NOTES ON SOME METHODS FOR THE EXAMINATION OF SOIL PROTOZOA.

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(With Plates II and III.)

I. Introduction.

During the last three or four years, the protozoa of the soil have been the object of a considerable degree of interest, and investigations into their occurrence and importance have been made by workers here and elsewhere. The aim of the present paper is to indicate what we know of the life of the protozoa in the soil, and to furnish descriptions of certain methods which have been found useful in work on this subject.

When attention was directed to the protozoan inhabitants of soils, it was quickly found that protozoa in great numbers and variety were easily obtained by inoculating soil into a suitable medium. Setting out from this fact, investigators have frequently been led to describe the forms found in cultures from a soil as the fauna of the soil, thus making the more or less tacit assumption that every form found in cultures from a soil was leading a trophic life in the soil at the moment when the culture was made.

Unfortunately, what may be termed the "cultural fauna" of a soil is of relatively little value in forming an idea of the protozoa actually living in the soil. On the one hand the cultural fauna consists in part of protozoa which were present in the soil only as cysts; on the other, some forms relatively important in the soil, notably the thecamoebae, appear very late, or not at all, in cultures on the ordinary media.

The protozoa in any soil may occur in the active (trophic) state, or enclosed in cysts. We propose to call the former the "active fauna," and the latter the "cyst fauna"; and we would emphasize the necessity of keeping these two classes clearly distinguished.

Under the varying conditions which obtain in a soil there must be continually changing relations between these two faunas, but at any moment only the members of the active fauna of that period can exert any effect on the soil. To guard against possible misunderstanding, it may be well to state that it is very improbable that the line between the active and the cyst fauna of a soil is one between species and species. There is little doubt that under most conditions a species represented in the active fauna will also be represented in the contemporaneous cyst fauna.

Since the cultural method fails to distinguish between the above two categories, and even leaves unsettled the question of whether an active fauna is present at all, recourse has been made to other methods of examination, which are fully described in the next section. By their aid it has been completely established that an active fauna does exist in a variety of soils ranging from the unmanured plot on Broadbalk field at Rothamsted to sewage-farm soil, leaf mould, and soil from a cucumber border. Some of the results obtained by the examination of these soils will be found in section III.

As regards the forms found, it is improbable that many are generically new; most of them seem to have been described by the older workers on protozoa. Of recent years a very large amount of the literature on protozoa, including the more recent textbooks on protozoology, have been devoted almost exclusively to parasitic forms, so that a worker on soil forms must refer back to the excellent papers of the older authors. References to some of these works will be found in the literature list.

Before the effect of protozoa on the soil can be adequately discussed, it is necessary to gather information about the life led by the protozoan fauna. In particular the effect exercised on the active fauna by the water content, the density of the bacterial flora, the temperature, etc., must be investigated.

Now whilst soil temperatures can readily be determined with sufficient accuracy, the evaluation of the other two factors presents considerable, and in part unsolved, difficulties, which arise largely from the heterogeneity of the soil.

Thus the present method for determining water content deals usually with samples taken to a depth of nine inches. It is clear, however, that if in dry weather a crust has been formed on the surface of the soil, the protozoa may be active at a lower level which might still have a relatively high water content, so that the figure obtained

for the water content of the whole soil would give no indication of the actual minimum quantity of water in which protozoa could remain active. This difficulty would be felt even if the soil were a homogeneous mixture; but unfortunately this is far from being the case, and it is certain that in a relatively dry soil the fragments of manure and of decaying plant roots would hold a far larger amount of water than is indicated by an ordinary determination of the water content, so that if, for example, one kilogramme of soil contained 950 grammes of soil particles and 50 grammes of decaying organic matter on which protozoa were flourishing, the figure given by the estimation of the amount of water present in the soil would give no indication as to the actual amount of water in the space where these protozoa were leading an active life.

Another important question is the difference between a coarse grained and a fine grained soil with an equal percentage of water. It would seem quite possible that an active protozoan fauna would be found in the large water spaces in the former at a time when the latter would exhibit no free forms.

Further, when conditions in different soils are to be compared, it is preferable from the biological point of view to express water content as percentage by true volume rather than as percentage by weight.

As regards bacterial counts, all the points which have been urged in connection with the heterogeneous nature of the soil carry here even more weight. In the first place, it is probable that the bacteria are concentrated in groups round decaying organic matter, and it has been found in the examination of fresh films from the soil that the bacteria are present as colonies, and are not scattered singly like currants through a cake. It is obvious that the bacterial count must very largely depend on the degree to which these colonies are broken up during the process of dilution. It is well known, also, that the numbers obtained are dependent upon the medium adopted, and on the conditions of culture.

When the heterogeneity of the soil is taken into consideration, it would seem impossible to hope for an accurate method for the estimation of the active protozoa present in a soil. It is, however, possible that practicable approximate methods may be devised, but before they can be considered satisfactory as a basis for extended experiments, it is very necessary that the range of their probable error should be known.

Up to the present, the only method proposed for the enumeration of the soil fauna is the dilution procedure described by Rahn (11). The work of Cunningham and Löhnis (3) on the thermal death-point of the

active, and of the cyst fauna, has been used by Cunningham (4) as the basis of a method of determining the active fauna. He estimates the total fauna of a soil, and, in a second sample, the cyst fauna; the difference between the results is taken as a measure of the active fauna.

Unfortunately, the results obtained by a dilution method will almost certainly be vitiated by the incompleteness of the cultural fauna. As has already been pointed out, present cultural methods fail to indicate, or indicate very late indeed, an important class of soil protozoa, the thecamoebae. Again, the manipulative errors of the successive dilutions, together with the serious risk that shaking will not result in an even distribution of the protozoa through the suspension, introduce a cumulative series of inaccuracies into a troublesome and complicated method. Finally, in common with any other numerical method, it encounters the weighty difficulty of the heterogeneous nature of the soil.

On the whole, therefore, it seems to us that this type of method will be liable to introduce a specious appearance of accuracy into a subject which bristles with difficulties[1].

A very rough, but still valuable, idea of the relative abundance of active protozoa in soils is given, however, by the richness of the fresh fixed films obtained as described on p. 112. In comparing different types of soil only the most striking differences can be regarded as significant, but in considering the variations in the active fauna of one particular soil under changing conditions of temperature, moisture, etc., it is probable that the index of richness of the films obtained will prove a sound basis for general conclusions, although no hope can be entertained of reaching numerical results by this method.

II. METHODS.

It is exceedingly difficult, in an examination of any ordinary soil, to get an adequate idea as to the abundance and nature of the active fauna, and for this reason we have thought it well to describe some of the methods we have found helpful in this work.

By far the simplest method of fixing and staining soil protozoa, whether in cultures or on fresh films from the soil, is by means of coverslip films. We have usually stored the films in small corked tubes of height $1\frac{1}{2}''$ and diameter $1\frac{1}{4}''$, and these tubes have been found very convenient for purposes of fixation and staining.

[1] This criticism does not apply to Cunningham's paper, where it is recognised that precise numbers cannot be given.

If ordinary coverslips are used for this work it is often difficult to decide which side of the coverslip the film is on, particularly if the films have been stored for some time in 70% alcohol. For this reason the coverslips described by one of us in a previous paper ("A note on the protozoa, etc., from sick soils," *Roy. Soc. Proc.*, Vol. 85, 1912, p. 395) will be found very useful. These are oblong coverslips of which one angle has been cut off, and they can be procured from Messrs Frazer, of Edinburgh, Messrs Zeiss, or Messrs Angus. It is obvious that no mistake can arise if it is arranged that the film is always on the lower surface of the coverslip when the long sides point away from the worker and the cut corner separates the right long side from the distant short side.

The methods for the examination of soil protozoa can be divided roughly into three categories, (1) methods for the detection and examination of the active fauna in life, (2) methods for the examination of the active fauna on fresh fixed films of a soil, (3) cultural methods.

(1) *Detection of active fauna in life.* Up to the present we have found no reasonably successful method for the collection and examination of the active fauna of a soil in a living state. Any method which depends upon the addition of water to the soil must admit of very rapid execution, otherwise there is the danger of protective cysts present in the soil opening, and thus giving a false impression as to the constitution of the active fauna. This danger is probably a very real one in the case of the small flagellates, and especially the resting forms of some green algae, in the case of which a few minutes' immersion in water may make the difference between a resting and an active form. Another difficulty seems to be to obtain films adequately rich in comparison with the films got by fixing the fresh soil by the methods described below, and in this respect it is found that methods which give fair results with one type of soil may break down completely with another.

All the methods we have used with any success up to the present depend upon the possibility of collecting and retaining some of the protozoa on a surface film. They all seem uniformly bad, and the only consolation in their use is that the other methods we have tried, including the use of the centrifuge, have up to the present given worse results.

With some rather dry, clay soils, at Rothamsted, fair results were obtained by crumbling a soil into a dish of water, and removing the surface film for the purpose of examination either by floating coverslips on it, or by means of thin wire formed into a circular loop of about $\frac{1}{2}''$ diameter.

In the rather coarse, sandy soils, at Abergavenny, fair results were obtained in the case of small flagellates, thecamoebae, and small amoebae, by allowing a stream of water to flow from the tap on to a quantity of the soil in the dish, until the soil was just covered, and then examining the surface films collected as above.

In the case of rather dry, clayey soils at Rothamsted, fairly large amoebae, with a thick pellicle, were obtained by the bubbling process described below.

A glass tube of internal diameter $1\frac{1}{2}''$ and length about $2'$ is provided with a singly-bored rubber cork at the lower end. Through this passes a glass tube drawn out to a jet. Connection is made with some form of airblast, so that a stream of air can be blown through the jet. The tube is clamped upright and a newly made suspension in water of the soil to be examined is poured in until the water level nearly reaches the top. Three hooks (conveniently made of bent strips of "tin") are hung round the rim of the tube in such a way as to furnish a support for the coverslip. The coverslip is placed in position about $\frac{1}{4}''$ above the water level. Air is now blown through the jet so as to produce a stream of fairly small bubbles rising through the suspension and breaking on the lower surface of the coverslip. The water level can be adjusted within small limits by regulating the air-flow.

After about 30 seconds the air-stream is stopped, and the coverslip lifted off and examined under the microscope. It is frequently of advantage to place a thin sheet of agar jelly on the lower side of the slip before commencing the bubbling, as the protozoa adhere more readily to this substance than to the glass. If this be done, the coverslip is placed for examination, agar side up, on a slide, and another slip is dropped on to the agar surface.

By this method there were obtained from a Rothamsted soil certain amoebae whose presence in the active fauna the other methods had failed to reveal.

Very fair stained preparations of any of the animals obtained by one of the above methods can be made by the ordinary processes in use in the zoological laboratories for making preparations under a coverslip. The easiest method is probably to fix by running a drop of Fleming's solution under the coverslip for a few seconds, then washing through with water, followed by picro-carmine five to ten minutes (this renders the process of staining after the Fleming fixation much easier), washing through again with water, staining with alum carmine for half an hour, washing through again with water, then alcohol up to

absolute, followed by terpeneol and balsam. Terpeneol will be found very convenient for this purpose as it clears from a much lower percentage of alcohol than oil of cloves or oil of cedar.

(2) *Examination of active fauna in fresh fixed films.* In the preparation of fresh films from soil to which a fixative has been added we once again depend upon the surface films. For some obscure reason not yet understood, if certain fixatives are added to a quantity of soil a surface film is formed which contains an unknown but probably variable proportion of the active fauna of the soil, cysts, diatoms, moulds, algae, and bacteria. In the production of this result, it is certain that the contained air in the soils exercises a favourable influence in bringing the animals to the surface film, and really good results cannot be expected by this method from a soil which is absolutely water logged. Of the fixatives we have tried up to the present, picric alcohol (*i.e.*, 50 % saturated solution picric acid in water, plus 50 % rectified spirit), and corrosive alcohol (*i.e.*, 50 % saturated solution corrosive sublimate in water, plus 50 % rectified spirit) have given us the best results.

The best method appears to be to place the soil in a porcelain dish, and pour enough of the fixative through a funnel to the bottom of the soil layer until the soil is just covered. The film so obtained can be taken off on coverslips floated on the surface of the liquid.

Of these two fixatives picric alcohol appears to give richer and more abundant films, particularly as regards small organisms, in sandy soils, whereas corrosive alcohol appears to work better on clay soils, and is more efficient in collecting thick-pellicled amoebae.

The efficiency of the film formation is frequently increased by slightly shaking the dish immediately after the addition of the fixative. The following is a good method for staining and mounting the film so obtained.

Picric Films	
Corrosive—2 minutes	Corrosive Films
70 % alcohol plus a few drops of I_2 in KI	5 minutes
Distilled water	5 minutes
Haemalum	5 minutes
Tap water	Till blue
70 % alcohol	5 minutes
Eosin in absolute alcohol	3 minutes
Absolute alcohol I	1 minute
Absolute alcohol II	1 minute
Xylol I	2 minutes
Xylol II	1 minute

The over-staining in eosin will be found of great assistance in searching rather poor films for active forms, especially in the case of flagellates.

These methods have been found to give very fair results as regards small flagellates, small amoebae, and thecamoebae. Up to the present we have only very rarely found large flagellates and ciliates, but to this question we return in a later part of the paper.

(3) *Cultural Methods.* It would we feel be premature at present to attempt a formal list of the culture media on which soil protozoa flourish. In all cases of cultures of soil protozoa, so far as we are aware, as Vahlkampf clearly insisted in his paper on the biology, etc., of *Amoeba limax*, the protozoa feed upon the bacteria of the culture, and hence almost any culture media on which soil bacteria flourish will probably support a large number of protozoa.

Therefore in those cases in which the expression "pure animal culture" is used we only wish to indicate that the culture contained only one form of protozoon, though of course it contained large numbers of bacteria. It may of course be possible in the future to obtain cultures of some saprozoic protozoa free from bacteria, and in certain cases we have found indications that certain amoebae show a distinct preference for certain culture media, though here, again, this effect may be a secondary one due to the encouragement of a certain type of bacteria.

Up to the present we have mainly used solid media for our cultures, as we find that they are far more convenient for isolating any given form. We used two types of culture media, one an ordinary agar made up of 1000 c.c. meat extract and 15 grm. of agar; but we have found a culture medium of Friedberger and Reiter described in Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*, vol. I, gives very good results for most soil protozoa; it consists of a horse-dung agar made up of three lumps of horse dung and 500 c.c. of water, this mixture is boiled for one and a half hours, then filtered through cloth, and finally about 8 grm. of agar is added. In many cases where it is used to get a very strong growth of protozoa it is advisable to add a small amount of water or dilute albumen to the culture plates to about a depth of 2 mm. This addition of water seems to obviate the vacuolated appearance which some workers have noted as characteristic of culture amoebae on plates.

The stock cultures are made up by adding a little soil directly to the plates. If these stock cultures are examined from time to time it will be found that in any given culture there is a more or less definite

succession of animal forms. By selecting the time and method of culture it will probably be found possible to get pure animal cultures of any of these forms.

The question how far the dominant active forms in a soil are represented in the cultures depends largely, firstly, on the condition of the soil, and, secondly, on the condition of the cultures. We return to this question below, but it may be pointed out here that in the case of most soils the conditions on the cultures mentioned above seem rather rich for some members of the active fauna, with the result that these forms appear very late in the cultures. A certain check can be obtained on these results by means of cultures in which a small amount of water is added to the soil.

III. Some Results.

So far the soils which have been examined by the methods described above are relatively few in number, but of varied types.

In three cases, where the soil was taken respectively from a cucumber border, from a fertile garden plot, and from the site of an old manure heap, the soils were probably far richer in farmyard manure than even the most richly manured fields; and correlated with this there was a higher capacity for holding water. As would be expected, all the indications were that these soils supported a far denser protozoan fauna than was found in the poorer soils examined.

In the cucumber border, the dominant protozoa were amoebae—one of the limax type (*Vahlkampfia soli* n. sp.) and one of the lamellipodian type (*A. cucumis* n. sp.). Thecamoebae, notably a species of Euglypha and a Trinema, could be detected in live films, though they were fairly rare on the fixed films, and were probably the next most numerous protozoa. Flagellates and ciliates were present only in small numbers.

The garden soil, and the soil taken from the site of an old manure heap (both at Abergavenny), contained many amoebae, but a great preponderance of thecamoeban forms. The similarity between their fauna is probably not accidental; it is very likely that the dominance of the thecamoebae in the garden soil was a persistence of the dominance of these protozoa in the manure heap with which the garden had been enriched.

In culture these thecamoebae did not appear in considerable numbers until two or three weeks at least after the culture had been started.

From a consideration of cultural results alone, it would have been imagined that flagellates, both large and small, and amoebae had been the dominant forms.

In a not very rich soil from a cauliflower seedling bed the picric acid method gave a considerable variety of protozoa, no one form of which appeared to have become predominant. It was fairly clear that the density of the fauna was relatively low. It is interesting to observe that this rather poor soil contained many more species than *e.g.* the soil from the cucumber border, though the latter had many more individuals. This suggests an interesting analogy with results obtained on the grass plots at Rothamsted, where the untreated (poor) plot gives a large number of species, whereas on plots which have received a large quantity of manure for many years the number of species is considerably curtailed. A similar phenomenon is shown in rich infusions, in which as a rule at any given moment one or other protozoon has got the upper hand, whilst in ordinary fresh-water pools the fauna is far richer in number of species, but far poorer in number of individuals.

The three Rothamsted field soils (Broadbalk dunged plot, Broadbalk unmanured plot, and a fallow plot on Agdell) also contained protozoa very sparsely, small amoebae being the most numerous, though thecamoebae were also represented. Flagellates were very rare, and ciliates were not found at all in the active state.

In culture, amoebae of the two types found in the cucumber border were prominent, together with a great variety of flagellates and many ciliates. The amoebae on the fresh films seem to be of a type different from either the limax or the lamellipodian amoebae.

Rather large amoebae of two sorts, both with a thick pellicle, were obtained from the dunged plot on Broadbalk (14 tons farmyard manure per acre each year since 1843) by the bubbling method. It is possible that these were more resistant to a comparative degree of drought than the more delicate types which flourished in the wet cucumber soil and came on strongly in cultures from the field soil.

By far the most abundant results were obtained with samples of these soils collected in November, 1913, when the moisture content of the dunged plot was given by the usual method as 22 %. In the dry summer of 1914 when the moisture on this plot varied usually between 13 % and 10 %, very poor results were given by all methods of investigating the active fauna. There is a distinct probability that here the water content is a limiting factor in determining the density of the active fauna.

In the case of the Abergavenny garden soil no clear correlation of this kind was observed; observations were, however, only made in the summer (June) before and after rain.

To get an idea of the fauna of a soil very rich in humus, a deposit of black leaf-mould in a wood near Abergavenny was sampled. Here thecamoebae were again very numerous, amoebae were slightly less numerous, and small flagellates and some ciliates were easily detected. As a further example of a soil rich in organic matter, samples were taken from a sewage bed at Abergavenny. Sewage had been led on to this, and allowed to percolate through. When the samples were taken the bed had dried sufficiently to allow of the deposit being scraped up into heaps ready for removal. Enormous numbers of phytoflagellates (forming a green film on the surface) were present, and thecamoebae and amoebae were very plentiful. Ciliates were not uncommon, and the smaller flagellates were fairly well represented.

As far as these results go, it appears that the numerically most important types of soil protozoa are thecamoebae and amoebae. Flagellates and ciliates are relatively rare. Of the flagellates found, it is very noticeable that the larger forms, such as Bodo and Copromonas and their allies, appear so far to be of very little importance in the active fauna. The most successful soil flagellates are small monads. This is a result which is not revealed by cultural methods, when the larger flagellates assume a much more prominent position. Sherman (14), using a dilution method, found small flagellates to be the most abundant protozoa in the soils with which he worked[1]. Though our observations have not, so far, supported his, we cast no doubt on the substantial accuracy of his results.

The results of examination of the Broadbalk dunged plot in winter and in summer suggest that normal variations in water content may have a considerable effect on the active fauna of the soil, but in the present stage of our investigations we feel it would be premature to lay too much stress on this point.

[1] Cunningham (4) arrives at a similar result.

IV. CONCLUSIONS.

It seems probable from the work that we have done up to the present that there are always some free living protozoa present in a trophic state in even relatively dry, poor soils.

In manuring on ordinary soil with farmyard manure, a large number of protozoa are introduced into that soil, and if the conditions of culture are such as to necessitate a high water and a high manurial content, the protozoa may well get the upper hand to such an extent as to produce a well-marked deleterious effect on the crop, resulting in the condition known as soil sickness (*e.g.*, in cucumber beds, sewage farms).

The nature of the protozoan fauna seems to vary to a certain extent with the soil under examination. It is probable that this is largely due to actual difference in the fauna of different soils, but it may be partially due to another factor. As is well known, if some soil is added to a hay infusion or other suitable culture medium, the fauna shows a tendency to run in cycles (*e.g.*, at first the dominant forms would be found to be small flagellates; these are usually followed by larger flagellates and amoebae, and these are succeeded by ciliates). It is possible that such cycles may occur in the soil, and it is possible therefore that two soils with a similar water content may show quite different active fauna, depending on the point of the animal cycle at which that soil had arrived. The dominant protozoa found in a trophic state in a soil may be the dominant form found in the cultures, as was probably the case in some sick cucumber soils; but it of course depends on the suitability of the medium, and the culture method adopted. It is probable that the richer the soil and the higher the water content at the time of examination, the greater the probability of the dominant culture form being the dominant trophic form in the fresh soil. A possible exception to this rule is furnished by the thecamoebae, which usually only appear late under present cultural conditions.

It will be seen that up to the present the dominant active fauna of the soil, as shown by the fresh films, consists mostly of amoebae, thecamoebae and small flagellates.

In this connection there is one point which requires further investigation, and that is the frequent prevalence of relatively large flagellates in soil cultures (*e.g.*, Prowazekia and Copromonas), whereas in fresh films the only flagellates found are very small monads. It may perhaps be found that the Prowazekia are present in the trophic state only in

groups on the decaying organic matter in the soil, possibly only for short periods, and that the encysted forms present in the soil are favoured by the condition of the culture at the expense of the smaller flagellate forms, or it is possible that these large flagellates are contented with a very short trophic life in the soil at a time when the water content is high and there are large quantities of decaying material in the soil.

Under these conditions it is not unlikely that the ciliates so frequently found in soil cultures lead a trophic life in the soil.

There is another factor which must be reckoned with in this connection, and that is the possibility that the present methods for the examination of fresh soil films do not give a fair account in regard to these large flagellates, which may be caught up by their flagella amongst the soil particles.

None of these possibilities is mutually exclusive, and it seems from recent work on cultures of soil to which water alone has been added that the last explanation is not very probable.

In conclusion, it seems to us that there are three categories under which the protozoan population of any soil at a given moment should be studied, (1) the active fauna, (2) the resting fauna (in cysts), and (3) the cultural fauna. In the immediate future better methods must be devised for the detection of the active fauna, a complete study is needed of the possible seasonal variations which might result in a transfer of certain forms from the resting fauna to the active fauna, and a more careful study must be made of cultural conditions, so that it may be possible to cultivate at once any desired member of the active fauna of a soil.

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DESCRIPTION OF PLATES.

PLATE II.

FIG.

1. *Euglypha* sp. from fresh fixed film (see p. 112) of cucumber bed. A thecamoeba.
2. *Chiloden* sp. from fresh fixed film of cucumber bed. A ciliate.
3. Flagellate from fresh fixed film of cucumber bed.
4. Dividing *Vahlkampfia soli* from fresh fixed film of cucumber bed. A limax amoeba.
5. *Euglypha* sp. from fresh fixed film of cucumber bed. A thecamoeba.
6. *Chlamydophrys* sp. from fresh fixed film of cucumber seedling bed. A thecamoeba.
7. *Amoeba gobanniensis* from fresh fixed film of cucumber seedling bed. A lamellipodian type of amoeba.
8. *Amoeba* sp. Do.
9. *Amoeba* sp. Do.

PLATE III.

10. *Vahlkampfia soli* from fresh fixed film of cucumber bed. A limax amoeba.
11. *Vahlkampfia soli* stage in division.
12. *Amoeba cucumis* from young culture. A lamellipodian amoeba.
13. *Amoeba cucumis* late stage in division.
14. *Bodo caudatus* from a culture. A flagellate.
15. *Bodo caudatus* stage in multiple division.

Note. These illustrations are designed to assist bacteriologists and others who are interested in soil protozoology to refer the species they will encounter to the general type. It is hoped in particular that the organisms vaguely referred to as "Amoebae" may be more definitely distinguished at least into Thecamoeba and Amoeba. The limax and the lamellipodian type of amoebae will almost certainly be among the most successful amoebae found in cultures, and it would be of interest to distinguish them from one another and from other less defined types. The sizes of the protozoa shown varied from 15 to 50 μ; but the figures were not drawn to the same magnification.

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