A METHOD FOR ESTIMATING THE NUMBER OF ACTIVE PROTOZOA IN THE SOIL.

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INTRODUCTION.

The present writer has pointed out in a previous paper (4) the importance of finding means for estimating the number of protozoa in the active-noncystic-condition in a soil sample. Up to the present, with few exceptions, the total number of protozoa, irrespective of their condition, has been ascertained, and no method has yet been evolved giving an approximately accurate estimate of the number in an active condition.

PREVIOUS WORK.

Martin and Lewin (9) were the first to show that in normal soil there existed an active trophic [1] protozoal fauna. This they did by two methods. In the first, soil was stirred into picric acid contained in a porcelain dish. A film rose to the surface containing protozoa, which could be collected on cover glasses. This method gave fair results as regards small flagellates, small amoebae and thecamoebae. The second method consisted in bubbling a stream of air through a suspension of the soil to be tested and allowing the bubbles to break on to cover glasses fixed at the surface of the suspension. Thus they showed that Rothamsted soil contains active amoebae and flagellates.

These observations are interesting and important in demonstrating the presence of active soil protozoa, but give no clue as to their numbers.

Cunningham (3) attacked the problem in a somewhat different manner. As cystic protozoa are more resistant to high temperature than active ones, he suggested heating to 58° C. in order to kill all active organisms and leave cysts uninjured, thus allowing a distinction to be made between these two conditions. This was adopted in combination with a dilution method. Two sets of dilutions were made, the first with untreated soil and the second with soil which, in the 1/100 dilution, had been heated to 58° C.

[1] Active means capable of movement and trophic means capable of feeding. As the states usually synchronise protozoologists use the terms synonymously.

Experiments indicated, however, that a temperature of 58° C., while absolutely essential if one wished to be certain that all active forms were killed, also kills a large number of encysted protozoa. The method was therefore impracticable.

Drying the soil or treating it with 0·5 per cent. caustic potash, though showing that an active protozoal fauna existed, proved unsuitable for estimating the number of cysts and active forms.

Koch (7), Itano and Ray (6) and Kopeloff, Lint and Coleman (8) have described direct methods for doing this, but none are entirely satisfactory for routine work.

HYDROCHLORIC ACID METHOD.

The enumeration of the active forms can be obtained by the use of hydrochloric acid, in conjunction with the dilution method in use at Rothamsted.

Cropper and Drew (2) have already found that the cysts of a soil amoeba—species not given—are able to withstand 48 hours action of a 2 per cent. solution. The HCl used was B.P. pure 31·8 per cent. Goodey (5) has also shown that hydrochloric acid does not dissolve the cyst wall of *Colpoda cucullus*. In the present investigations mixed cultures of protozoa from the soil were used, including the following species: *Cercomonas* sp., *Oicomonas termo*, *Monas vulgaris*, *Amoeba glebae*: *Vahlkampfia* sp., *Colpoda cucullus*, *Colpoda steinii*, *Gonostomum affinis*. In all cases it is found that the active forms are killed while the cysts are unaffected. Counts made before and after treatment, therefore, give estimates of the respective numbers in the two conditions.

EXPERIMENTAL DETAILS.

Control Experiments.

Microscopic examination. These experiments were to determine the effect of varying strengths of hydrochloric acid on protozoa in the active condition. Cultures of the various flagellates, amoebae and ciliates in the active stage were subjected to the action of HCl, of the strengths 0·5 per cent., 1 per cent., 2 per cent., of the ordinary variety of 1·15 sp. gr.[1] In all cases the organisms were almost instantaneously killed as shown by their sudden cessation of movement, followed by disintegration. There is no doubt that hydrochloric acid of the strengths stated kills all the active forms of protozoa investigated. Barratt (1) has also shown that

[1] The titration value is as follows: 10 c.c. 2 per cent. requires 18·9 c.c. N/10 NaOH.

0·0004 N. HCl will cause the death of Paramoecia in one minute. Cysts appeared unaffected by the treatments, but they might have been killed without showing any outward sign. To test this two methods were used: a rapid eosin method, to which, however, some exception might be taken on theoretical grounds, and a more detailed excystation method, to which we believe no exception can be taken. Kuenen and Swellengrebel state that dead protozoan cysts are coloured red under the action of dilute eosin while living ones remain colourless. This was found to be the case with the cysts of *Entamoeba histolytica* by Wenyon and O'Connor (10) and the present writer. Such a rapid method of detecting dead cysts is naturally of great use and was therefore tested on the cysts of soil protozoa. Cysts were tested with a 0·125 per cent. watery solution of eosin. In some cases the cysts remained colourless for as long as half an hour. Experiments at longer intervals were not made. Excystation showed that these colourless cysts were alive. Cysts from the same cultures were then killed either by boiling or heating at 85° C. for one hour, or else they were placed in normal solutions of strong acids for a quarter of an hour. Excystation experiments demonstrated that such treatment caused the death of cysts. When these dead cysts were placed in the watery solution of eosin they at once became uniformly coloured. In the next series of experiments cysts of amoebae, flagellates and ciliates treated with 0·5 per cent., 1 per cent., and 2 per cent. hydrochloric acid (sp. gr. 1·15) for 12 hours and 24 hours respectively were tested for viability by this eosin method. The cysts did not take up the stain and were therefore regarded as living.

Microscopic examination therefore showed that active forms of amoebae, flagellates and ciliates were killed by the action of hydrochloric acid in strengths ranging from 0·5 per cent. to 2 per cent.; but that the cystic forms, or at any rate the large majority of them, could withstand this treatment for at least 24 hours. The different reactions of dead and living cysts to dilute eosin obviously depend on changes in the permeability of the cell membrane. As our knowledge of these changes is scanty and somewhat chaotic, perhaps too much reliance should not be placed on the method. Nevertheless it is very useful on account of its simplicity and rapidity in giving results, and if checked by other experiments is well worthy of general use.

Excystation tests. In these experiments suspensions containing a known number of active and cystic amoebae, flagellates and ciliates were made. The number per cubic centimeter was calculated by the method previously described (4). The suspensions were then treated with 1·5 per

cent. HCl (sp. gr. 1·15) acting overnight and the following morning suitable dilutions of 1 c.c. of the suspensions were plated on to agar. It was found unnecessary to wash the protozoa entirely free from acid after the treatment as excystation will take place in the presence of ·02 per cent. of the hydrochloric acid, an amount considerably in excess of that left after the dilutions have been made. In Table I are given the results of some of these experiments. It demonstrates very clearly that, by the addition of hydrochloric acid in suitable strength to a suspension of protozoa, the active forms are killed, leaving the cystic one alive, and that this occurs in types of protozoa common to the soil.

Table I.

Suspension	Number of organisms per c.c. found by direct count			Number of organisms after treatment as found by dilution method	
1.	Amoebae	(cysts)	35,000	Amoebae	30,000
	,,	(active)	20,000		
	Flagellates	(cysts)	170,000	Flagellates	150,000
	,,	(active)	680,000		
2.	Amoebae	(cysts)	80,000	Amoebae	70,000
	,,	(active)	50,000		
	Flagellates	(cysts)	45,000	Flagellates	40,000
	,,	(active)	266,000		
	Ciliates	(cysts)	45,000	Ciliates	35,000
	,,	(active)	35,000		
3.	Amoebae	(cysts)	40,000	Amoebae	35,000
	,,	(active)	100,000		
	Flagellates	(cysts)	250,000	Flagellates	250,000
	,,	(active)	400,000		
	Ciliates	(cysts)	75,000	Ciliates	60,000
	,,	(active)	50,000		
4	Amoebae	(cysts)	40,000	Amoebae	30,500
	,,	(active)	25,000		
	Flagellates	(cysts)	175,000	Flagellates	170,500
	,,	(active)	0		
	Ciliates	(cysts)	12,500	Ciliates	10,000
	,,	(active)	5,000		
5.	Amoebae	(cysts)	25,000	Amoebae	20,000
	,,	(active)	100,000		
	Flagellates	(cysts)	200,000	Flagellates	160,000
	,,	(active)	150,000		
	Ciliates	(cysts)	50,000	Ciliates	45,000
	,,	(active)	30,000		

It will be noted, however, that in each experiment the number of organisms recovered alive after the treatment is usually a little less than

was expected. A discussion as to the reason for this is given later. The final series of control experiments were made upon soil containing protozoa. Ordinary Rothamsted soil was sterilised in the autoclave under 15 lbs. pressure for half an hour, and to this soil was then added a counted suspension of protozoa in all stages of development.

It was then treated with 2 per cent. HCl[1] overnight. Suitable dilutions were made and plated on to agar. Fifteen such experiments were performed, a representative five of which are given in Table II.

Table II.

Sample	Number of organisms per gram of soil			Number of organisms after treatment as found by dilution method	
1.	Amoebae	(cysts)	25,000	Amoebae	25,000
	,,	(active)	40,000		
	Flagellates	(cysts)	100,000	Flagellates	95,000
	,,	(active)	100,000		
	Ciliates	(cysts)	32,500	Ciliates	30,000
	,,	(active)	5000		
2.	Amoebae	(cysts)	96,800	Amoebae	95,000
	,,	(active)	50,250		
	Flagellates	(cysts)	560,000	Flagellates	500,000
	,,	(active)	130,000		
	Ciliates	(cysts)	10,000	Ciliates	10,000
	,,	(active)	35,000		
3.	Amoebae	(cysts)	15,000	Amoebae	12,500
	,,	(active)	20,000		
	Flagellates	(cysts)	45,000	Flagellates	40,000
	,,	(active)	90,000		
	Ciliates	(cysts)	15,250	Ciliates	10,000
	,,	(active)	35,000		
4.	Amoebae	(cysts)	2500	Amoebae	2250
	,,	(active)	4000		
	Flagellates	(cysts)	1500	Flagellates	1000
	,,	(active)	6500		
	Ciliates	(cysts)	25,400	Ciliates	20,000
	,,	(active)	5000		
5.	Amoebae	(cysts)	645,000	Amoebae	600,000
	,,	(active)	537,000		
	Flagellates	(cysts)	1000	Flagellates	900
	,,	(active)	1500		

[1] In all experiments the carbonate content of the soil was estimated and sufficient 2 % HCl of sp gr. 1·15 added to leave an excess of acid.

HYDROCHLORIC ACID METHOD OF DISCRIMINATING BETWEEN CYSTS AND ACTIVE FORMS.

The control experiments described above show that the hydrochloric acid method is capable of giving approximately accurate counts of the active protozoa of the soil. The method now in use in this laboratory is as follows.

On one part of the soil sample total protozoal counts are made by the dilution method described below. Another sample—10 grams of soil—is then treated with sufficient 2 per cent. HCl (see p. 139, footnote) to neutralise the carbonate present and still leave an excess of unchanged 2 per cent. acid. The acid is allowed to act overnight. After treatment the number of protozoa in the sample is ascertained by the dilution method; this gives the number of cysts since the acid has killed all the active forms, leaving most of the cysts unharmed. The number of cysts subtracted from the total number of organisms given by the first count gives the number of active protozoa per gram of the soil sample[1].

The details of the dilution method, devised by Cunningham, modified by L. M. Crump, and used in my laboratory, are as follows:

10 grams of soil are passed through a 3 mm. sieve and then added to 100 c.c. of sterile tap water or physiological salt solution. This gives a 1/10 dilution. From it further dilutions are made as shown below.

No. 1.	10 gm. soil	in	100	c.c.	H_2O	=	1/10	dilution
„ 2.	10 c.c. No. 1	„	90	„	„	=	1/100	„
„ 3.	5 „ „	2	„	45	„	„	= 1/1000	„
„ 4.	20 „ „	3	„	30	„	„	= 1/2500	„
„ 5.	20 „ „	4	„	20	„	„	= 1/5000	„
„ 6.	30 „ „	5	„	15	„	„	= 1/7500	„
„ 7.	30 „ „	6	„	10	„	„	= 1/10,000	„
„ 8.	20 „ „	7	„	30	„	„	= 1/25,000	„
„ 9.	20 „ „	8	„	20	„	„	= 1/50,000	„
„ 10.	30 „ „	9	„	15	„	„	= 1/75,000	„
„ 11.	30 „ „	10	„	10	„	„	= 1/100,000	„

Nutrient agar is poured into sterile Petri dishes. When the medium has solidified, the dishes are inoculated in pairs with 1 c.c. of each dilution. Incubation at 20° C. is continued for 28 days, and the plates examined at intervals of 7 days, 14 days, 21 days and 28 days. This long period of incubation is necessary in order to ensure accurate results.

[1] As further controls after treatment the titration value of the acid solution over the soil is estimated and the fluid allowed to act on a culture of active protozoa in order to prove that the acidity is sufficient to cause the death of all active forms.

The hydrochloric acid method has been in use for a short time only, but sufficiently long to show that it gives concordant and apparently accurate results.

A few of these are given in Table III.

Table III.

Date sample taken	Manurial treatment of plot	Number of protozoa per gm. (cystic and active)			Number of active protozoa per gm.			Water content
		A*	F	C	A	F	C	
July 29, 1919	Farmyard manure	1000	25,000	100	900	17,500	90	17·52
	Unmanured	2500	2500	0	2400	1500	0	14·07
Aug. 18, 1919	Farmyard manure	100	1000	10	90	900	10	3·08†
	Unmanured	100	5000	10	90	4900	10	4·81
Oct. 8, 1919	Farmyard manure	2500	25,000	10	0	17,500	10	15·48
	Unmanured	7500	7500	0	2500	5000	0	13·24
Oct. 27, 1919	Farmyard manure	2500	5000	10	1500	4000	0	19·11
	Unmanured	100	1000	0	90	950	0	12·87
Nov. 13, 1919	Farmyard manure	500	2500	10	400	0	0	22·27
	Unmanured	100	100	0	50	0	0	16·17

* In the above table A = amoebae; F = flagellates; C = ciliates.

† This value is remarkably low; the sample was taken after a long period of drought.

The experimental plots investigated are part of Broadbalk field where wheat has been grown continuously since 1843. One of the plots has received no manure since 1843; while the other has had applied to it 14 tons per acre of farmyard manure yearly since 1852.

DISCUSSION.

Table III demonstrated that in the soils investigated there are normally present a number of active trophic protozoa, even when the moisture content of the soil reaches the unusually low proportion of 3·08 per cent. or 4·81 per cent.

As shown in a previous paper(4) it is probable that protozoa are usually resident on the soil particles, from which it seems to follow that the organisms are in a moist environment even when the soil is low in water content.

The hydrochloric acid method has not been in operation sufficiently long to allow of discussion upon the proportion of active to cystic protozoa, which apparently varies from month to month; nor of the action of external factors upon the ratio. It is obvious, however, that interesting results will follow the application of the method over a long period.

In Tables I and II it will be noted that the number of protozoa recovered after treatment with the acid is in most cases somewhat less than

was expected. On no occasion were more recovered. This may be due to two causes—experimental error, and death of certain cysts. Experimental error will doubtless explain a certain amount of discrepancy, but the whole cannot be ascribed to this cause, for on such an assumption one would expect the numbers to be both greater and less than expectation, and not only in one direction.

Certain cysts, however, will probably be killed by the hydrochloric acid, thus giving a uniformly lower count after treatment than before it. Protozoa at the beginning of encystation, or in the last stage of excystation are much less resistent to the action of acid and would probably be killed by such treatment. As these processes are continually going on in the soil a certain proportion of cystic protozoa will therefore be killed by the hydrochloric method. Nevertheless the error so produced is not a serious one, for organisms in such conditions would in the one case be active a short time before the soil sample was taken, and in the other case would become active immediately afterwards. These cysts therefore could be regarded as active forms. Finally, it must be remembered that the word cystic is used for two quite distinct conditions—a point most soil protozoologists seem to have ignored. Physiologically there are two types of cysts—the reproductive and the resistent ("dauercysten"). Our knowledge of the reproductive cysts of soil protozoa is extremely scanty, both as regards the species which produce them and as to their resistance to unfavourable external conditions. It may well be that treatment with hydrochloric acid kills some or all of such cysts. However, a thorough investigation of these questions is in hand. Notwithstanding these possible sources of error, the hydrochloric acid method gives sufficiently accurate results to warrant its application to the counting of active soil protozoa[1].

SUMMARY.

1. A method is described by which it is possible to estimate the numbers of active protozoa in a soil.

2. The total number of protozoa is first found by a dilution method. A fresh portion of the soil is then treated with 2 per cent. HCl (sp. gr. 1·15) overnight. By this means all active forms are killed. A second count by the dilution method gives the number of cystic protozoa in the soil. From these results the number of active forms can be ascertained.

[1] While this paper has been passing through the press Collett has published an interesting account of the toxicity of acids to ciliates (*Journ. Exp. Zool.* **29**, p. 443, 1919).

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