THE QUALITATIVE AND QUANTITATIVE EFFECTS OF FOOD ON THE GROWTH OF A SOIL AMOEBA (*HARTMANELLA HYALINA*)

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(With Three Text-figures.)

Among the free-living protozoa, excluding those forms which contain chlorophyll, two types of nutrition are found, holozoic and saprozoic; in the soil commonwealth there is an ample food supply for the saprozoic forms without reference to the other members of the micro-organic population, but the holozoic species present a different problem, since in the great majority of cases their food consists of the bacteria or other smaller organisms which occur with them in the soil solution. The idea that a normally holozoic animal might be induced to become saprozoic arose with the introduction of a suitable pure-culture technique. Among the earlier workers Beijerinck (1896), Frosch (1897) and Tsujitani (1898) all obtained "pure mixed cultures" of amoebae, and record their failure to grow these except in the presence of bacteria or yeasts, though Tsujitani was able to grow his amoebae with bacteria killed by heating them to 65° or 70° C.; later Mouton (1902) records that an amoeba, a form obtained from soil, would not grow except in the presence of living bacteria, and Oehler (1916, 1924), who has perhaps worked more in this field than any other observer, has experienced great difficulty in obtaining any protozoan growth in cultures which do not contain bacteria or yeasts, either dead or alive, though successful growth was obtained in the case of *Colpoda* by feeding the culture with ground-up fish or powdered egg albumen. It further appears from Oehler's work that, while the holozoic ciliates and flagellates are practically omnivorous provided that the food is supplied to them alive and of a suitable size, the amoebae tend to be very much more fastidious in their acceptance of food. All these writers seem to have been impressed by the enormous amount of bacterial food that a successful culture of protozoa can consume, and a quantitative study of this problem by Cutler and Crump (1924) showed that in the case of *Colpidium colpoda* in pure mixed culture the reproductive rate varied from 0·0 to 5·3 in 24 hours according as the number of bacteria per individual ciliate varied from 250 to 1,024,000; similar results have been obtained by Vieweger (1923)[1], (1924).

Ever since the study of soil protozoa received its initial impetus from the work of Russell and Hutchinson (1909) this group of organisms, and more especially the

[1] Although the interesting work of T. Vieweger was published in 1923 it has only recently come to our notice.

amoebae, have been under suspicion of preying upon the bacteria and seriously reducing their numbers, thus constituting a possible menace to soil fertility. It has been established that in soil the numbers of active amoebae to a very marked extent vary inversely with those of the bacteria (Cutler, Crump and Sandon, 1922); further, that the numbers of protozoa present in any soil increase directly with the increase of organic matter (Sandon, 1927), in other words, with the crude supply of bacterial food. It seemed desirable therefore to get more direct information concerning the relationship of these soil amoebae to their food supply by a study of their behaviour in pure mixed culture. For this purpose a small limax amoeba *Hartmanella hyalina* was used, since it is a ubiquitous soil form and one of the dominant amoebae in normal field soils.

Methods. Pure mixed cultures of *Hartmanella hyalina* were obtained by the method which, with slight modifications, has been used by all earlier workers. A mixed culture of the amoeba is inoculated on to an agar plate either at one end of a streak of a pure culture of the desired bacteria or surrounded by a circle of them; after three or four days when the amoebae begin to appear at the other end of the streak or on the outside of the circle, a subculture is made in exactly the same way, and the process is repeated until the amoebae are obtained free from all but the requisite species of bacteria. The process can be facilitated and the number of contaminating bacteria reduced by washing cysts of the amoebae with 2 per cent. hydrochloric acid (sp. gr. 1·15) or some other suitable reagent for several hours. With certain bacteria, such as *B. prodigiosus* and *Azotobacter chroococcum*, we have been entirely unsuccessful; Oehler (1916) also records a failure to grow his amoebae with *B. prodigiosus* on bouillon agar owing to the production of trimethylamin, but states that they grow successfully together on a plain 1 per cent. or 2 per cent. agar. In the case of *Azotobacter* it seems probable that the size and consistency of the bacteria make it difficult for the amoebae to ingest them, though Cutler and Bal (1926) record the ingestion of dead *Azotobacter* by the amoebae.

The observations on the effect of food supply on *Colpidium* were carried out on mass cultures, but the same method is not applicable in the case of an amoeba, for it has been found impossible to obtain a good distribution of the animals in liquid culture owing to their tendency to crawl on the walls of the tube, concentrating in younger cultures just below the surface of the liquid in a band from which it is difficult to dislodge them. Single individuals were therefore isolated in ruled counting chambers covered with cover-glasses on which a drop of medium containing bacteria was suspended. The size of the drop was measured by the method detailed in an earlier paper (Cutler and Crump, 1923) and the amoebae could readily be counted under the low power of the microscope. In enumerating the bacteria, sample squares were counted and the assumption was made that the bacteria were uniformly distributed throughout the drop. If there was reason to believe that there was not this uniformity, even distribution was ensured by rotating the cover slip so as to mix the fluid before the bacteria were counted. The medium used in the experiments was soil extract prepared as follows: one kilogramme of soil was boiled for one-and-a-half hours with two litres of tap water and the mixture allowed

to settle, the supernatant fluid was poured off, made up to a litre, and filtered through two thicknesses of filter paper. The medium was then adjusted to a *p*H value of 7·2 and sterilised by autoclaving for 20 minutes at 115 lb. pressure. This medium has proved most successful for all the soil protozoa which have been grown in it; it is, however, open to the serious objection that repeated sterilisation renders it toxic, at least to *Hartmanella hyalina*. It is unsafe therefore to autoclave it more than once or at the most twice.

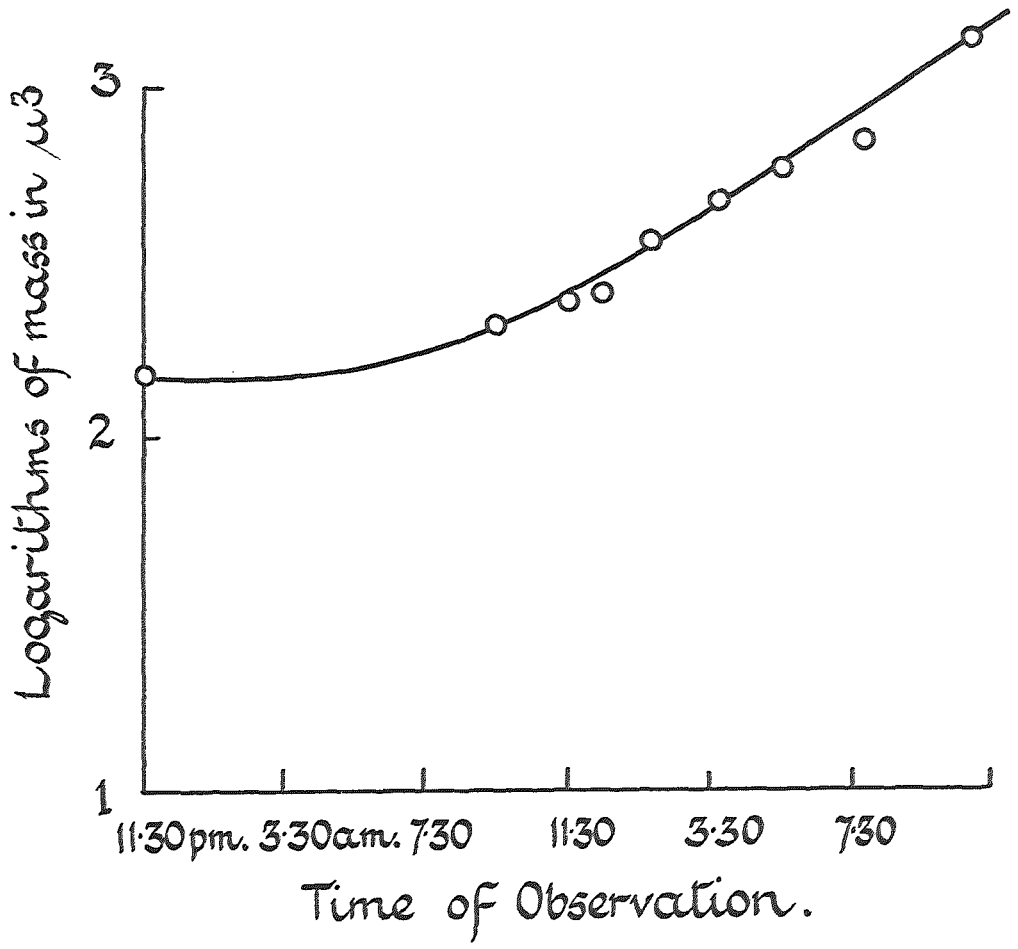

Fig. 1. Increase in mass of *Hartmanella hyalina* starting from a single specimen.

For the measurement of the actual increase in protoplasm as distinct from the increase in numbers, a camera lucida was used; ten drawings of the individual under observation being made as rapidly as possible on millimetre squared paper. The average number of squares in the ten outlines divided by the magnification gives the approximate area of the animal, and the thickness was regarded as 1μ. This method of measurement gives fairly consistent results (Fig. 1) but discrepancies may be found when the animal is stationary, as it tends to be thicker than when actively moving; in consequence the drawings were always made of moving animals. At a very early stage in the investigation it was obvious that the amoebae grew more readily with certain bacteria than with others; three species therefore were selected with which to obtain pure cultures, one giving extremely good growth, another poor growth, and a third—*B. mycoides*—which lay on the whole between the other two; all these species came originally from Rothamsted field soils.

Description of bacteria.

"YB." Rods, 1·6–2·0μ × 0·8μ, occasionally in pairs, non-motile, gram positive; colonies on nutrient agar—round, convex, buff, smooth shining, edge entire; on Thornton's

medium: round umbonate, white, smooth shining, edge entire; nutrient agar stab: nail-head, line of stab filiform; liquefies gelatine.

"SE." Short rods $1{\cdot}6 \times 0{\cdot}8\mu$, occasionally in pairs, non-motile, gram negative; colonies on nutrient agar: round convex, white, smooth shining, edge entire; on Thornton's medium: punctiform, white, convex, smooth shining, edge entire; nutrient agar stab: nailhead, line of stab filiform; does not liquefy gelatine.

Experimental Results. For purposes of comparison with the reproductive rates of the amoebae the numbers of bacteria may be calculated in two ways, either the number present per unit area may be made the basis of comparison or the total number present in the whole drop; in the first case the unit area being one square of the counting chamber, that is 0·0025 sq. mm. In the majority of cases either method of calculation would give the same results since in general the bacterial population is correlated with the size of the drop; but cases do occur where the total numbers of bacteria are the same but on the one hand the drop size is large and the bacteria are sparsely distributed and on the other hand the drop size is small and the bacteria densely distributed. Hence in the present work both methods of presentation have been used.

Table I. *Reproductive rates of* Hartmanella hyalina *with three species of bacteria.*

No. of bacteria per square of 0·0025 sq. mm. area	Reproductive rate of amoeba for 24 hours from one individual					
	With "YB" bacteria		With *B. mycoides*		With "SE" bacteria	
	No. of cases	Average	No. of cases	Average	No. of cases	Average
0–12	12	2·7	19	1·8	8	0·6
12–24	32	3·3	55	1·6	43	0·8
24–36	37	3·8	49	2·1	44	1·6
36–48	67	3·7	36	2·4	44	2·0
48–60	63	3·9	25	2·6	22	2·0
60–72	44	4·2	6	2·4	18	2·4
72–84	22	4·5	—	—	12	2·6
84–96	19	5·0	—	—	6	3·0
96–108	12	4·9	—	—	1	3·3
108–120	4	4·9	—	—	—	—
120–132	4	5·1	—	—	—	—

A further reason for adopting both methods is that the question of food supply is not necessarily identical in each case: for there can be no doubt but that when there is a small drop densely populated the food is more accessible to a relatively slow moving organism such as an amoeba, than when the volume of the drop is big and the bacteria widely separated. Discrepancies due to this effect are eliminated by adopting the unit area as the basis of comparison, but by using this method the total available food supply is ignored. In every case the number of bacteria given is the average between the number present at the beginning and at the end of the 24-hour period. The results obtained with the three bacterial types (ignoring the drop size)

are shown in Table I, from which it is obvious that there is a definite correlation between the numbers of bacteria per unit area and the reproductive rate of the amoebae and, further, that the species "YB" induces considerably increased reproduction, the greatest rate in "SE" being only slightly greater than the lowest rate in "YB," though the sizes of the bacteria are nearly the same. *B. mycoides* lies intermediately as a source of food, but this species is hardly comparable with the other two, since it tends to form chains during its growth, and therefore one "mycoides" will not of necessity be equivalent to one "YB" or "SE." The size of the total bacterial population and its effect on the rate of reproduction of the amoebae is given in Table II, where a gradual increase in rate corresponds to an increasing food supply. These results are of course in effect the same as those given in Table I, but they are of use in showing the actual number of bacteria required by each amoeba in order to give any particular rate of reproduction.

Table II. *Reproductive rates of amoebae for 24 hours from one animal.*

Total bacteria per amoeba in thousands	"YB" bacteria		*B. mycoides*		"SE" bacteria	
	No. of cases	Average	No. of cases	Average	No. of cases	Average
0–200	9	3·2	31	1·7	10	0·7
200–400	25	3·4	44	1·9	24	1·4
400–600	21	4·1	41	2·1	29	1·4
600–800	19	4·4	21	2·2	26	1·6
800–1000	25	4·4	12	2·4	29	1·8
1000–1200	15	4·7	6	2·2	18	2·1
1200–1400	7	4·8	—	—	16	2·4
1400–1600	3	4·6	—	—	7	2·5
1600–1800	4	5·1	—	—	5	2·9
1800–2000	1	5·8	—	—	3	2·8
over 2000	4	4·6	—	—	3	2·3

It was thought possible, though not likely, that, although the rate of reproduction was markedly different in the "SE" and "YB" types of feeding, yet the total volume of amoebic protoplasm produced would not be very different. To test this possibility the experiments on the rate of growth of amoebae were carried out.

As is shown in Table III the "YB" bacteria gave a greater increase in volume than did the "SE." Unfortunately at the beginning of the experiment the numbers of bacteria in the "YB" culture were greater than in the "SE," while the amoeba in the "YB" culture was smaller than in the "SE" one. However from 3.45 p.m. onwards the bacterial populations were practically the same in the two cases, but the increase in the total volume of the amoebae present was 30 per cent. greater in the case of the "YB" culture.

The effect of feeding was also well shown by a series of observations recorded in Table IV. Two parallel cultures were started, one of which was well fed with "SE" bacteria, while the other had only the bacteria supplied with the isolation fluid and

was therefore in a relatively starved condition. This animal steadily decreased in size and after 24 hours it had shrunk to only $83{\cdot}5\mu^3$. At this point food was introduced with the result that after five hours the animal had divided into five and the total volume had been more than six times increased. The well-fed amoeba on the other hand showed a steady increase in volume throughout the experiment.

Table III. *Growth of* Hartmanella hyalina *with different bacteria.*

Time of observation	Fed with "YB"			Fed with "SE"		
	No. of amoebae	Mass in μ^3	No. of bacteria per 0·00025 mm.^{3}	No. of amoebae	Mass in μ^3	No. of bacteria per 0.00025 mm.^{3}
Feb. 22						
10.15 a.m.	1	104·4	16	1	135·2	9
12.15 p.m.	1	104·4	21	1	124·0	10
2.15 p.m.	1	134·4	25	1	132·4	18
3.45 p.m.	1	168·8	27	1	143·6	23
5.45 p.m.	1	158·0	26	1	146·0	32
7.45 p.m.	1	182·8	36	2	178·8	39
Feb. 23						
7.45 a.m.	8	1496·0	56	5	758·8	62

Table IV. *The effect of starvation and feeding on mass in* Hartmanella hyalina.

Time of observation	Unfed		Fed	
	No. of amoebae	Mass in μ^3	No. of amoebae	Mass in μ^3
10.30 a.m.	1	248·7	1	159·4
11.30 ,,	1	246·0	1	185·7
12.30 p.m.	1	222·0	1	210·6
2 ,,	1	177·8	1	234·1
3 ,,	1	200·8	1	266·5
4 ,,	1	185·2	1	226·7
5 ,,	1	156·0	1	288·0
6 ,,	1	162·2	1	272·5
7 ,,	1	201·3	—	—
8 ,,	1	204·3	2	364·3
9 ,,	1	179·7	2	395·1
9·30 a.m.	1	143·4	5	895·2
*10.30 ,,	1	83·5	—	—
11·30 ,,	1	159·2	5	901·6
12.30 p.m.	1	192·8	—	—
2.30 ,,	4	465·4	—	—
3.30 ,,	5	561·7	—	—

* The culture was well fed after this observation was made.

Earlier work with the ciliate *Colpidium colpoda* gave a similar demonstration of the effect of food upon starved organisms. In Fig. 2 line drawings, each of 20 individual *Colpidia* taken at random are shown. The small organisms were taken from a starved culture, which on being fed contained within 24 hours animals similar to

the large ones seen in the drawing. Further the experiments on growth in mass show that the rate of growth is very variable after feeding, the starved animal having a much more rapid percentage increase in size per hour than is seen in the progress of a consistently well-fed animal. In comparing the effects of feeding either in respect to the species of bacterium used or to the quantity of food available it is found that the size at which the amoebae divide varies within wide limits, but is connected with both these conditions. For example amoebae well fed with "YB" bacteria on the average divide at 274·0μ^3: while those sparsely fed with the same species divide at 191·6μ^3. Using "SE" as the source of food well-fed animals divide at 190·0μ^3.

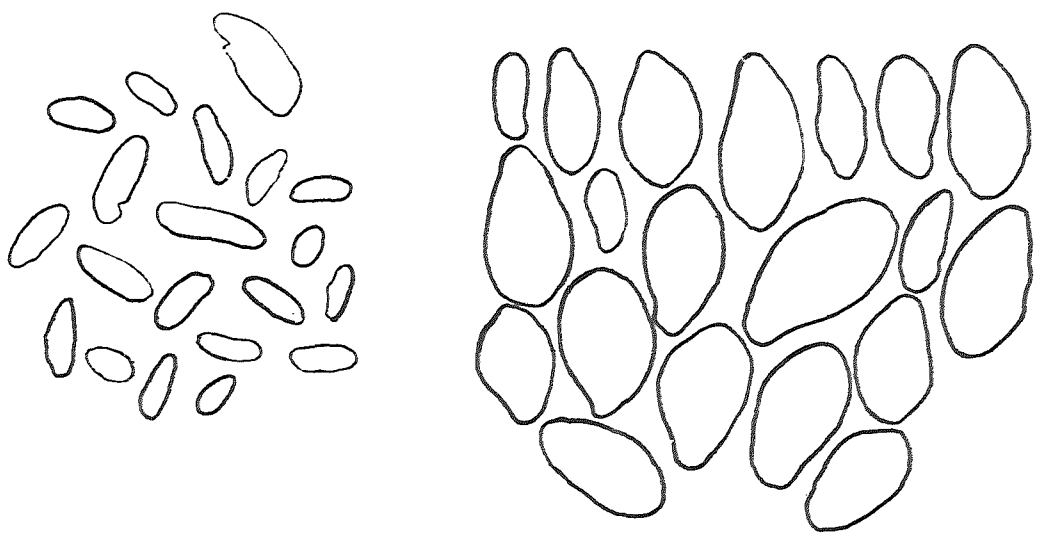

Fig. 2. Twenty individuals of *Colpidium colpoda* taken at random from a starved culture and from the same culture 24 hours later after heavy feeding with bacteria [× 250].

There are several records of differential feeding effects in amoebae and other protozoa. Frosch (1897) found that when he inoculated bacteria-free cysts of amoebae into pure cultures of various species of bacteria from soil he obtained very different degrees of growth. Tsujitani (1898) grew his amoebae successfully with a variety of bacteria, including *coli*, *fluorescens liquefaciens* and *non-liquefaciens*, *Staphyloccus pyogenes aureus*, *pyocyaneus*, typhus and cholera, but was unable to make them grow with anthrax bacilli or with yeasts. Mouton (1902) also found that *B. anthracis* gave poor cultures of amoebae, and records that *B. coli communis*, *Staphylococcus aureus*, the cholera bacillus and one or two other forms were suitable for his amoebae, as was a small yeast, *Saccharomyces exiguus*. Hargitt and Fray (1917), working with *Paramecium aurelia*, found that on the whole single species of bacteria did not give satisfactory cultures, while a mixed flora was successful, *B. subtilis* being the only bacterial species which gave good results in pure culture; Phillips (1922), working on the same lines and using three different bacteria, showed that alone these gave different degrees of growth, and in every case a mixture provided a more suitable food supply than did any single species.

Oehler (1916), in reviewing the subject, asserts that, on the whole, gram-negative bacteria are more readily eaten by amoebae than are the gram-positive species. Working with five distinct species of amoebae the following interesting feeding peculiarities were observed: two of his forms would eat *Saccharomyces exiguus*, while the other three would not; two of them would eat *coli*, *Sarcina* and

yeasts that had been autoclaved at 130° C. for an hour, while the other three scarcely grew at all under these conditions. One of these three would however readily accept *coli* killed at a temperature of 56° C. for one-and-a-half hours, and another would grow with *Bacterium fluorescens* killed by heating to 45° C. for one-and-a-half hours; the fifth species refused all these.

These different results obtained with different bacteria may be due to any of four causes: a species may be of a superior food value, or more suited to the digestive processes of the consumer, or possessed of toxic properties which inhibit growth, or of such a structure that for physical reasons it cannot readily be ingested. To decide between the first and second of these alternatives is by no means simple, but the third is obviously accessible to experimental attack. "SE" bacteria and *B. mycoides* were therefore grown in liquid cultures in soil extract medium, and on successive days 20 c.c. were taken from the mass cultures and filtered through porcelain candles; the filtrate was then diluted with an equal quantity of sterile, fresh soil extract and amoebae were isolated into the fluid in counting chambers in the usual way and fed with "YB" bacteria. The results of these experiments are given in Table V which shows that when extracts from a young culture are used there is no difference between the control and the treated cultures, but that the extracts from older cultures of "SE" bacteria have a bad effect. The same thing also occurs when extracts from an old culture of "YB" bacteria are used where no question of the bacteria having toxic properties can arise, therefore it appears that the inhibiting factor present in the older cultures of "SE" bacteria is due to extrinsic and not to any intrinsic properties of the bacteria themselves.

Table V. *Effect of bacterial extracts on reproduction of amoebae.*

Reproductive rates of amoebae for 24 hours all fed with "YB" bacteria

Control		Extract		Type of extract
No. of cases	Average	No. of cases	Average	
9	4·1	10	4·2	*B. mycoides* (2 days old)
12	3·8	12	3·7	,, (3 days old)
15	5·0	14	4·9	"SE" bacteria (1 day old)
14	1·9	12	1·8	,, (2 days old)*
12	2·2	13	1·9	,, (3 days old)*
13	4·4	14	3·1	,, (4 days old)
11	4·2	11	3·6	,, (5 days old)

* These cultures were incubated at 15° C. instead of 21° C.

Owing to the methods of counting employed in soil investigations it is difficult directly to apply the results outlined above to soil economy. Enumeration must be done in an indirect way and even when such a count is made it is incomplete since no one medium supports the whole bacterial population. Ammonifying species of bacteria predominate in these counts and many important species do not appear at all. Under these conditions it would not be likely that any direct ratio such as was

found in the fluid-culture experiments would emerge; but by employing only species of bacteria capable of growing on the culture medium used comparable results may be obtained. An experiment designed for other purposes, in which sand was inoculated in one case with "YB" bacteria and *Hartmanella* and in the other case with "SE" bacteria and *Hartmanella*, gave results which could be used in this connection. In Table VI the rates of reproduction of the amoebae are set out against the numbers of bacteria per amoeba. It is found that the rate of reproduction is consistently lower than it is in liquid cultures containing the same bacterial ratio, but that the correlation between reproductive rate and food supply still holds good, together with the lower food value of the "SE" bacteria.

Table VI. *Reproductive rates of* Hartmanella hyalina *in sand cultures.*

Successive days	Bacterial ratio	Reproductive rate
With "YB" bacteria		
1	1,800,000	3·0
2	124,000	0·5
3	34,000	0·0
4	190,000	0·5
5	127,000	0·0
6	250,000	0·0
7	260,000	2·5
With "SE" bacteria		
1	14,000,000	2·0
2	4,000,000	1·5
3	1,300,000	0·0
4	3,900,000	1·0
5	1,700,000	1·0
6	490,000	0·0
7	1,000,000	0·5

The lower rate of reproduction in soil or sand cultures would, however, be expected since the distribution of the food supply is such as to render it less available than in the liquid cultures used for the experiments. Here the bacteria are uniformly distributed and the whole of the liquid is accessible to the amoebae; in the soil on the other hand the bacteria are irregularly distributed through the film of water surrounding the soil particles and the amoeba is confined to the surface of the particles and not free to wander through the water film.

The figures in Table VI give some idea of the rapid changes that occur in the distribution of protoplasm among the constituent groups of the soil micro-organisms. For example, in the sand containing "YB" bacteria and amoebae, where on one day there are 7600 active amoebae, the 11,000 found on the next day represent a consumption of 1,444,000 bacteria. This figure is obtained by assuming that 0·5 division requires 190,000 bacteria per amoeba and therefore that 7600 amoebae will need the number of bacteria given above. An actual count of the bacterial numbers on the successive days showed a reduction of 1,134,000 bacteria, which is in keeping with the calculated result. When it is remembered that the bacteria are undergoing rapid divisions the large bacterial numbers required to keep up the protozoan population are not so unreasonable as might at first appear to be the case.

Where "SE" bacteria are the only available forms the number required to maintain the same sized amoebic populations as occur with "YB" bacteria is of course even greater. An increase of 400 amoebae to 600 (0·5 division) requires 480,000 bacteria; but if the numbers of amoebae had been 7600 as in the previous case, and had risen to 11,000, the bacterial consumption would have been 798,000,000,000. Interestingly enough, in our experience the numbers of amoebae have never risen above 900 when "SE" bacteria have been the sole source of food.

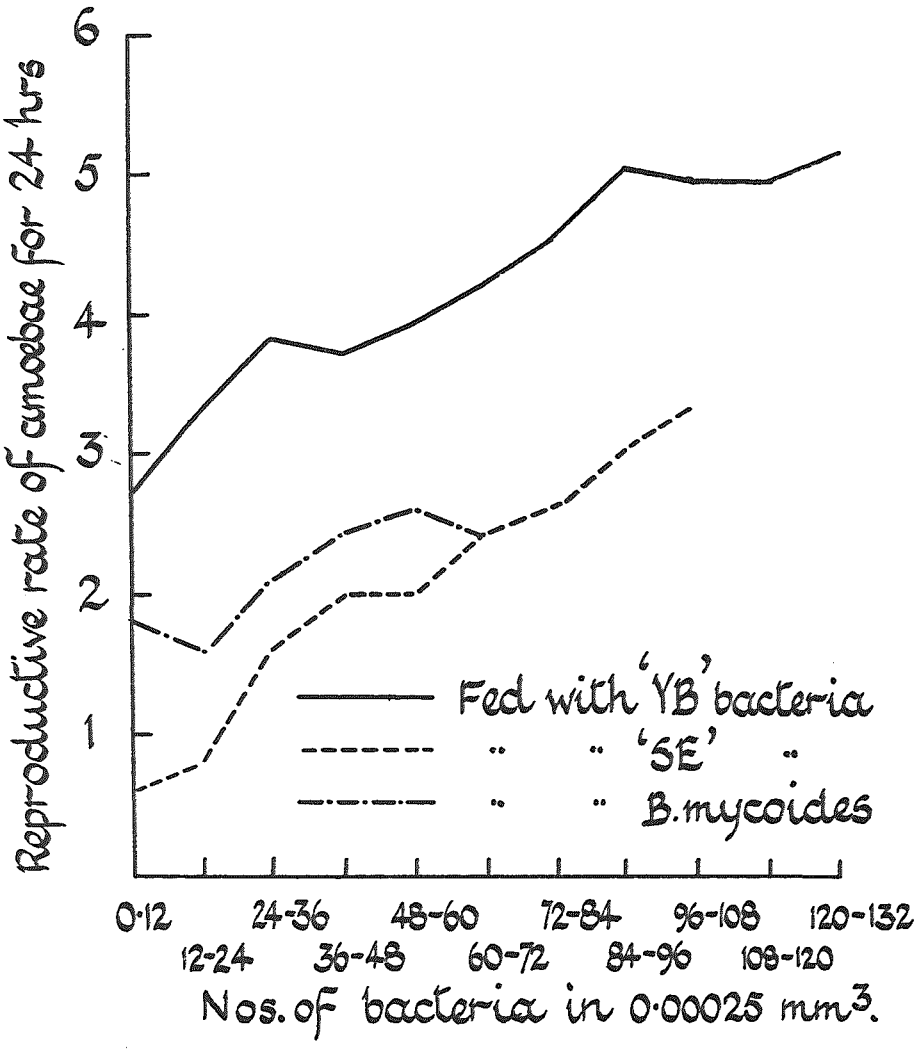

Fig. 3.

These results also have a bearing on the curious daily changes of the amoebic population which were recorded in a previous paper (Cutler, Crump and Sandon, 1922). It was found over a year's examination of soil samples taken daily from the field that both the numbers of bacteria and amoebae showed large fluctuations. The bacterial changes in numbers showed an inverse relationship with the amoebae, but the changes in the numbers of amoebae were inexplicable, as gross external conditions (rainfall, temperature, water content) taken alone had no effect upon them.

If, however, one assumes that the bacterial population undergoes changes similar to those found in plankton, in its constituent species as well as in numbers, an assumption for which there is much evidence—and that the feeding values of the various species are as different as is the case with the soil forms "YB" and "SE," then it must follow that the size of the amoebic population must also undergo rapid

changes. For when a species like "YB" is in the ascendancy the numbers of amoebae will rapidly increase, but a change to the "SE" type of bacteria will cause the death of many amoebae unless the numbers of bacteria increase to a figure higher than is associated with normal soils.

SUMMARY.

1. A definite relationship between the reproductive rate of a soil amoeba, *Hartmanella hyalina*, and the available bacterial food supply has been demonstrated.

2. It has been shown that three species of soil bacteria have different feeding values not only in respect to the rate of division of the amoeba, but also in respect to the total increase in the amount of protoplasm.

3. The bearing of these results on the soil economy is discussed.

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