THE SUBSTANCES NEEDED FOR THE GROWTH OF A PURE CULTURE OF *COLPIDIUM COLPODA*. BY

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THOUGH considerable attention has been given to the nutritional needs of bacteria, the protozoa have largely escaped notice. This seems to be mainly for the reason that it has been generally believed that they required complicated chemical bodies for growth, if not actually bacteria. During the course of some other work, a pure culture of protozoa was wanted. Reference to previous work did not elicit any information upon the means of obtaining such a culture, free from bacterial and other organisms. Some work, however, by Hargitt and Fray(1), shows how paramœcia may be kept alive upon definite strains of bacteria, after freeing from other organisms.

The following paper is an account of the means by which a strain of *Colpidium colpoda*, reared from one individual, has been kept alive for about ten months, free so far as can be ascertained from other organisms. The minimum needs of this strain in inorganic and organic substances have been sketched as a preliminary to work upon a larger scale.

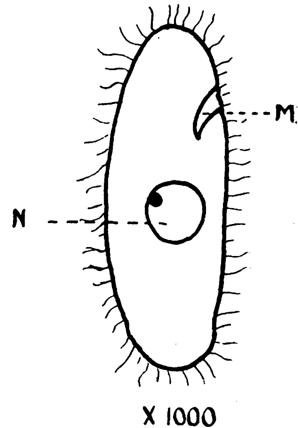

Fig. 1. *Colpidium colpoda* (Ehrbrg) Stein. × 1000. Specimen fixed in osmic acid. *N* = macronucleus, one micronucleus inside. *M* = mouth.

The ciliate organism finally used belonged to the variety usually known as *Colpidium colpoda* (Ehrbrg) Stein[1]. It was obtained from a hay infusion. It has a mean length of about $50\,\mu$, and has one micronucleus (cp. Fig. 1).

Methods. In a preliminary communication(2)[2] the medium has been described in which the original isolations were made. The method of starting the cultures was to a large extent

[1] I am indebted to Mr Clifford Dobell, F.R.S., for kindly identifying the organism for me and for some helpful criticism.

[2] The organism was here erroneously described as paramœcium.

that used by Hargitt and Fry. A Colpidium was isolated from a hay infusion culture, in a drop of sterile medium upon a sterile microscope slide. A capillary pipette made of drawn-out glass tubing and fitted with a rubber teat was used both for this operation, and for the subsequent transfer of the organisms. The protozoa were kept under observation during these operations by watching the results with a stereoscopic binocular microscope. The isolated Colpidium was washed through several changes of sterile culture medium upon sterile microscope slides. Between each washing the pipette was carefully heated in order to keep it sterile. (The operations should be performed in a room with as little draught as possible, to avoid the chance infection of the drops upon the microscope slide. The quicker the operation can be made, the greater the chance of success.) After about six washings, the organism was transferred to a fresh drop of sterile medium, in a two inch depression block covered with a small sterile cover-glass, both of which had been previously sterilised by heat. A trace of vaseline was usually placed between the block and the cover-glass to prevent evaporation of the drop. The process is rather tedious as there is always the chance of injury during the operations as well as other disturbing factors. However, by a process of trial and error, organisms were finally obtained which divided well in the sterile fluid. As the number of colpidia increased, more culture medium was added to the block, taking care not to expose the sterile culture to the air more than was necessary. When about 20 organisms had been obtained, the whole culture was transferred to a small tube, previously sterilised and fitted with a plug in the ordinary bacteriological way. After a good active culture had been grown in this way, it was found possible to subculture into ordinary size, $6 \times \frac{5}{8}$ inch, test-tubes fitted with cotton wool plugs and containing about 10 c.c. of sterile medium. One or more drops of protozoal culture were subcultured.

Some stress is laid upon the relation of the volume of the medium to the number of colpidia growing in it at these early stages, as it is believed that this is an important factor. Many attempts have been made to grow individuals isolated in small test-tubes in about 1 c.c. of medium, all of which have been failures. It is suggested that in order that growth should proceed, the organism has to modify its environment, probably by adding to it some synthesised substance in sufficient concentration for growth. The matter must be closely related to the so called "bios" effect with yeast. When a sterile medium containing 20–40 organisms has been obtained, the subsequent subcultures are easily made.

Out of several strengths of the medium the following one, Table I, gave a successful culture.

TABLE I.

	p.c.		p.c.
Sodium chloride	·06	Glucose	·03
Potassium chloride	·0014	Histidine	·01
Calcium chloride	·0012	Arginine	·01
Basic sodium phosphate (Na_2HPO_4)	·0001	Leucine	·01
Acid potassium phosphate (KH_2PO_4)	·0001	Ammonium lactate	·003
Magnesium sulphate	·001	Ferric chloride ...	Trace
Sodium bicarbonate	·002	Potassium iodide ...	Trace
Phenol red	Trace	Manganous chloride	Trace

The substances were made up with glass-distilled water. The constituents were autoclaved separately, and the final mixture sterilised by heating to 80° C. on three successive days. The reaction was adjusted (3) to $p.H = 7{\cdot}4 \pm 0{\cdot}4$ with NaOH N/100. The indicator, phenol red, was added to show any changes in reaction occurring during growth. It has a convenient range round about the neutral point and does not affect the protozoa.

Preliminary trials in hay infusions of various reactions showed that the organism grew best when the reaction was initially $p.H = 7{\cdot}0 - 7{\cdot}4$, about the reaction of human blood, though variations of 0·6 did not seem to make much difference. D. Dale (3) has shown that the limiting acid and alkaline reaction for the growth of *Paramœcium caudatum* is between $p.H$ 5·0 and 9·0.

In the later work a silica pipette was used in place of glass, made of drawn-out ¼-inch silica tubing. It had the great advantage of standing heat and does not contribute doubtful substances to the media. In all cases, unless stated, glass distilled water was used. In the earlier experiments this was obtained by distilling tap-water to which baryta had been added. In the later experiments where it was desired to eliminate the possibility of organic nitrogen, tap-water was distilled from alkaline potassium permanganate, and the middle portion of water coming over used. The method of obtaining the special water used in the potassium research is described below. The chemicals were Kahlbaum's practically throughout. In the experiments upon the inorganic constituents, Kahlbaum's "Zur Analyse" salts have been used.

The successful culture was tested for bacteria in the ordinary bacteriological way. Drops from the growing culture were transferred with a platinum loop to the following four media, 1 nutrient broth, 2 nutrient agar, 3 glucose agar (anærobic) and 4 litmus milk.

Preparation of media. 1. Nutrient broth. 1 pound of bullock's heart was minced and 1 litre of tap water added. The mixture simmered for one hour, was boiled for 30 minutes,

filtered, neutralised, with N soda. Peptone was then added to a strength of 1 p.c. and NaCl to ·5 p.c. It was then heated, neutralised with N soda to $p.H = 7{\cdot}4$ and heated, filtered, put up into test-tubes and sterilised on three successive days at 100° C.

2. Nutrient agar. The above + 2 p.c. agar added.
3. Glucose agar 2 + 1 p.c. glucose (ærobic).
4. Litmus milk. Separated milk + neutral litmus.

Subcultures upon 1 and 2 were left in the cold (15° C.) for two days, when they showed no sign of bacterial growth. They were then incubated at 37° C. and no growth was obtained in a fortnight. On the second day the broth cultures were subcultured upon 2, and put in the incubator at 37° C. showing no growth after a week.

No growth could be obtained with media 3 (anærobic) and 4 in the cold or heat. Control cultures on these media in cold and heat, made from the original hay infusion from which the paramœcium was isolated, showed a good growth in 16 hours.

After the failure of growth on the ordinary culture media, some slant tubes of media made from the amino-acid culture medium stiffened with agar were set up. Subcultures upon these from a growing culture produced no result.

A further control experiment was done in the following way. Colpidia will pass through one filter paper, but not through two. A filter funnel containing two filter papers was sterilised. A growing culture of colpidia was then filtered through the sterilised filter papers into a sterile test-tube. The filtered medium contained no visible colpidia. About 1 c.c. of the filtered medium was put into a fresh tube containing 10 c.c. of the same medium. This tube was fitted with a cotton-wool plug, and covered loosely with tinfoil to avoid the entry of dust. (Previous experiment has shown that colpidium will grow under similar conditions.) After a month no change could be detected in the medium. Seeing that the filter papers used were large enough to pass colpidia in one layer and would also let through *B. subtilis* cultures in two layers it seems unlikely that any other organism would not come through. This therefore indicates that there are no other organisms present which can be cultured apart from the colpidia. Microscopic examination using the ordinary bacteriological stains failed to show any bacteria, even after crushing the protozoa.

We were, however, puzzled for a time over the following fact. In certain cases (especially at the beginning of the work) an examination of the living culture in a hanging drop preparation under the 1/12 oil immersion lens showed the presence of rod-shaped, apparently motile bodies. Upon cursory examination they rather resembled bacteria. When watched carefully, however, it could be seen that their movements were restricted at one end, they appeared to be fixed to the cover slip. Often they occurred in groups. They were never observed to divide. They could not be fixed by heat. When fixed by osmic or iodine vapour in

chloroform, they were difficult to stain. It was suggested in the preliminary account that they were knocked off cilia, and this explanation has received confirmation.

Firstly. After subculture in the ammonium glycerophosphate medium for four months, an examination of the living cultures showed none of these bodies. This was not understood until a chance experiment upon a phosphate deficient medium gave a clue to the matter. In this medium, on the seventh day after sowing, it was noticed that the protozoa were apparently rounding off and forming cyst-like bodies. An examination of the culture in the hanging drop preparation showed the presence of many of the rod-shaped bodies, which were not present in the control culture. This seemed confirmatory evidence of protozoal origin.

Secondly. It was found that if a hanging drop preparation from a colpidium culture containing none of these bodies was made in a chamber through which CO_2 could be passed, and if CO_2 was passed through the chamber while the preparation was observed under the 1/12, as the protozoa disintegrated, these bodies appeared. This disintegration in some cases leads to the splitting off of patches of epithelium with cilia attached to them. The direct observation of disintegration seems to afford the strongest proof that the bodies observed arose from the protozoa themselves. The fact that the disintegrating action of CO_2 produces these bodies so quickly seems to put out of court a possibility that they might be symbiotic or parasitic organisms, similar to those described by Petenschko (4) and others for paramœcia. The possibility that they are some hitherto unknown form of colpidium itself seems unlikely. For if they were, growth of colpidia might well have been expected in the filtered culture.

The weight of the evidence is that the cultures really contain no other organisms than the one in question. This is to a certain extent confirmed by the appearance of chance infections during the course of the work. At times cultures have been found to contain bacteria when tested upon nutrient agar. They are in most cases easily picked out by the naked eye as they show the cloudiness usually seen in a bacterial culture. Cultures of colpidia generally look fairly clear unless they contain exceptional numbers of the organism (10,000 or more per c.c.).

It must be confessed that it has been a surprise to find that it was possible in an ordinary laboratory to keep a strain of protozoa sterile for so many months in spite of the many subcultures made. The technique has been carried out carefully with bacteriological methods, but even then it would seem that there must have been some other element at

work—either the killing of small chance infections by the colpidia themselves, or the fact that the media used were not on the whole suitable for the growth of organisms like *B. subtilis*. This was certainly true for the amino-acid media. A sudden clouding of the medium with bacteria is usually followed by the disappearance of the protozoa. This type of effect has been observed by others. Thornton and Smith (5) found that they obtained the best growth of Euglena by sowing into a culture medium in which the bacteria could not multiply fast. An attempt, however, to find out whether colpidia could eat up bacteria when added to the culture was not successful. The bacteria were still present at the end of some days and the protozoa were much diminished[1].

Nutrition. The growth of colpidium in culture media containing only simple inorganic and organic substances raises the interesting question as to the mode of nutrition in such cultures. Mr Dobell informs me that it is generally accepted that ciliates feed upon solid food. In these cultures, the question of nutrition could not be settled without experiment directed to the point. Ultimately it seems clear that the food material comes from simple substances in solution. Is it absorbed from the inside or the outside? A growing culture usually contains after some time a mass of debris, excreta and otherwise, from the colpidia. This may well serve as a source of nutrition, in the sense that substances in solution condensed in the particles may be swept into the organisms; but the very great growth in clear media (up to 20,000 per c.c. in some cases) would suggest that there is also some direct absorption. That they are not growing upon the bodies of killed bacteria in the distilled water from which the media was made up, is proved by the following considerations. First, if they were, selective growth on different carbon sources should not take place. Secondly, the distilled water was in most cases used directly, and experiments upon the ammonium glycerophosphate medium, in which it was filtered before sterilising did not give different results. Thirdly, distilled water put up in the ordinary way and kept for a fortnight when tested for bacteria gave no growth on planting out 1 c.c. portions upon plate culture media.

The cultures have been grown at laboratory temperature ranging from 15–24° C.

Change of reaction during growth. During the process of growth the

[1] Attempts to apply the same method of culture to *Paramœcium caudatum* have not yet been successful. Though this organism has been freed successfully from bacteria, it has not been possible to free it from a small flagellate. Whether this is a case of symbiosis or parasitism has not yet been determined.

reaction in most cases changed. A medium containing ·06 p.c. NaCl and ·06 p.c. ammonium glycerophosphate for instance goes acid during growth. On the other hand if the medium contains ·03 p.c. NaCl and ·03 p.c. NH_4Cl instead of ·06 p.c. NaCl, the reaction goes alkaline during growth. In the former medium the protozoa tend to bunch at the bottom of the culture tube and in the latter at the top. This must be in some way an attempt to get to the neutral zone because, if a tube of the latter medium is inoculated and not disturbed, and the initial reaction is made slightly acid, the top of the medium will turn pink before the lower part changes. (Phenol red is yellow when acid and pink when alkaline.) The colpidia will be seen in the greatest numbers at the point where the pink joins the red fluid. The orientation of these protozoa to hydrogen ion concentration seems to show that they are not so markedly affected by oxygen concentration, as has been observed to be the case for certain other flagellate organisms.

Methods of estimating growth. It was found at a comparatively early stage that the following medium (NaCl ·06 p.c., KCl ·001 p.c., $CaCl_2$ ·002 p.c., $MgSO_4$ ·001 p.c., ammonium glycerophosphate 0·06 p.c., phenol red trace) made with glass distilled water and put up in amounts of 10 c.c. into test tubes would suffice for the needs of growth. In this medium ammonium glycerophosphate serves as the nitrogen, carbon and phosphorus source. It will be known throughout as the "ammonium glycerophosphate medium." The strain of colpidia have been kept going upon it for a period of four months by frequent subculture, so that it apparently contains all the essential constituents for growth. The colpidia in each subculture do not get appreciably smaller as judged by the length at the time of maximum growth. If a test-tube containing 10 c.c. of this medium is inoculated with a drop from a sterile culture, growth will take place in the course of two or three days. The growth will vary a bit with the temperature tending to be rather faster at 23° C. than at 18° C.

In order to get comparative results, it was necessary to obtain a technique for estimating the growth. Two methods were used throughout the research.

1. *Approximate method.* An approximate idea of the growth was obtained by shaking the culture tube and placing it in a parallel-sided glass trough almost the same size as the test-tube. The trough was then filled with water and the contents of the test-tube examined with a binocular microscope, using a 1-inch objective. With some practice a rough judgment of the growth can be made. The degree of growth can

be marked out of 10, where 10 represents the maximum growth possible. The results are taken by one observer and marked down by the other so that in this way an independent judgment is got. The daily results are then charted. Fig. 2 shows such a chart, upon the ammonium glycerophosphate medium. The ordinates represent growth 10 divisions = 10 (maximum growth). The abscissæ represent days. The vertical arrows represent the making of a new subculture. It was found that the organisms did not subculture certainly unless there were 20 or more transferred in the drop to the new culture tube of 10 c.c. Cultures marked as 2 or more by judgment were usually good enough to subculture.

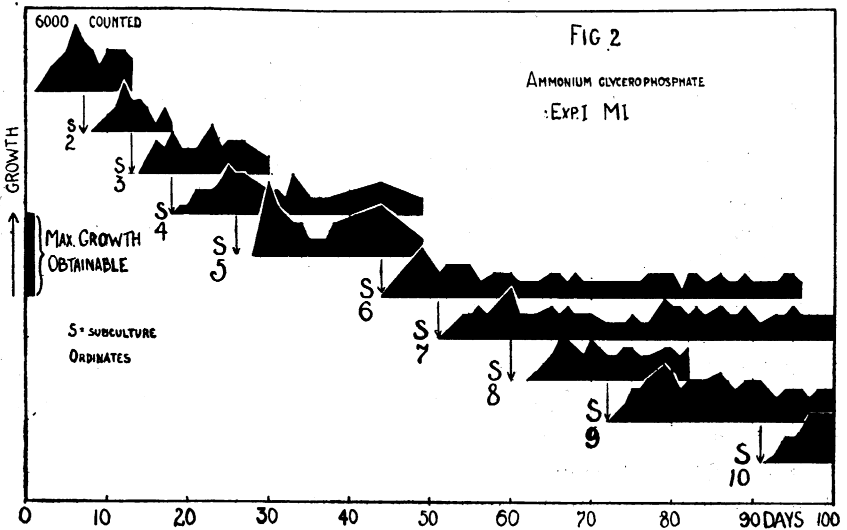

Fig. 2. Exp. 1. Series of subcultures on ammonium glycerophosphate medium (M 1).

Those marked 1, and it was the practice to mark them "one" if they showed life, did not always subculture.

There are two main sources of error, (1) the personal equation and (2) the fact that the colpidia sometimes bunch together and so are apt to give a false idea of the number present. In many cases, if this was expected, a count by method 2 was also made. A mark of two and under was usually under 2000 per c.c. when checked by counting. A mark of three to about six tended to increase from 2000 to 6000 or 7000 per c.c. Above this 10 meant 10,000 or over per c.c. The maximum ever observed by counting has been 40,000 per c.c.

In spite of its roughness, the method is quite good enough to judge of the time for subculturing. Further it has the great advantage of

speed, and of avoiding any extra risks of contaminating the culture liable to occur if the tube is opened and some of the fluid withdrawn.

Fig. 2 shows the following things. For any given subculture there is a rise in growth to a maximum, and then a gradual fall to what is apparently a constant condition. The colpidia are gradually dwindling in this period in size. A few organisms will survive for a long time in one of these sterile cultures. They have been observed after a period of 6 months. In order to analyse what was going on more carefully, about 100 c.c. of the medium was put up in 200 c.c. flasks fitted with cotton wool plugs, and the organisms were counted regularly by the method described below.

In many of the figures showing the daily growth as judged by the ocular method, the culture appears to die down temporarily and then recover. This is especially noticeable in the media which were deficient in some important constituent (*e.g.* M 31, 2, Fig. 10). The result is not purely artificial, as is shown by daily counts of flask cultures, where very large variations in the numbers may occur from day to day. It would seem that growth takes place up to a certain limit, then there is some kind of failure. Some organisms survive and are able to use the disintegration products of the others. This is supported to a large extent by the results of the deficient medium M 31, for instance.

There may also be the additional factor of cyst formation contributing to this result. Cysts have often been observed on the walls of the test-tubes during the course of the work.

2. *Accurate method.* The test-tube was shaken, the plug carefully withdrawn and some of the culture removed with a sterile silica pipette. Holding the pipette vertical two drops were allowed to fall respectively into two separate drops of strong iodine in potassium iodide previously placed upon the microscope slide. The iodide both fixes and stains the protozoa, which can then be easily counted under a 1-inch or Zeiss *A* objective. Successive counts in most cases show surprisingly good agreement. The volume of the average drop delivered by the pipette in a vertical position can be found by weighing, and a simple multiplication gives the number of organisms per c.c. of the culture. Duplicate counts do not as a rule differ by more than 10 p.c. At the same time that the counts are made, measurements of the length can be made with a micrometer eyepiece. This should be done immediately before counting, as the organisms are apt to shrink. The average length was taken by estimating the length of 10 organisms taken at random. It has been shown by other observers that length gives the best idea of the size of the organisms. Broad organisms usually mean that division will soon take place.

The course run by a growing culture, Exp. 2, ammonium glycerophosphate medium, upon which counts have been made regularly, is

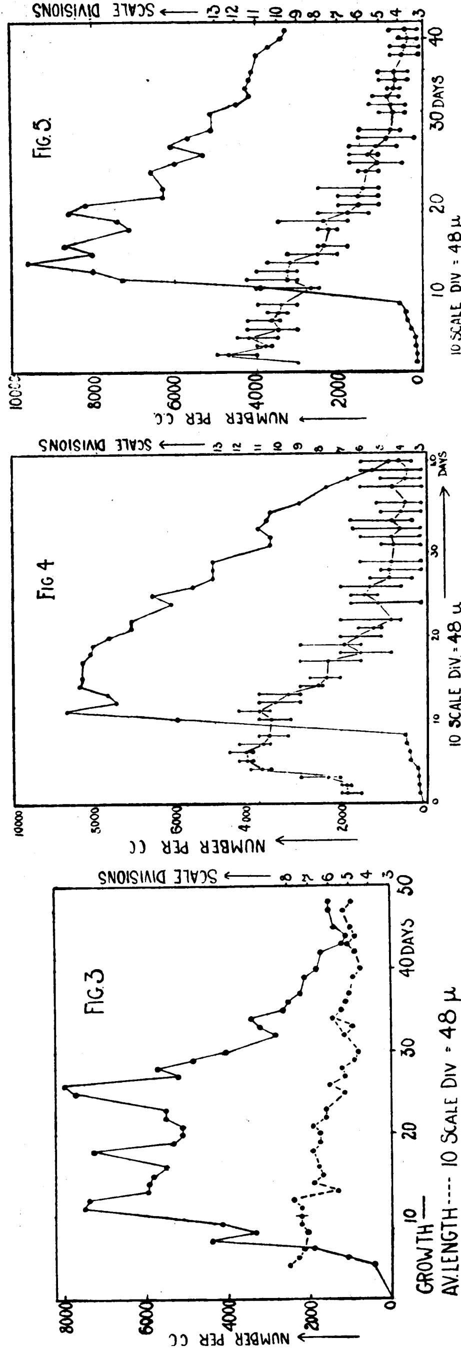

Fig. 3. Growth curve of colpidium culture upon ammonium glycerophosphate (M 1).

Fig. 4. Growth of colpidium culture on Medium 2.
Fig. 5. Growth of colpidium culture on Medium 2.

• Maximum length.
• Average length.
• Minimum length.

shown in Fig. 3. This flask was studied over a period of 48 days. It will be seen that, broadly speaking, the growth can be divided into three periods: (1) 1–12 days inclusive, (2) 13–28 days and (3) 29 days to the end. The first phase shows a gradual increase in the number of organisms up to a maximum of 7500 per c.c. During this time the length kept between 7·2 and 7·9 scale div. During the second phase the number of organisms tends to fluctuate between 5100 and 8000, with a gradual and variable diminution in the length. During the third phase the number of organisms gradually fell until it remained constant at about 1500

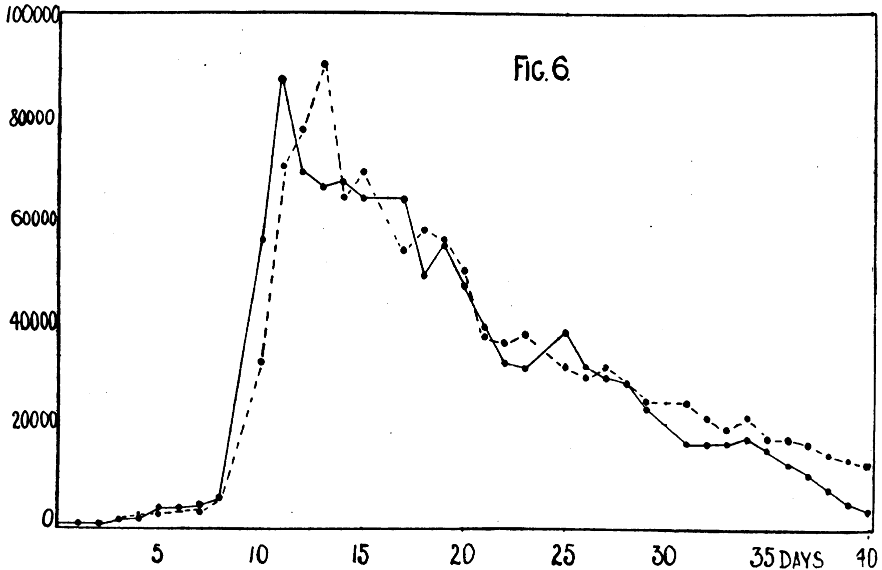

Fig. 6. Variation in living material in colpidium cultures (Exp. 3 and 4). Ordinates = product (no. per c.c. × mean length).
—— Exp. , Fig. 4. - - - - Exp. , Fig. 5.

per c.c. while the length averaged about 5 S.D. The extreme variations found in the lengths of organisms tended to be more in the later stages.

In order to find out what variations were likely to appear between two cultures treated in the same way, two parallel experiments were done with a medium in which a substitution of ·03 p.c. AmCl, ·01 p.c. ammonium phosphate, ·02 p.c. glucose, ·02 p.c. sodium maleate, and ·01 p.c. leucine was made for the ·06 p.c. ammonium glycerophosphate of the ammonium glycerophosphate medium. Figs. 4 and 5 show the results. The curves are very similar to Fig. 3. Fig. 5 lags behind Fig. 4 by about a day, probably due to a slight difference in the number of

organisms originally sown. In Fig. 6 the values (number per c.c. × average length) for Figs. 4 and 5 have been plotted to obtain an idea as to the average content of living material from day to day. It will be seen that the curves for this product follow one another quite closely. They reach a maximum on the 10th–12th day after which there is a gradual fall. An inhibiting factor appears upon the 12th day, which may be due to many causes. It might be due to lack of food material (the most likely cause), or to the accumulation of toxic material in the medium, or to a balance being established between the number of organisms growing or the number of organisms dying. In a large 5-litre culture the inhibiting factor appeared at about 2000 organisms per c.c.

When the subsequent or middle phase starts, the numbers of organisms no longer increase and there is a gradual decrease in size. As the size is decreasing, there are temporary increases in the number of colpidia present per c.c. This would seem to prove that growth is taking place at the expense of the living material already present. For supposing that when the maximum was reached, the organisms merely dwindled in size due to inanition, it would be expected that the curve would gradually come down owing to the accidental death of the individuals. This does not happen. After dropping it rises again, without increase in protoplasm as judged by Fig. 6.

Hence it may be taken that in the organism increase in protoplasm and cell division are two factors capable of being dissociated. Dr E. C. Grey has told me that he noticed the multiplication of bacteria in a poor medium, as they dwindled in size. He thought that there might be division going on at the expense of the protoplasm. These colpidium curves would seem to prove it. A phenomenon of this sort is apparently common in the dividing ovum. Dr Shearer tells me that the first divisions take place without increase in size of the whole mass. The diminution in size due to lack of food material has been observed by many. The most elaborate studies seem to have been made by Jennings(6) and Wallengren(7) on paramœcia. Jennings has shown that diminution in size may be due to several factors among which the most important in this connection is the food material, other causes are of course mainly connected with heredity.

That in the cases of these sterile cultures, there was no permanent change in the size of the organism was readily shown by the following experiment. Colpidia were taken from three old cultures, in which the paramœcia were seen to be small with the microscope. The average length for ten organisms was taken. Subcultures were then made into

three fresh tubes of the same medium. After 5 days the average length of the organisms in the new cultures were taken. Table II shows the result.

TABLE II.

Culture	Length in old culture	Length in new culture
A	3·9 S.D.	8·5 S.D.
B	5·3	10·0
C	5·3	9·0

10 scale divisions (S.D.) = 48μ.

The minimum needs of Colpidium colpoda. With the present technique, it has not been possible to do more than outline the substances which must be added to the water to get growth. Traces of other substances were probably present throughout the research both in the water and in the pure salts employed. This does not really affect the question here, as the object is to find out what substances must be added. If growth will not proceed under the conditions of the experiments without the addition of a given substance, that substance may be considered to be essential. Taking the ammonium glycerophosphate medium as a standard, each of the constituents has been omitted in turn, replacing the missing constituent if necessary with some other substance to adjust the osmotic pressure. It has been found that small changes of osmotic pressure (as represented by the range, ·06 p.c.–·12 p.c. NaCl) are compensated for by the organism. Owing to the time required it has been impossible to do daily counts of all the cultures. In most cases charts of the growth have been made by the ocular method. The real criterion is whether successful subcultures can be made, because the first and probably the second culture upon a new medium will still contain traces of the missing constituent from the original medium. The character of the growth as compared with the control, and whether it was found necessary to resow or not (marked *R* upon the charts) also gives valuable information. In some cases, *viz.* potassium and carbon compounds, the effect of certain substitutions have been tried.

The technique followed was in practically all cases the same as that described for the medium in Table I, 10 p.c., 1 p.c., or 0·1 p.c. strengths of the constituents being made up as was most convenient. It was usually found convenient to make up 50 c.c. of a new medium. From this, four 10 c.c. amounts were transferred by pipette to cleaned and sterilised test-tubes. The test-tubes were cleaned with soda, and nitric acid, washed out with ordinary distilled water and then with several changes of the water used for the experiments. They were then dried, excluding dust as far as possible, fitted with cotton wool plugs and

sterilised at 150° C. for an hour. The test-tubes containing the medium were sterilised in the ordinary way, by steaming on three successive days.

In cases where a batch of media varying in only one or two constituents had to be made up, these latter were made up as a basal mixture ten times the strength of the final medium, to secure uniformity of the constant parts of the medium. 5 c.c. of the basal mixture were then transferred to a 50 c.c. flask, the extra constituents added and the whole made up to the mark with the necessary water.

The four tubes of any new medium were labelled 1, 2, 3, 4, and stored in a dust proof way for use. A subculture was made from a standard or other medium by transferring a drop from the standard tube to tube 1 of the new medium in the ordinary bacteriological way. When the first culture reached a mark of three by inspection, tube 2 was sown from tube 1 and so on with tubes 3 and 4. In the second tube the percentage of the original medium for a drop of 1/25 c.c. would be 0·04 p.c. Anything added to the medium by the colpidia in the course of their growth is not eliminated by this method. This could only be done by washing several times with the centrifuge, a proceeding which would largely increase the chance of infection and disturbance of conditions.

The inorganic basal diet. The following ions had to be investigated, anions Cl, SO_4, PO_4, kations NH_4, Mg, Na, K, Ca.

In the earlier experiments, the mixture of $FeCl_3$, $MnCl_2$, and iodide was added (called iron mixture). Later this was omitted when it was found that the organism could be taken through six subcultures in a medium to which this mixture had not been added. As it has been pointed out above, the fact that these substances did not have to be added does not say that they are not essential to the manifestation of life. Traces of them may quite well have been present in the pure salts used in the work. To obtain cultures it was not necessary to add them specifically. Fig. 7 shows the results of chloride, sulphate, phosphate and ammonium deficiency in duro glass test-tube cultures, together with a control culture (M 3) on the ammonium glycerophosphate medium containing the iron mixture.

Chlorides. M 3, the control grew well and subcultured. In M 4 the chlorides were reduced to the small amounts present in the iron mixture by substituting for $CaCl_2$ and KCl, Na bicarbonate ·02 p.c., KH_2PO_4 ·002 p.c., $CaCO_3$ ·001 p.c., and raising the concentration of ammonium glycerophosphate to ·10 p.c. Growth was not good, in fact the last subculture hardly grew. In M 5, the traces of chloride in the iron mixture were removed by leaving out the iron mixture from M 4. A subculture

could not be obtained upon resowing. That this is not due to any toxic effect of the new medium seems apparent from the fact that when the first subculture was made growth was obtained.

Phosphate. M 6 and M 7, in which ammonium glycerate, ·08 p.c., was substituted for ammonium glycerophosphate, show the effect of leaving out phosphate, M 6 contained the iron mixture, M 7 did not. M 6 did not grow at all in the first two tubes even after a resowing. In the third tube growth was obtained, though the fourth tube, sown from tube 3, would only grow upon resowing, suggesting the carrying over of

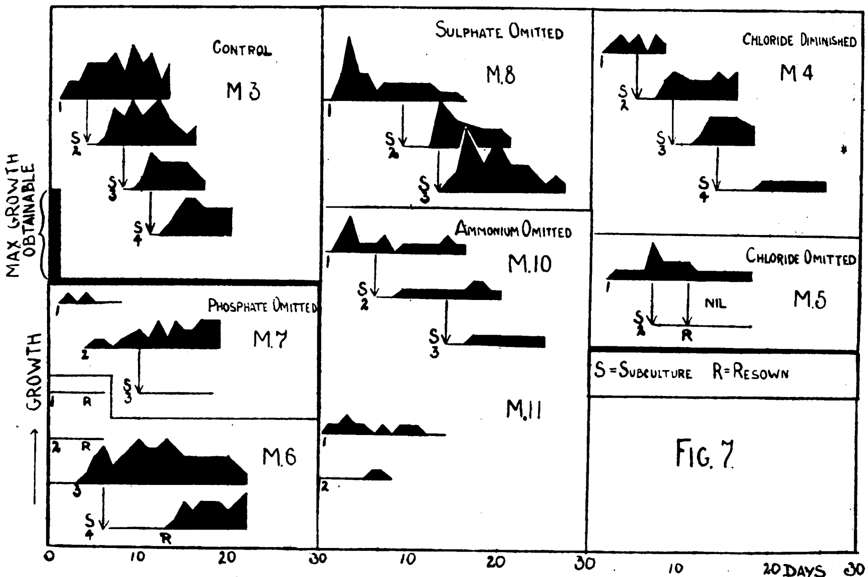

Fig. 7. Effect of deficiency of the medium in chloride, sulphate, phosphate and ammonium. Scale same throughout.

traces of phosphorus from tube 3 to tube 4. In M 7 no growth was obtained in tube 1, though the organisms lived a day or two. A second tube sown grew, but gave no subculture. The contrast with the control is striking. In some later experiments it was found that lack of phosphate led to apparent disintegration of the organisms.

Sulphates. M 8 and M 9, in which $MgCl_2$ was substituted for $MgSO_4$, show the effect of leaving out sulphates. M 8 contained iron mixture, M 9 did not. On the whole the organism seems to get along without any added sulphate. This experiment does not prove whether sulphates are necessary for growth or not, as it is not possible in laboratory conditions

at the moment to be sure that minute traces of sulphur are really excluded. Further experiments with M 9 in quartz tubes made up with the special water described later elicited the same result. There is no doubt, however, that sulphur has been reduced to a very small quantity, without interfering substantially with growth. Other observers (see Czapek) have observed a similar result for certain bacteria.

Ammonium. M 10 and M 11, in which sodium glycerophosphate was substituted for ammonium glycerophosphate, shows the effect of leaving out ammonium. As would be expected growth will not take place. The ammonium is the nitrogen source. (M 11 contained no iron mixture.)

Potassium and calcium. It is convenient to consider these two metals together. It has been considered somewhat as an axiom that for the functioning of certain physiological mechanisms, there should be a certain ratio between K and Ca. At the beginning of the work therefore, the solutions were prepared with the ratio of K/Ca the same as in ordinary frog's Ringer. The results of later experiments showed that this was not necessary.

A series of experiments were made with various relative concentrations of KCl and $CaCl_2$ (40 in all), the range of variation was for KCl from ·00037–·0026 p.c. and for $CaCl_2$ ·00062–·0022 p.c. Between these limits the ratios followed were 0·6, 0·8, 1·0, 1·2, 1·4, 1·6, 1·8, 2·0. There was no substantial difference for growth in the different concentrations.

It was then found that growth would take place quite well in the presence of solid calcium carbonate. Again, keeping the percentage of calcium constant at ·0013 p.c., extra KCl was added to make the ratio $KCl/CaCl_2$, 2·75, 3·5, 5·0, 6·6, 12·0. Growth was normal so far as could be judged though rather slower in starting in the higher concentrations, probably due to an osmotic effect. It would appear therefore that for this organism the concentrations of KCl and $CaCl_2$ can be altered within wide limits. The changes in the medium compatible with growth, argue a wide independence of environment upon the part of this strain.

There followed next the question as to whether potassium and calcium were necessary for growth. In the case of the higher plants, these two elements are certainly essential, and this is evidently the case also for the algæ, and for molds and bacteria (8, 9).

Fig. 8 shows a series of four experiments (Media 12 to 19) in which growth on a control medium is compared with growth on the same medium with the potassium omitted. M 12, M 14, M 16, M 18 were the ammonium glycerophosphate medium without the KCl. M 12–M 15

inclusive contained iron mixture, M 16–M 19 did not. Phenol red was omitted in these and subsequent experiments on potassium, uranium,

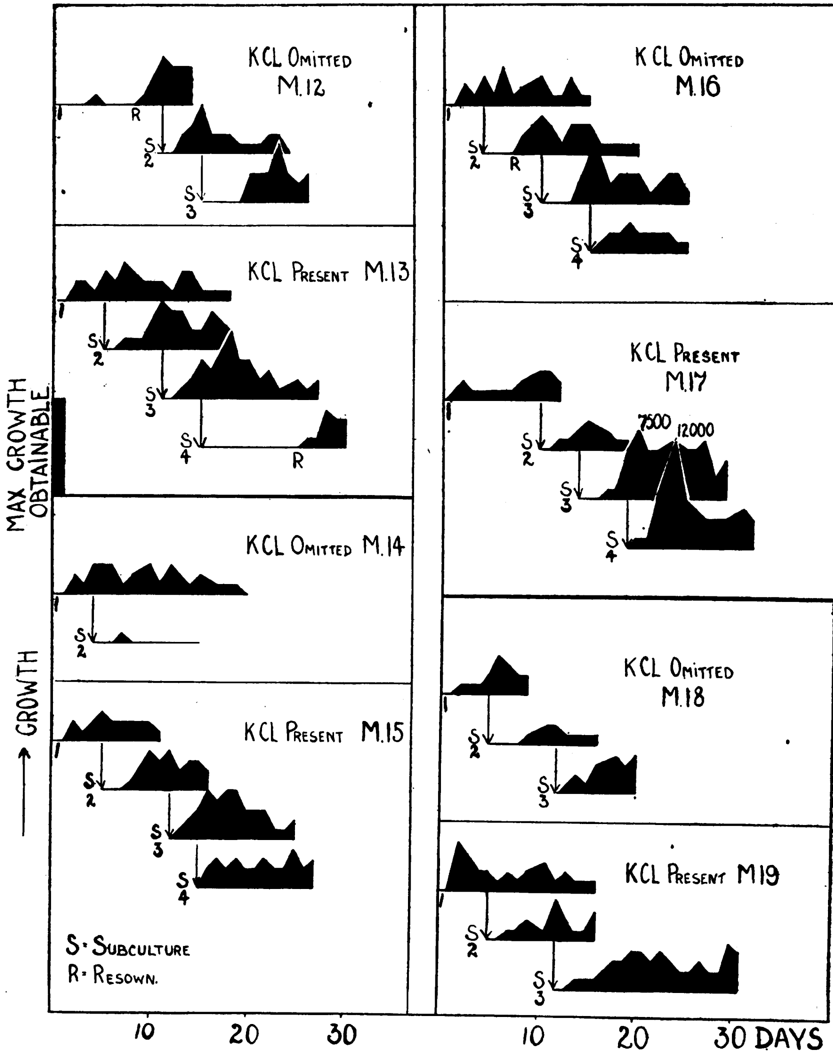

Fig. 8. Effects of potassium deficiency in glass test-tube cultures. Description as Fig. 7.

calcium and sodium. The experiments were made in duro-glass test-tubes. The controls to the series were M 13, M 15, M 17, M 19. It will be seen that the results are somewhat variable. For instance M 13 sub-

cultured well, M 12 subcultured after a resowing. M 15 subcultured, whereas its fellow without potassium would not subculture. M 17 subcultured very well, M 16 not so well. M 19 subcultured well, M 18 was difficult to start in the second culture, so much so that another tube was subcultured in case the culture should be lost. Judging from these results alone, it would be concluded that the organisms grow better with potassium, but could get along without it. The variability of the experiments suggested that there was some other factor at work, and the natural source of error to suspect was the glass.

The experiments were therefore repeated with silica apparatus throughout. Muslin was used to cover the cotton wool plugs and so reduce the chances of traces of cotton-wool finding their way into the media. The media were made up with water distilled through a silica condenser from a Jena flask. The aim in the preparation of the water was to get rid of dust and of metals other than ammonium.

Tap water, treated with alkaline permanganate, was distilled from a distilling flask fitted with a Liebig condenser, and set up in the way described by Grey[1]. The middle third of the water coming over was collected and stored in Jena glass flasks, fitted with corks covered with tin foil. The cork and neck of the flasks were covered with tin-foil and paper to exclude the dust. The water so obtained was then made just acid with HCl and distilled from a Jena distilling flask, 2000 c.c. capacity, into which fitted a Jena glass stopper. The side tube of the distilling flask was passed into the silica tube which had the condenser fitted to it. The silica tube was of $\frac{3}{8}$-inch bore and 30 inches long. Over the silica tube was fitted a Liebig condenser. A piece of tin-foil served to keep the dust from entering the join between the flask and the silica tube. The first portions of water coming over were neglected. The distillation was made when the water was wanted, and the water distilled direct into the vessel used for making up the solutions. In most cases a silica flask was used for this purpose. As far as possible every precaution was taken to prevent the entry of dust during the making and subsequent handling of the media.

The salts used were Kahlbaum's, first grade KCl, $CaCl_2$, $MgSO_4$. Phenol red was not used. The uranium acetate used later was a Kahlbaum sample. The ammonium glycerophosphate was a Kahlbaum sample, but not guaranteed. The solutions of KCl, $CaCl_2$, $MgSO_4$, were stored in silica tubes. The ammonium glycerophosphate was measured direct into

[1] I am able to take the opportunity of thanking Dr Grey for his valuable advice upon the preparation of the water.

the flasks by a drop method from the silica pipette. Any washings needed were done with some of the water stored in a silica flask.

When the experiments upon potassium deficiency were repeated in silica test-tubes, the differences were striking. Fig. 9 shows the results. The series M 21, 1, deficient in potassium, and the control; M 22, 1 and 2, were made up from the same batch of solution, the only difference being the addition of the potassium to the medium for M 22, after

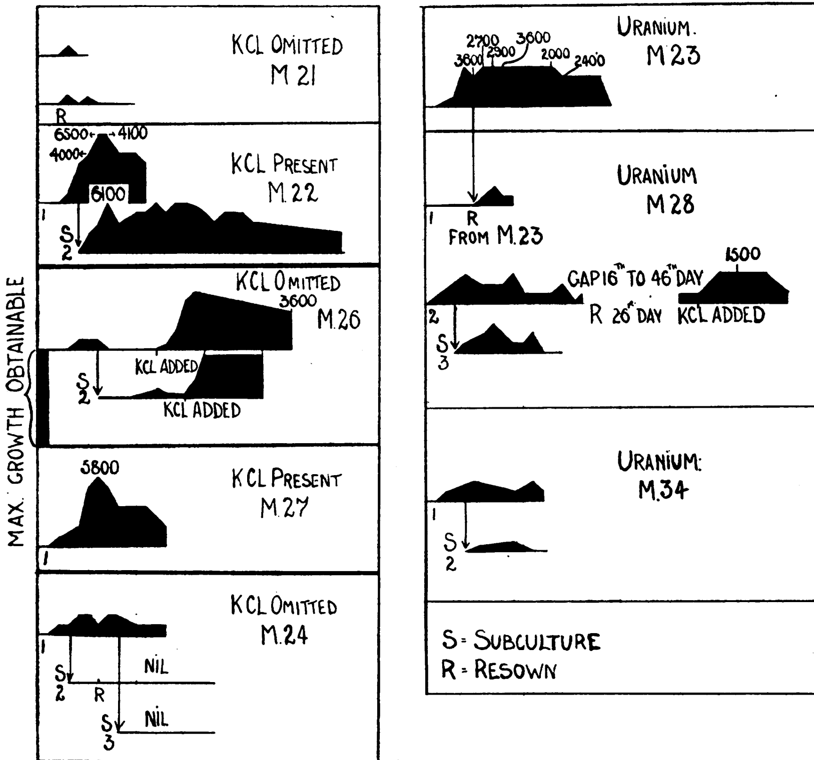

Fig. 9. Effect of potassium deficiency and of uranium substitution in quartz test-tube cultures.

having filled the M 21 tubes. M 22, 1 grew normally and gave a good subculture into M 22. On the other hand in M 21, 1, the colpidia merely survived a few days and then died. M 21, 2, was then sown, and the colpidia died. The latter was resown and the colpidia lived in a poor way for nine days without apparent division. Their movement in the potassium-free medium was very abnormal. They moved very slowly and were inclined to come to a complete standstill upon the bottom of the

tube. Possibly this may indicate a need of potassium for ciliary movement, but at any rate the difference is emphatic.

Another set of tubes—M 26, deficient in potassium, and M 27, the control—showed similar differences. M 27 grew well. M 26, 1, lived in a feeble way for six days after which a subculture was made into M 26·2,

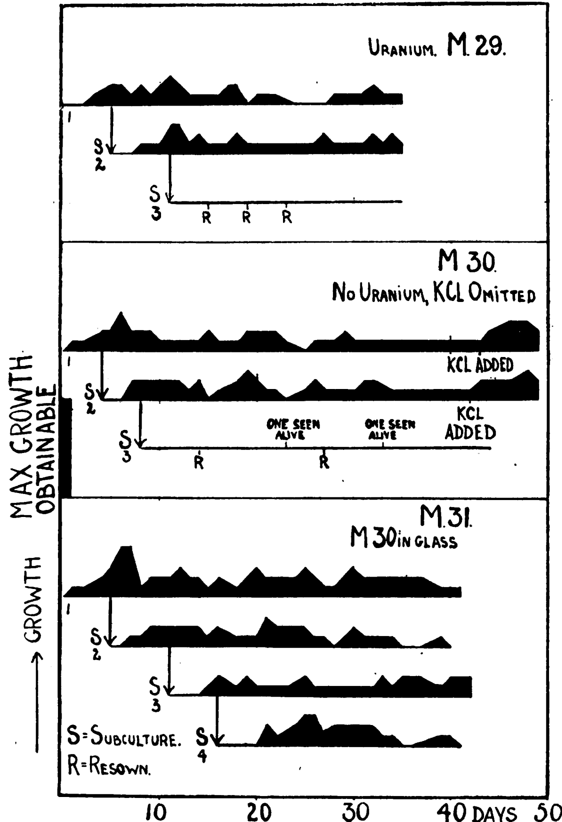

Fig. 10. Effect upon growth of (*a*) uranium addition to a potassium deficient culture in quartz with its control, (*b*) a similar potassium deficient culture in quartz without uranium, and (*c*) the same medium, *viz.* (*b*), in glass. NOTE. KCl was added to M 29·1 at end of period producing the rise of growth shown.

which survived in a feeble way for a few days. Upon the eleventh day after sowing M 26, 1, a trace of KCl was added to it. This was followed by a development of the colpidia up to 3600 per c.c. The experiments prove that potassium is essential.

Experiments were done in which the same potassium deficient medium was put up in both quartz and glass. The remainder of the

medium left after filling tubes M 21, was put up into glass tubes M 24. Fig. 9 shows the effect. There is growth in the first subculture.

M 30 and M 31 (Fig. 10) are another pair of experiments in which the effect of leaving out potassium in glass and silica respectively was tried. The difference in the character of the growth is quite striking, but M 30 in silica did better than the other non-potassium culture in silica (*viz.* M 21 and M 26). This can perhaps be attributed to the contact of the fluid with the glass measuring-flask, though the contact was more than one hour.

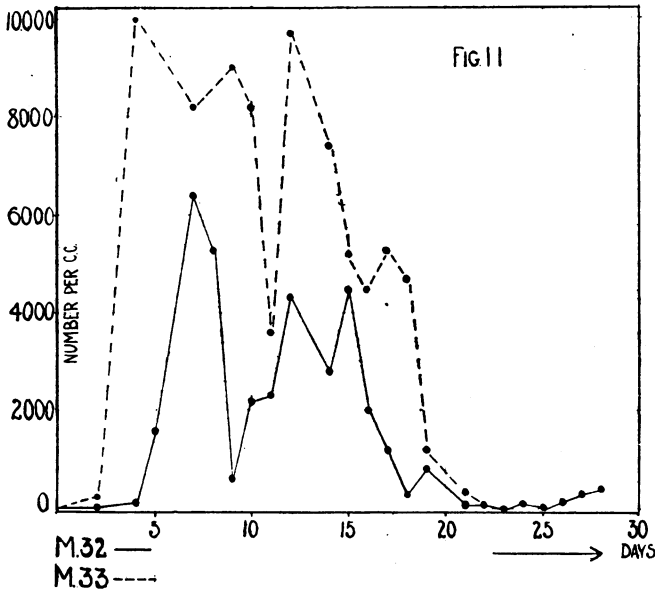

Fig. 11. Potassium deficiency. M 33. Jena glass flasks.
—— M 32 potassium deficient. ---- Control containing potassium.

As a further check upon these results, 100 c.c. respectively of a medium containing potassium and a medium not containing potassium were put up in Jena flasks. The idea was that a difference, that would not show up in test-tubes, might show up in a flask where the ratio of glass surface to solution was smaller. The flasks were counted at regular intervals, and the results are shown in Fig. 11 (M 32 deficient in potassium, M 33). The ordinates represent numbers per c.c. and the abscissæ days. There is a marked difference between Exp. 5, and Exp. 6, though some potassium was apparently being obtained from the glass.

The evidence is that when we really reduce the concentration of

potassium below a certain point growth will not proceed. This brings the protozoon organism into line with the algæ molds and bacteria in this respect.

Uranium. As the result of his experiments upon the heart, Zwaardemaker(10) has concluded that the reason why potassium is essential to the normal activity of the heart is that it provides a source of radio-activity. In the absence of potassium, this source can be provided by other radio-active elements substituted in the right proportions. The idea that in the organism potassium provides a source of radio-activity is a fascinating one. Potassium is necessary for growth, and apparently in its absence colpidia do not move properly. If it could be shown that the function of potassium in the growth process could be taken on by other radio-active elements such as uranium, it would extend Zwaardemaker's view of the effect for the radio-activity of K to other functions of K than those given by him.

The cultures obtained in the course of this work gave a good opportunity of testing whether such a substitution was possible. The history of the work is worth sketching because it shows how an original observation in favour of the replacement being possible was shown to be wrong as the work progressed. A substitution of uranium nitrate for potassium chloride was originally made in four strengths: ·00002, ·0005, ·0001, ·0002 p.c. Good growth and good subcultures were obtained, in fact exceptionally fine growth in the ·0002 p.c. The ratio of uranium to the original potassium for this strength was as 1: 4, almost the same proportion as Zwaardemaker had used. The experiment was repeated with ·0001, ·0002 and ·0003 p.c. uranium nitrate, substituting ammonium phosphate for potassium phosphate. The same success was obtained, and good subcultures. Moreover the cultures were so striking that they could be easily separated from the others with the binocular microscope. A fresh experiment gave the result again. Upon measurement the colpidia were rather larger than normal.

A large number of tubes of two media were then prepared, one M 34*a* containing ·0002 p.c. uranium acetate, glucose ·04 p.c., ammonium lactate ·02 p.c. in place of ammonium glycerophosphate, and KCl; and the other M 34*b* ammonium glycerophosphate. The latter medium was the same as ammonium glycerophosphate medium with the exception that ·0002 p.c. uranium acetate was substituted for the potassium chloride. Subcultures from the uranium culture started these two new batches. M 34*a* grew exceptionally well. It has given consistently higher counts at its maximum than any of the other cultures.

On one occasion a culture containing 40,000 organisms per c.c. was obtained. The colpidia in it were also above the normal size, some, on one occasion were 17 scale div. Upon the medium 34*b* results have been consistently bad, not as good as the results with potassium M 1.

When the effect of potassium dissolved from the glass became known, it was clear that all the above media really contained potassium plus uranium. Some experiments were therefore done in silica tubes. The results are shown in Figs. 9, 10, in which uranium acetate ·0002 p.c. was substituted for KCl. The more carefully the experiments were done, the less evidence was there that uranium would replace potassium. M 23 grew in the first culture differing in this respect from its control M 21. In the second culture M 28, 1, growth was poor, suggesting the potassium deficient cultures. M 28, 2 (control M 27, 1) was sown from a uranium culture in glass containing therefore very little potassium.

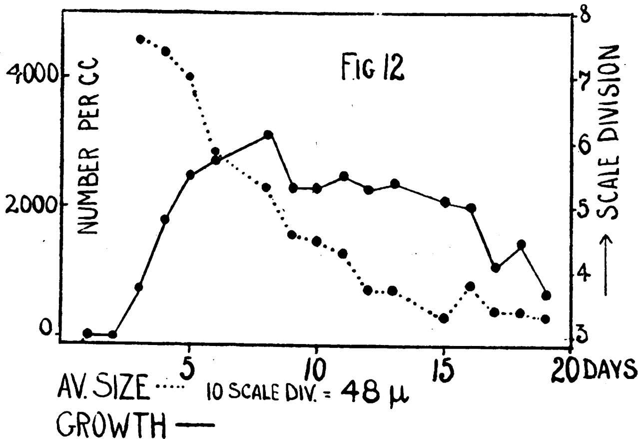

Fig. 12. Uranium containing culture deficient in potassium (control Fig. 3).

The reason why a potassium deficient culture was used in this case was that it might have been argued that it took some time to accustom the organisms to a change from potassium to uranium. (Zwaardemaker always perfuses with potassium-free Ringer first.) Growth was very poor as will be seen from the diagram. A subculture into M 28, 3, died. Before it died a subculture was made into M 29, 1. This dragged on a feeble life, until potassium was added to it, producing increased growth. When subcultured into M 29, 2, the organisms lived without growth so far as could be judged, and a subculture from them into M 29, 3, did not grow even when resown several times. Another experiment was done in

which quartz tubes were sown from a culture grown first in the uranium medium in glass test-tubes M 34, 1, 2, Fig. 9. In this case the organisms grew fairly well in the glass. When transferred to the quartz, there was a very slight growth in the first tube and none in the second.

Some experiments in Jena glass flasks upon a larger scale were also done by daily counting. M 35 is one of them. Fig. 12 shows the results. It will be seen that the behaviour of M 35 (Fig. 12) is very different to that of Fig. 3, which was a control to this experiment. The maximum growth obtained was never so great. Again M 23, and its control without potassium, Fig. 13, show not much difference between the potassium deficient and the uranium containing culture. At any rate there is no

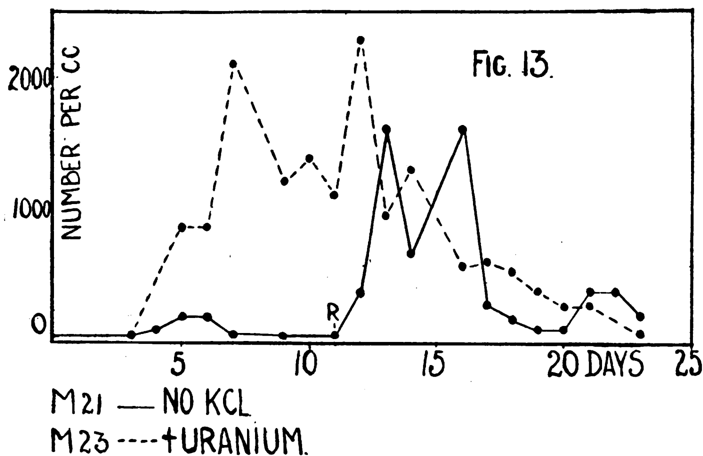

Fig. 13. Growth of culture deficient in potassium with and without the addition of uranium.

effect comparable to that of having potassium present. A further test was made in a silica flask to avoid any glass errors. A sample of M 29 was put up into a 100 c.c. silica flask. This was sown from the flask in M 35, Fig. 12, to allow for any possible acclimatisation effect that might be needed. The organisms as judged by counting lived for a few days without increasing in numbers.

The fact that subcultures cannot be made upon the uranium medium, where uranium is substituted for potassium, shows that uranium will not replace potassium in the growth process of this organism.

Is there any evidence for a partial replacement? A reference to the charts shows that M 23, 1, grew better than the control without potassium, M 21, 1. The same happened for M 28, 1, the control being M 26, 1.

There was not much difference, however, between M 29, 1, and M 30, 1. In the flask cultures the same thing tends to appear, a slightly better growth in the first subculture with the uranium than without it. The effect is not much but it is fairly constant, and unless there are traces of potassium in the uranium acetate sufficient to affect the cultures, it appears that uranium is having a slightly stimulating effect in the first subculture. This cannot be regarded as a replacement.

The fact that uranium cannot replace potassium in the growth process of *Colpidium colpoda* shows that Zwaardemaker's conclusions as to the function of K in connection with the heart and other specified functions cannot be extended directly to all biological functions of this element. If the radio-activity of K is the property which gives it peculiar value in the growth process, we should have to believe that this element is specially adapted for supplying the radiant energy. It would be necessary to postulate a two-fold function for potassium, (1) radio-activity, (2) chemical and physical. In the absence of any evidence upon this point, it seems better to conclude that K is of use *per se* in growth. This is a similar conclusion to that reached by J. Loeb (11) in a paper published after the completion of this work. Radio-activity will stimulate growth. Redfield (12) has shown that a moderate stimulus of β-rays stimulates division in Nereis eggs. A larger dose has the opposite effect.

The same type of observation has been made by Richards and Good (13) for Cumingia eggs.

The fact that the best cultures have been obtained by the addition of traces of uranium (M 34 *a*) suggest some stimulating effect. This has been observed by other workers empirically. Sasaki (14), for instance, added traces of uranyl phosphate (as a growth catalyst) to his media when working with *B. coli*. The increase in length of the protozoa in the uranium and potassium cultures suggests that uranium is stimulating growth, and slowing division rate.

Calcium. As in the case of potassium, experiments in glass test-tubes showed that it was not necessary to add calcium. The experiments were then repeated in quartz. The omission of calcium from the media set up in quartz tubes with silica distilled water was without effect. In one case organisms were taken through four successive subcultures in quartz and then into 70 c.c. of the calcium deficient medium in a quartz flask. After 16 days' growth in this flask there were 6000 colpidia per c.c. in the culture. The culture medium and protozoa were then evaporated in the quartz flask down to a bulk of ½ c.c. and a spectroscopic wire test

for calcium done, which proved negative, indicating the presence of less than $\frac{1}{10}$ mgm. of calcium.

It therefore appears that down to the limits of experiment, it is not necessary to add calcium to get growth. As this is hard to believe, it seems well to consider the amount that might reasonably be expected to be necessary.

The length of a colpidium is about 60 μ and the breadth 20 μ (taking rather high values), from which it may be calculated that 100 c.c. of a 10,000 colpidia per c.c. culture cannot contain more than ·02 gm. of organism. The amount of potassium in blood corpuscles is given as about ·2 p.c., so that we might on the same basis expect to find ·2 p.c. $\times$ ·02 gm. of K in our culture, or ·00004 gm. K p.c. If the calcium percentage in the cell is of the same order as potassium, about ·00001 gm. p.c. Ca.

It is clear from this type of calculation that experiment upon the lines indicated can hardly settle whether calcium is necessary or not. It only seems remarkable that it should have been possible to demonstrate the need in the case of potassium. We must remember that there are two salt needs to consider: (1) the amount needed for structure, etc., inside the organism and (2) the amount needed in the environment immediately outside the organism for the maintenance of function at the surface layer.

Magnesium. Several experiments were made upon the effects of leaving out magnesium in glass test-tube cultures. One with M 36, Na_2SO_4 substituted for $MgSO_4$, shown in Fig. 14, is a good example. The cultures grew normally. Other experiments were M 37 in which $MgSO_4$ was left out, and which did not subculture well. A rise of concentration of $MgSO_4$ to ·01 p.c. was without harm. A repetition of the experiments under quartz conditions, gave distinctly poor results in magnesium deficient media, so that it appeared that the limit for magnesium deficiency had been reached.

Sodium. Fig. 7 shows the effect of leaving sodium out, ·02 p.c. ammonium chloride being substituted for NaCl, in media set up in Jena glass tubes. It will be seen that bad effects did not follow the cutting down of the sodium from ·06 p.c. NaCl to the traces not removed from the medium. In two media M 42, and M 43, the concentration of NaCl was ·01 p.c. and ·20 p.c. M 42 did well, showing a count of 9000 per c.c. in the second tube. M 43 did not subculture well and showed abnormal movement in the second culture (probably owing to change in the osmotic conditions).

In quartz a sodium-deficient culture was taken through two quartz

cultures and showed a growth of 6000 organisms per c.c. (full length) in the second tube. The limit for Na deficiency has therefore not been reached.

The inorganic requirements. Summarising the inorganic experiments, the experiments conducted in glass show that to obtain a growth of *Colpidium colpoda* ammonium, phosphate, chloride, must be added to a medium to get growth. The experiments in quartz, show that potassium

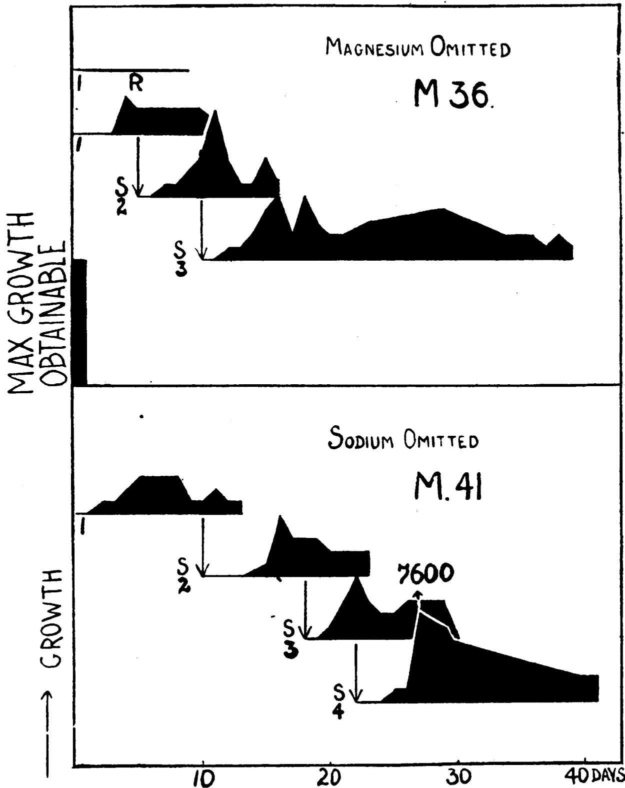

Fig. 14. Effect of magnesium and sodium deficiency in glass.

and magnesium must also be added. The necessity of sodium, of sulphate and of calcium has not yet been demonstrated. In all the experiments silica has been a constant factor.

We have therefore arrived at some knowledge of the basal inorganic constituents for this organism. Traces of other substances (in amounts below the accuracy of the work) are most probably required by the

organism, and when the work is conducted upon a larger scale, it may be found necessary to add other substances possibly supplied by the glass or water or air when working upon the small scale. All that has been proved is that from a practical standpoint, when working upon a scale not exceeding 500 c.c. cultures, if the above elements are present, any other impurities which may be required by the organism for its growth will be present too.

It is interesting to note that Lockemann (15) (for the tubercle bacillus) and Benecke and others (8) (9) (in the case of molds and other bacterial species) have found it unnecessary in some cases to add calcium and sulphur to their culture media. The results for colpidium agree with their experiments, but in view of the minute quantities of such substances possibly needed, further experiment is required to settle how far these deficiencies are real.

Nitrogen. The first media used contained amino acids. They seem to be well used by the organism. Three media were tried containing leucine as the nitrogen and carbon source, in strengths of ·05 p.c., ·1 p.c. and ·2 p.c. In the first two strengths good subcultures were obtained. In the last strength, the last subculture was not successful, probably owing to the concentration. When amino-acids are present in a culture, it would seem that growth starts rather quicker. They are also apparently used by the protozoa with greater ease than by bacteria of the *B. subtilis* group so that their use has an additional advantage.

The organic requirements. Several experiments have been done upon the carbon compounds necessary for growth. Table III gives a summary of the results.

This table cannot be considered as more than preliminary. Certain features may however be remarked upon. Growth has not been obtained upon sources of carbon under three carbon atoms. Life however was maintained upon all the media tried so that there was no obvious poisoning effect. Lactate alone did not give growth. Glucose alone gave variable but rather poor growth. Glucose + lactate gave remarkable growth, better than the growth in the standard glycerophosphate mixture.

For future studies upon organisms of this nature, it would appear most promising to work with glycerate, glycerophosphate, or glucose + lactate media, with perhaps the addition of a small amount of an amino-acid such as leucine. Before the ground has been more completely mapped out it would be unwise to attempt generalisations.

In spite of the different media on which this organism has been grown during the course of the 10 months' work upon it, the colpidium does not appear to have changed. Diet has altered the size temporarily;

TABLE III.

Carbon source	Growth	Medium: Variations in place of ammonium glycerophosphate in standard medium
Nil (dissolved CO_2)	Nil	Am. phosphate ·06 p.c.
Monobasic acids.		
Formate (Na and Am.) ...	Nil	Am. phosphate ·0002 p.c., am. formate ·02, ·04, ·06 p.c., Na formate ·06 p.c.
Acetate (Na)	Nil	Am. chloride ·02 p.c., Sod. acetate ·03 p.c., sod. phosphate ·01 p.c.
Proprionate (Na)	Fair	Am. phosphate ·001 p.c., Na proprionate ·06 p.c.
Caproate (Am.)	Fair	Na ·001 p.c., am. caproate ·05 p.c.
Palmitate (Na)	Fair	NaCl ·02 p.c., AmCl ·04 p.c., Na palmitate ·06 p.c., am. phosphate ·001 p.c.
Monobasic hydroxy acids.		
Glycollate (Na)	Nil	NaCl ·02 p.c., am. phosphate ·001 p.c., am. chloride ·02 p.c., Na glycollate ·06 p.c.
Lactate (Am.)	Nil	Am. phosphate ·001 p.c., am. lactate ·05, ·1, ·2 p.c.
Glycerate (Am.)	Good	Na phosphate ·001 p.c., am. glycerate ·05 p.c.
Pyruvate (Na)	Good	Am. phosphate ·01 p.c., Na pyruvate ·05 p.c.
Dibasic acids		
Succinate (Na and Am.) ...	Nil	(1) Na phosphate ·001 p.c., am. succinate ·05, ·1, ·2 p.c. (2) NaCl ·02 p.c., am. phosphate ·001 p.c., am. chloride ·04 p.c., Na succinate ·06 p.c.
Oxalate (Am.)	Nil	Am. phosphate ·001 p.c., am. chloride ·02 p.c., am. oxalate ·06 p.c.
Tartrate (Am.)	Fair	Am. phosphate ·0002 p.c., am. bitartrate ·02, ·04, ·06 p.c.
Tribasic acids.		
Citrate...	Nil	NaCl ·02 p.c., am. phosphate ·001 p.c., am. chloride ·02 p.c., Na citrate ·06 p.c.
Various.		
Glycerophosphate (Am. or Fe)	Good	NaCl ·02 p.c., am. phosphate ·001 p.c., Fe glycerophosphate ·06 p.c.
Sugars, etc.		
Glucose	Poor	Na_2HPO_4 ·001 p.c., glucose ·06 p.c., am. nitrate ·01 p.c.
Glucose + succinate	Very poor	Na_2HPO_4 ·001 p.c., glucose ·04 p.c., am. succinate ·01 p.c.
Glucose + lactate	Very good	Na_2HPO_4 ·001 p.c., glucose ·04 p.c., am. lactate ·01 p.c.
Cane sugar	Good	Am. phosphate ·001 p.c., cane sugar ·06 p.c.

lack of food has led to a temporary diminution in size, but this has always been retrievable by fresh subculture into a standard medium, when organisms of average length have been obtained at the maximum of the new culture. Provided that the organism has grown, it has maintained the structure which it has reached in the course of its evolution. Change in chemical environment has not so far as can be ascertained produced real biological change. The factors, whatever they are, which

determine the structure of the organism, are apparently more deep-seated.

It is not possible to leave the subject without reference to the work upon the phenomenon of endomixis, worked out recently by Woodruff and Erdmann (16). According to their observations, paramœcia grown upon a uniform bacterial diet and isolated daily after division under conditions where conjugation is impossible, show periodically great changes in the nucleus. About every month a kind of splitting up of the nuclear material takes place followed by a reorganisation upon well determined lines. The process apparently replaces the sexual process, and after the phenomenon (termed endomixis), the paramœcia which divide, show much larger variation than the normal. In this way it is thought lies a means by which the organisms can adapt themselves to a fresh environment. Changing their environment causes endomixis to set in. It is of course not possible to say that this type of phenomenon has not occurred during the work done upon this colpidium. Many specimens have been stained and none of them have shown the characteristic changes described. It is rather likely that an alteration of osmotic environment would be the precursor to such a change and this factor has been kept fairly constant throughout.

Discussion.

In a current physiological text-book, the following statement is made: "the animal organism even in its lowest forms the protozoa, is satisfied with nothing less complex than glucose as a source of carbon (17)." It seems to be generally received opinion that most of the protozoa require bacteria as part of their food. This has been shown to be incorrect for *Colpidium colpoda*. A simple ammonium salt will supply it with nitrogen, and its carbon needs can be met with glyceric or proprionic acid. Up to the present the condition has not been realised in which this ciliate will utilise sources of carbon under three carbon atoms. Glycollic acid even has not been used, so that it is not merely a question of the presence of an oxidisable group. During the process of growth there is apparently a synthesis of fat and it is hoped to obtain some quantitative data upon this by working upon larger cultures.

It is clear that this protozoon organism is widely independent of its environment. From the biological standpoint this is largely what would be expected, as the colpidium must depend for its existence upon the chance and variable composition of rain puddles, etc. In these conditions from its nutritional needs it apparently is well equipped for

dealing with changing situations. What then is its place in nature? Apparently it is much more close to the autotrophic flagellate than many bacteria, but the absence of chlorophyll means that it is dependent ultimately upon the green cell for its needs. It is felt that the use of pyruvic and glyceric acids for growth, when contrasted with the non-utilisation of lactic acid suggests an unexpected relationship to the yeast cell, though the point cannot be pressed at present. The utilisation of ammonia by the cell in the synthesis of nitrogen compounds seems to be generally true for living cells (Boas (18)).

Comparative work upon the needs of other pure protozoal cultures should give material for fresh generalisations. Organisms of this nature are likely to prove of value for the study of several biochemical problems, such as the evolution of proteins, synthesis of fats, and possibly the relation between carbohydrate and fat. Though they do not multiply so quickly as bacteria, they have the great advantage of being easily kept under observation. Direct counts are easy and so are estimations of size. An extension of this type of work to the pathogenic protozoa should elicit more exact knowledge of the character of the toxins produced in disease, as well as giving a clear field for work upon life histories.

Summary.

1. Pure cultures in synthetic media of a ciliate protozoon, *Colpidium colpoda* (Ehrbrg) Stein, have been obtained from a single individual and studied for a year. Evidence is brought forward, which is regarded as conclusive that these cultures contain only the organism in question.

2. Upon a medium consisting of glass distilled water, calcium, potassium and sodium chlorides, magnesium sulphate, and ammonium glycerophosphate, test-tube cultures have been kept going by frequent subculture, without diminution in the size of the organisms. Rather better results are got by substituting glucose and ammonium lactate, as nitrogen and carbon sources.

3. By counts and measurements of size, it has been found that the average maximum for growth under these conditions is 8000–10,000 organisms per c.c. The growth curve shows three phases: (1) a rise to a maximum number, (2) a more gradual fall to (3) a plateau, during which life may continue for some time. Phases (1) and (2) last about 12 days. During phases (2) and (3) the organisms diminish in size. During phase (2) temporary rises in the number of organisms may take place.

4. Experiments upon the omission separately of the medium constituents, showed that ammonium, phosphate and chloride could not be

omitted from glass test-tube cultures without stopping growth. Phosphate deficiency in the medium led to apparent disintegration. The omission of potassium and magnesium in glass cultures was without effect, but in quartz test-tube cultures potassium deficiency led to failure of movement and death of the cultures. Magnesium deficiency also inhibited growth. No effect was produced by omitting the sodium, calcium or sulphate separately from the medium, though it is not claimed that traces of Na, Ca or S were not still present.

5. Uranium salts cannot be substituted for potassium salts in the growth process, thereby proving that the biological value of potassium is not solely a question of radio-activity.

6. Amino-acids will serve as nitrogen source in place of ammonium salts.

7. Growth has not been demonstrated on carbon sources containing less than three carbon atoms in the molecule. Glycerate is used, but not lactate or citrate.

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