described by FRANK* and BEYERINCK† in contra-distinction to larger non-motile rods. ZINSSER‡ describes the organism as a small actively motile bacillus, in which, however, he failed to detect either spores or cilia.

Probably the inconstancy in the motility of the organism is correlated with variations in the fluidity of the medium, though it must not be overlooked that a transient motile condition is found in other bacteria.§

In *plate cultures* of organisms obtained from tubercles of different genera of Leguminosæ no radical differences were noticed; a general description of these colonies will therefore suffice.

After two days' cultivation on nutrient gelatine at ordinary temperatures (15°—18° C.) cream-like colonies, varying in size from fine points to tiny drops, are visible to the naked eye. Under a $\frac{2}{3}$ obj. the plates show yellowish-brown colonies, circular, oval, or slightly irregular in outline, opaque, discoid with regular margins, and very faintly granular surface. After four days a distinction is obvious between the flat, pale yellowish-brown emerged colonies and the submerged ones which show a manubrium either central or on the margin, and are darker in colour and more opaque than the former. The submerged colonies frequently become confluent. No liquefaction of the gelatine had taken place after two months (figs. 13 and 14).

The colonies of the organism from nodules of *Desmodium gyrans* were paler in colour, and slightly more granular than those of the organisms of *Pisum*, *Lupinus*, and other Leguminosæ. This appearance was probably due to the larger size of the individuals forming the colonies. When about the size of a pin's head they become distinctly domed, even when viewed macroscopically (fig. 15).

In *streak cultures* slight differences in the mode of growth of the various types occur, but no corresponding microscopic differences could be seen. In describing these cultures I shall again use the letters A, B, C to denote the type organisms employed, as was explained on p. 56.

Streak cultures on nutrient gelatine at ordinary temperatures after five days:—

A. White pearly streak, shining, opaque, homogeneous, with regular margins, raised from the surface of the gelatine, which shows no sign of liquefaction (fig. 16 *a*).

B. Cream-like streak, shining, opaque, homogeneous, with regular margins, raised from the surface of the gelatine, which does not liquefy (fig. 16 *b*).

* Frank, *ibid.*, p. 23.

† Beyerinck, *ibid.*, p. 758.

‡ Zinsser, 'Prings. Jahrb.,' vol. 30.

§ *E.g.*, *B. Megatherium*, see Sturgis, 'Phil. Trans.,' B, vol. 191, 1899, p. 159.

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C. Somewhat pearly white streak, opaque, not quite homogeneous, because beginning to break up into heaped-up masses. Streak much thicker at the bottom, margin regular, raised from the surface of the gelatine, no liquefaction of the medium (fig. 16 *c*).

After 14 days the difference in consistency between A and B is much less marked, A having lost much of its pearly appearance. As growth continues this difference becomes less and less marked, until, in five weeks' time, no distinction can be made between the cultures.

C, after 14 days, showed a thicker heaped-up line down the centre of the streak, and from this, spreading over the surface, a less opaque thinner layer with wavy margins. These cultures gradually lose their pearly consistency and become cream-white in colour. No liquefaction occurred in B after two months; in A and C the gelatine had begun to soften along the sides of the streaks.

The initial differences in A and B were probably due to the change of media in the case of A, *i.e.*, from the commercial substance to pea-extract gelatine. On an *agar* medium (pea-extract, asparagine, $\frac{1}{4}$ per cent.; sugar, 1 per cent.; agar, 2 per cent.) at 15°—18° C. thin colourless or very pale cream streaks appear in two days. These grow moderately quickly, becoming more cream coloured, and spreading as a thick, slightly liquid layer over the surface of the medium, in which no liquefaction occurs (fig. 19).

At 35°—37° C. the growth on *agar* is at first more rapid (especially in tubes infected with organism A), but ceases after about 14 days. In 17 days (at 15°—18° C.) tubes C show denser, more solid portions, distributed through a cream-white layer, which entirely covers the sloping surface of the agar, and collects as a dense homogeneous mass at the bottom of the tube. In A and B this marked change in consistency has not occurred, though tiny granules are to be seen scattered in the semi-translucent layer which covers the surface.

Microscopic preparations from agar cultures show no differences except in the larger size of the individuals from C.

Stab cultures at 14°—18° C. in three days show small, whitish, cream-like, homogeneous patches on the surface of the gelatine, and a very faint line of growth down the tunnel.

At 20°—23° C. this amount of growth takes place in 24 hours. These patches gradually increase in diameter and thickness, becoming more or less domed, whilst down the tunnel are seen a succession of tiny drop-like colonies, diminishing in size and number the greater their distance from the surface. In 14 days (at 14°—18° C., or 20°—23° C.) the growth nearly covers the surface, and causes a slight depression of the gelatine from increased growth in thickness. The submerged colonies are still quite small, and almost entirely confined to the edges of the tunnel (fig. 17). No liquefaction of the gelatine occurs. As in the streak cultures, the surface growth in A was at first more pearly in consistency than in B and C.

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In a *liquid medium*, consisting of pure pea extract, growth is comparatively rapid. In a few days at ordinary temperatures a thin film forms on the surface, and if the tubes are kept quite still the rest of the liquid remains quite clear. On shaking, a cream-white deposit settles at the bottom.

This power of growth in pea extract was made use of to test by means of a *fermentation tube*, the aërobism of the organism, *i.e.*, to confirm the evidence of the stab cultures. After six weeks there was no sign of the formation of gas in the upper arm, whilst the free surface of the liquid was covered by a coherent film, and a similar whitish skin was seen floating in the liquid near the surface. Microscopic examination showed this to be a zoogloea-like growth, containing immense numbers of minute rodlets, and scattered amongst them bright spore-like bodies. Whether these bodies are in reality spores or merely rodlets lying above the surface has not yet been determined, but this question is now being further investigated.

On a medium consisting of silica jelly, and a mixture of inorganic salts,* with nitrogen in the form of ammonium sulphate, and a small percentage of sugar, the organisms from *Desmodium* and *Pisum* grew with comparative ease at the ordinary temperatures of the laboratory. Owing to the clear transparent nature of the medium, very minute colonies are extremely difficult to detect in plate cultures, but after about 10 days, bluish, more opaque areas are discernible, and these, under a $\frac{2}{3}$ obj., appear as pale yellowish very faintly granular colonies, quite comparable to those grown upon gelatine media, except that the colonies seem to spread over the surface of the jelly rather than become heaped up, giving rise to the domed appearance common in cultures on gelatine. If the plates are thickly sown with the organisms, the bluish opaque appearance spreads equally over the surface, making it impossible to trace the separate colonies.

In hanging drops of silica jelly, cultures of the organisms from *Desmodium* show that at a temperature of 17°—18°, colonies measuring 30μ in diameter will grow in about seven days.

By means of the Diphenylamine† reaction for nitrates some attempt has been made to determine whether these organisms have the power to convert ammonium salts into nitrites or nitrates. The results thus far obtained seem to show that no reaction of this kind has taken place, but much more work is required in this direction before a definite conclusion can be arrived at. These experiments are now being continued and extended to include an investigation of the effect of the presence of nitrates in the culture medium upon the action of the organisms.

* The actual salts employed in these cultures were :—

$(NH_4)_2SO_4$, ·4 gramme.	$CaCl_2$, trace.
$MgSO_4$, ·05 ,,	Na_2CO_3 = ·70 gramme.
K_3PO_4, ·10 ,,	Dist. H_2O = 100 cub. centims.
Saccharose = 1 gramme.	

† See Strasburger's 'Practicum.'

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As already stated, no growth of the nodule organisms took place in pure water, or in USCHINSKY'S medium. Negative results only were also obtained with a medium consisting of vejos (a preparation of yeast), 1 per cent.; saccharose, 10 per cent.; gelatine, 10 per cent.; and distilled water.

On *Broth agar* no growth occurred at 20° C., or at 25°—30° C.

On *Broth gelatine* at 20°, growth was extremely slow; the characters of the streaks agreed with those grown on pea extract gelatine, but the cultures died after about 14 days. BEYERINCK ('Bot. Zeit.,' 1888) stated that the "bacteria" grow very slowly on "Fleischwasserpeptongelatine," but rapidly on gelatine media containing a decoction of pea stems or leaves.

On *potato*, temperature 14°—18° C.

A. In three days a glistening dew-like streak, which increases but slowly. After 14 days cream-white watery patches, and a watery liquid draining over the surface of the potato.

B. After three days a cream watery streak, which gradually spreads over the surface, forming an irregular watery patch, with a paler coloured liquid of drainage.

C. Cream coloured growth as above, but less watery in consistency. The dry upper part of the patch tends to break into little heaps, such as occurred on gelatine cultures (fig. 18).

Potato tubes at 20°—25° C. showed no differences from the above.

In *milk*, temperature 15° C. No change after three weeks, either in consistency or in reaction to litmus.

Having followed the behaviour of these interesting organisms through the various stages of their life history, we come to a consideration of their systematic position in the vegetable kingdom.

It may now be assumed as proved beyond question that we are dealing with independent organisms which have become very specially adapted for life within the cells of Leguminous plants—a specialisation which varies apparently with different hosts. In the majority of cases, at any rate amongst the Papilionaceæ, a filamentous mode of growth from cell to cell has been adopted, whilst in other cases the formation of these filaments seems to have been wholly or partially dispensed with.

It is obvious from the characters here described that the organisms show many striking points of agreement with Schizomycetes—especially in their size, chemical reactions, behaviour when cultivated on artificial media outside the plant, their method of multiplication, and the nature of the tube formed within the cells of the host (viz., a filamentous zoogloea-like structure, consisting of numbers of straight rods lying parallel to the longer axis of the tube, and embedded in a colourless matrix, in all probability arising from the swollen cell walls of the embedded rodlets).

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As was pointed out, however, by MARSHALL WARD,* in 1898, "minuteness, staining reactions, rapid growth, and the characters obtained in plate-cultures," are not in themselves sufficient to prove that these organisms must be true bacteria. This quotation is especially significant when we consider the occurrence of distinct *branching*, either lateral or apical, by which the bacteroids are formed. It is true that similar branching processes have been described by various writers in organisms which they describe as true bacteria, *e.g.*, *Bacterium tuberculosis* and *B. diphtheriæ*. Besides these examples, SEWERIN† has recently described a rod-like denitrifying bacterium, which forms branched individuals which he compares to the bacteroids of Leguminosæ. In the same journal an example of branching "Nitrosobakterien" is described by RULLMANN.‡ On agar, blood-serum, broth, &c., this observer obtained straight rodlets only, but in streak cultures on gelatine microscopic examination showed the presence of shorter or longer "Fadenverbände." These cultures, when transferred to Nitritagar, or liquid inorganic media, formed in a few days simple branched threads, which, after being again transferred to ordinary media (*e.g.*, broth agar), again developed short rods only.

Apparently neither of the above authors has followed this branching process by direct observation in drop cultures, but they have made their figures from preparations taken from plate cultures.

RULLMANN failed to find the formation of any mould-like fungus such as STUTZER and HARTLEB§ had described. In March, 1899, Dr. ALFRED MOËLLER gave a detailed account of the characters and behaviour of another such organism in a paper entitled "Ein neuer säure- und alkohl-fester Bacillus aus der Tuberkelbacillengruppe, welcher echte Verzweigungsformen bildet." ('Cent. f. Bakteriol. u. Paras.,' March, 1899.) In this form the branched individuals have two long thread-like branches, or shorter branches with swollen ends.

The formation of small branches from rods still embedded in the gelatinous sheath seems to occur also in *Phragmidiothrix*.‖ It is, however, open to question whether these various authors are justified in classifying these organisms with the true bacteria, or whether this property of branching should not be regarded as a strictly non-bacterial character. If we retain the generally accepted definition of a Schizomycete we are forced to class the tubercle organisms along with such a type as MARSHALL WARD described in 1895 as a "false bacterium," and to regard them as possibly very minute oidial forms of a much reduced and in many ways highly

* Marshall Ward, "Some Thames Bacteria," 'Annals of Bot.,' vol. 12, 1898.

† Sewerin, "Zur Frage über die Zersetzung von salpetersauren Salzen durch Bakterien," 'Cent. f. Bakteriol. u. Paras.,' Abth. 2, vol. 3, 1897.

‡ Rullmann, "Uber ein Nitrosobakterium mit neuen Wuchsformen," 'Cent. f. Bakteriol. u. Paras.,' vol. 3, 1897.

§ Stutzer and Hartleb, "Der Salpeterpilz," 'Cent. f. Bakteriol. u. Paras.,' Abth. 2, vol. 3, 1897.

‖ Engler and Prantl, 'Schizomycetes,' 129, p. 3.

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specialised fungus, to which, with the data at our disposal, it is impossible to assign any exact systematic position. However, though it is now impossible to accept MARSHALL WARD'S previous theory that the organism of the Leguminous nodules might be classified with the Ustilagineæ, *because* of the resemblance of the filaments within the tubercle cells to the irregular, much swollen hyphæ so common in that group of fungi—a resemblance which has now been shown to be purely superficial—it must at least be confessed that it is impossible, as yet, to assign them to any other group, if not to the Schizomycetes.

On the other hand, if branched forms *are* to be included amongst true bacteria, in accordance with the views of the afore-mentioned investigators, the organisms [which it is convenient to continue to describe as *Rhizobium*] could certainly claim to be classified with them, and in this case the branched individuals would probably be regarded as involution forms. As, however, such a classification would involve a new definition of bacteria, it seems advisable to adhere to the view that the organisms are in reality either very primitive or very degenerate fungi.

In addition to the experiments described above, many others have been conducted in order to study the behaviour of the nodule organisms in their relation to the host plants. A full account of these experiments will not be given here, but the general conclusions to which this branch of the work has led are briefly as follow :—

i. That there is only one organism capable of forming nodules upon the roots of Leguminous plants, and that the difficulty, often experienced in securing successful inoculations of one genus, with organisms derived from nodules upon the roots of some other genus, not nearly allied, is probably due to special physiological adaptations to each particular host, giving rise to numerous race varieties, such as have been shown to exist in the rust fungi.

ii. That in the more practical problem of the value of applications of pure cultures of the organisms of the nodules as a fertiliser for Leguminous crops, the effect produced is directly controlled by the physical and biological conditions existing in the soil at any given time; consequently, in considering the importance of a supply of organisms to the soil, allowance must be made for these varying conditions and also for the action of the host plant in removing the products of metabolism from the field of action of the nodule organisms.

In conclusion, I desire to record my renewed thanks to Professor MARSHALL WARD, who, in addition to allowing me to continue this work in his laboratory, has given me, in every direction, his constant assistance and advice.

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Explanation of Plates.

PLATE 7.

Fig. 1. *Phaseolus multiflorus.* Section of tubercle, from material hardened in Abs. Alc.

a = crystal layer,
b = large empty cortical cells,
c = smaller cortical cells with starch grains,
d = bacteroid tissue, with starch grains.

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b. In Eau de Javelle. Obj. $\frac{1}{6}$.

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a. Fresh material, very young tubercle.
b. Fresh material after digestion for four hours in gastric juice.
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Fig. 10. Individuals from parallel cultures on nutrient gelatine of organisms from A. "Nitragin"; B. *Pisum sativum* tubercles, stained with carbol fuchsin. α. 24 hours', β. 48 hours', γ. 72 hours', δ. 5 days', ϵ. 7 weeks' cultivation on gelatine.

Fig. 10*c*. Commercial "Nitragin," stained in carbol fuchsin.

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Fig. 11. Stages in growth of colony of *Desmodium gyrans* in hanging drop of nutrient gelatine. Obj. $\frac{1}{6}$.

a. May 2, 3 P.M. T = 20°.
b. ,, 2, 4 P.M. T = 20°.
c. ,, 3, 10 A.M. T = 17·5°.
d. ,, 3, 3 P.M. T = 19°, diam. = 11μ.
e. ,, 4, 9 A.M. T = 14°, ,, = 15μ.
f. ,, 6, 10 A.M. T = 15°.
g. ,, 8, 10 A.M. T = 15°.

PLATE 8.

Fig. 12. Stages in formation of branched bacteroids, observed in hanging drops of nutrient gelatine. Organisms from pure cultures from *Desmodium gyrans*. *a*, *b*, *e*, *f*, obj. $\frac{1}{6}$; *c*, *d*, Hom. Imm. $\frac{1}{12}$ × oc. 2.

Fig. 13. A. Plate cultures on gelatine. Five days, ordinary temperature. Obj. $\frac{2}{3}$.

α = organism from "Nitragin."
β = ,, ,, *Pisum* tubercles.

Fig. 13. B. Colonies of organism from *Pisum sativum* on nutrient gelatine. Four days, ordinary temperature. Obj. $\frac{2}{3}$ camera.

Fig. 14. Plate cultures of *Lupinus* organism. Four days, ordinary temperature. Obj. $\frac{2}{3}$. Colony *a*, drawn with camera.

Fig. 15. Colonies of *Desmodium* organisms in hanging drop. Seven days. T = 20°—22° C. Obj. $\frac{2}{3}$. On seventh day colonies broke up, many individuals motile—motion ceased on eighth day. Individuals with obj. $\frac{1}{6}$.

Fig. 16. Streak cultures. Gelatine, six weeks, ordinary temperature.

Fig. 17. Stab cultures. Gelatine, 14 days. T = 14°—18°.

Fig. 18. Potato culture. Five days. T = 15°.

Fig. 19. Streak culture. Agar, five days. T = 14°—18°.

Figs. 16—19. A, B, C, see text.

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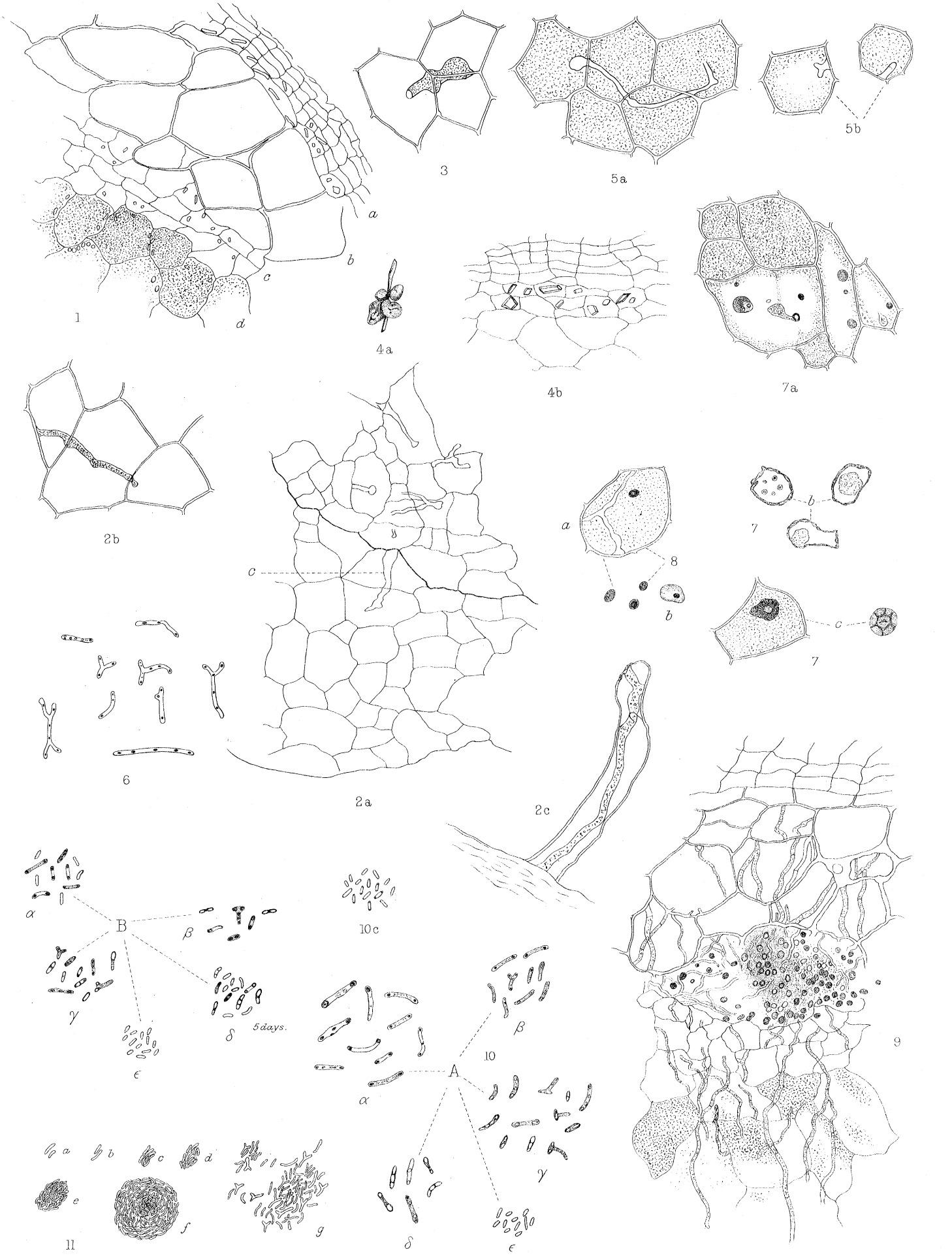

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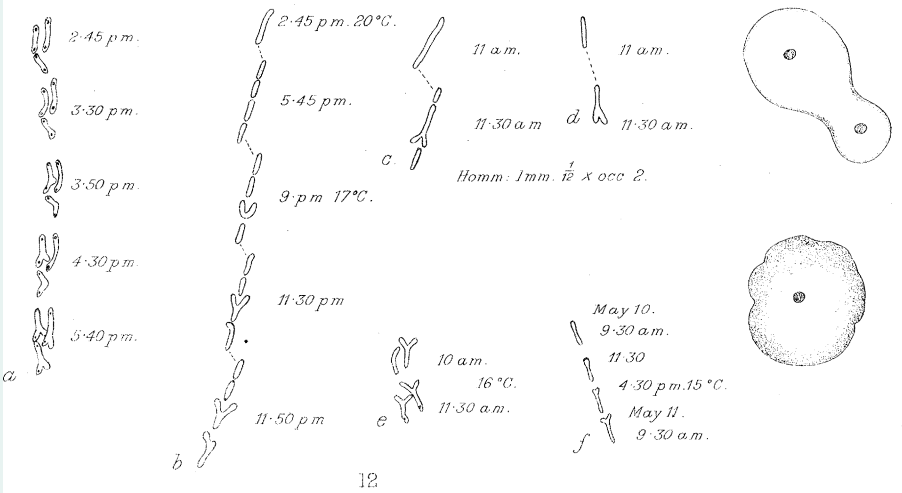

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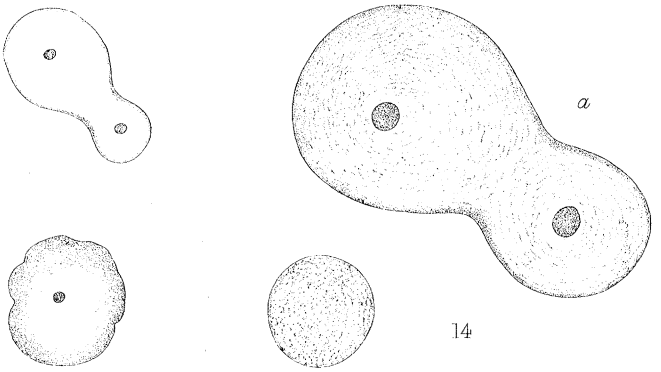

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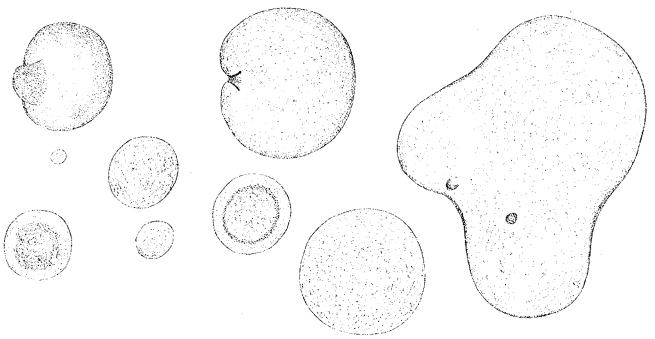

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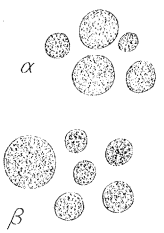

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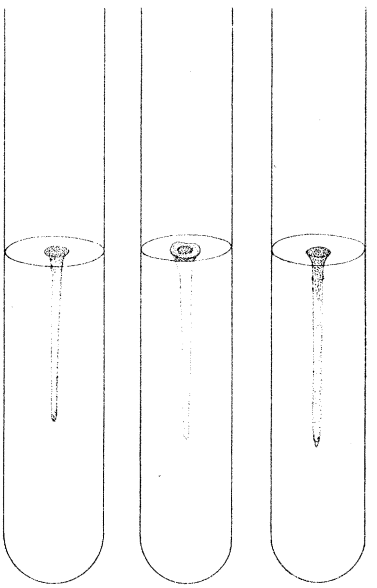

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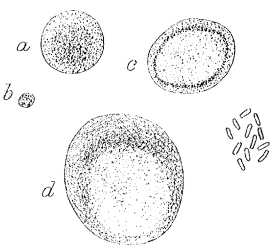

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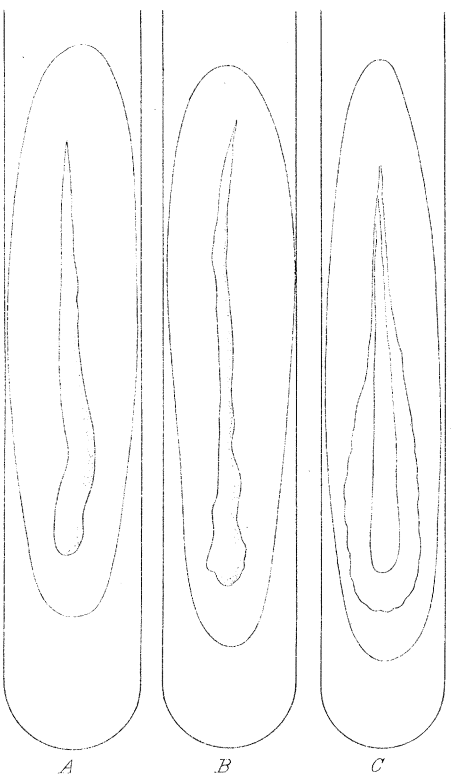

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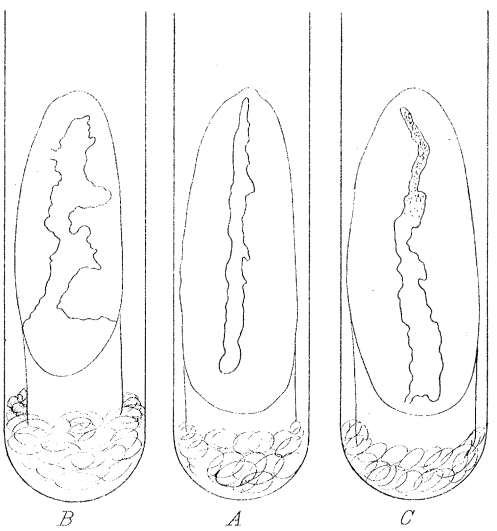

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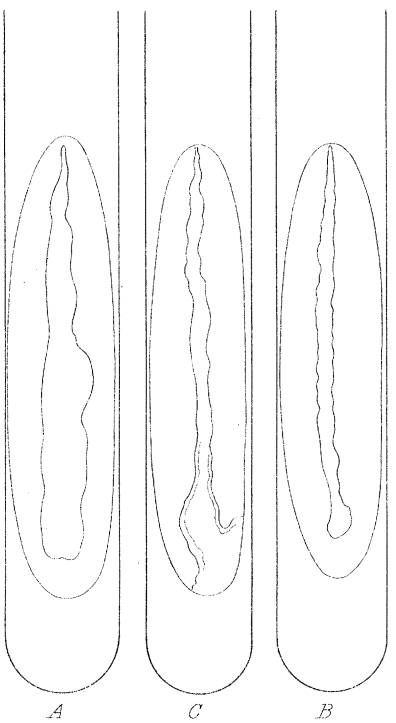

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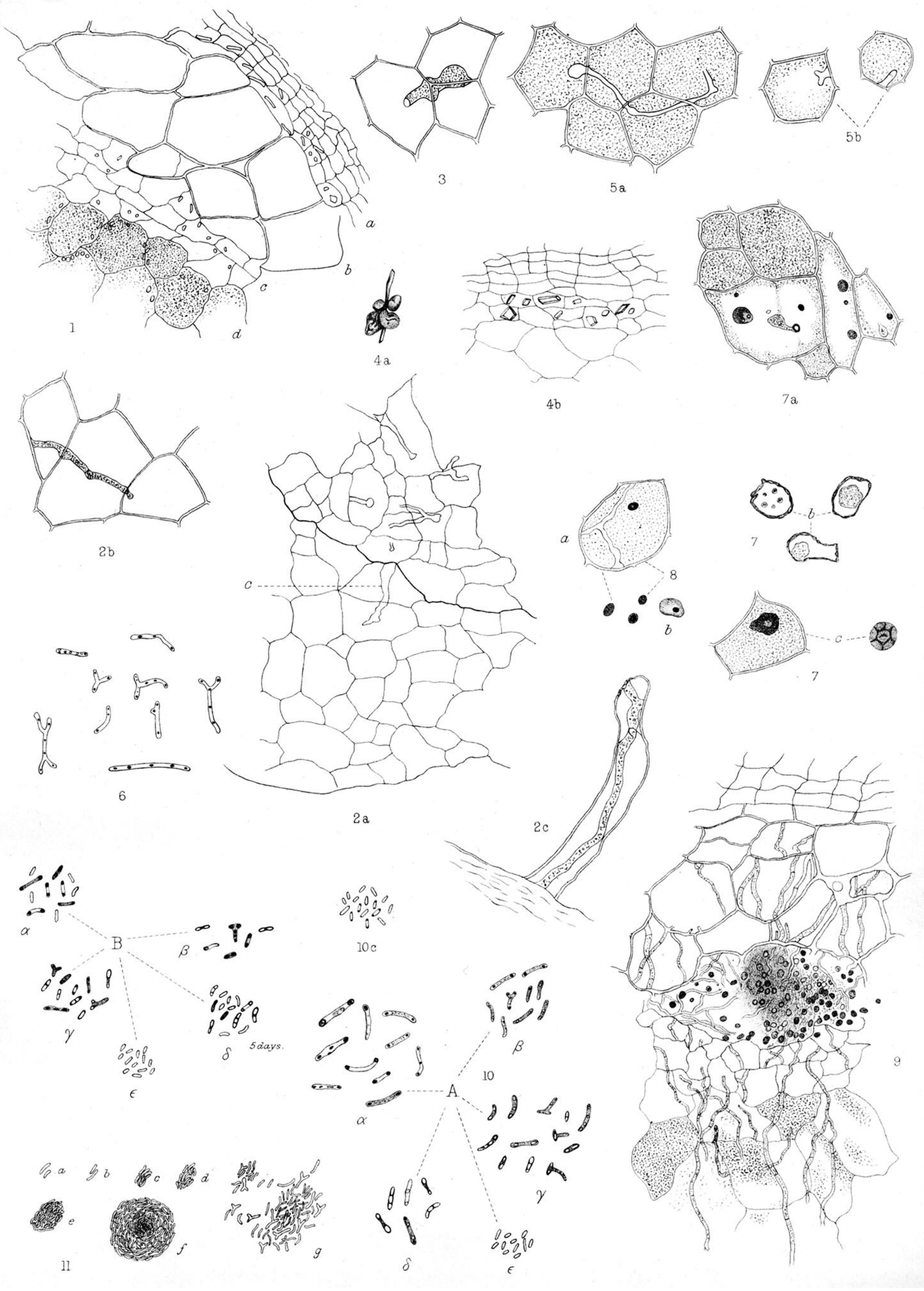

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a = crystal layer,
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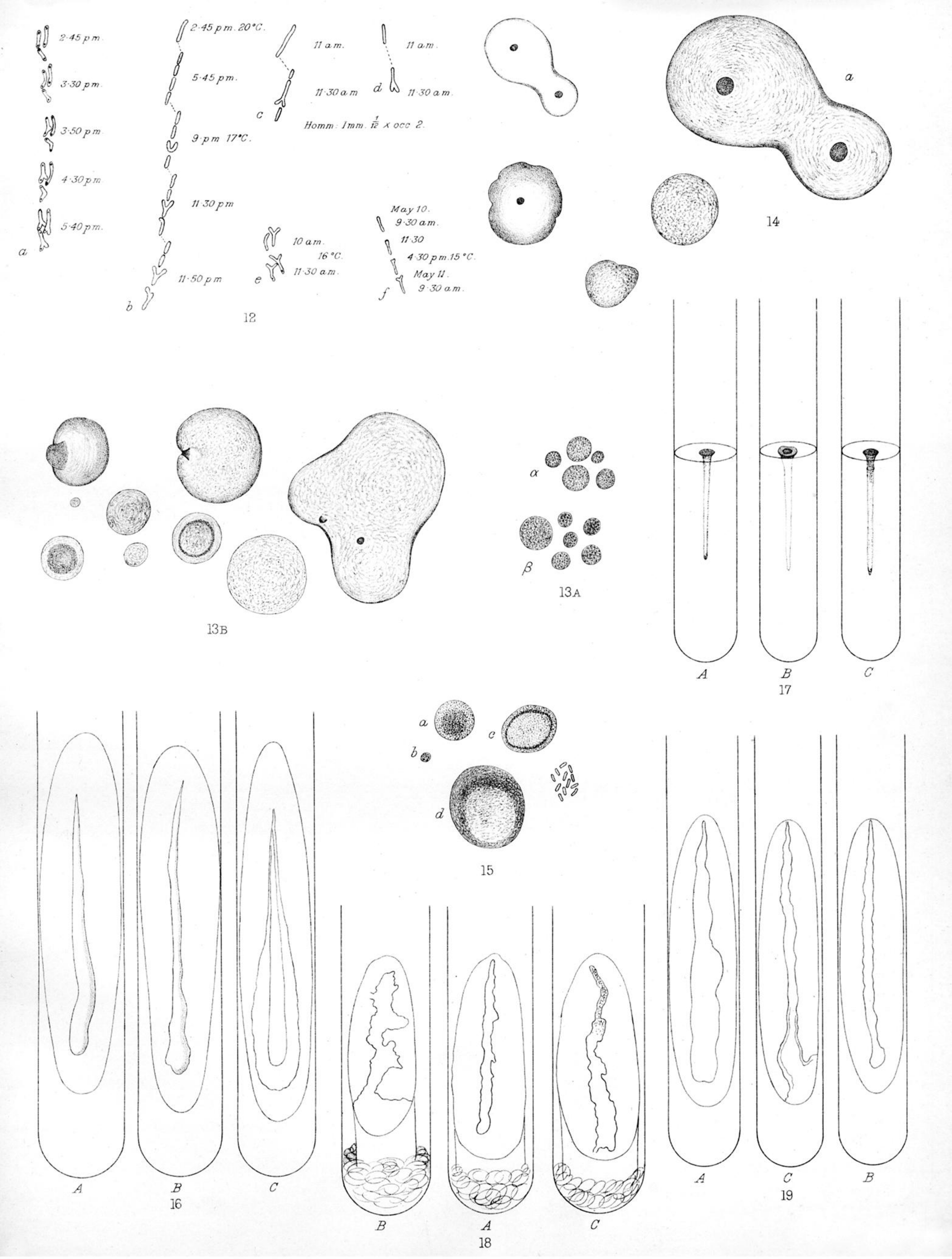

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