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NUTRITIONAL STUDIES OF PROTOZOA

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The question of nutrition in protozoa has held the attention of investigators for many years, although when compared to the advances made in bacteriology it is at once realized that little progress has been made in this phase of biology. It was not until bacteria were studied outside the host organism and in pure cultures that our knowledge was greatly enhanced in this field. Since Koch's initial experiments in pure culture work a great deal has been learned about the nutrition of bacteria which has been of infinite value in diagnosis and classification. It was early realized that if much was to be learned about the nutrition of protozoa they too must be grown in pure cultures. Difficulties were encountered from the very beginning because protozoa do not lend themselves readily to the pure culture methods employed in bacteriology. Furthermore, only a very few protozoa are causative organisms in human disease and therefore are not so extensively studied.

The protozoa are higher in the scale of living things than are bacteria and for that reason we might expect their nutrition to be somewhat more complicated. We might also expect to find transitional stages from autotrophic nutrition as found among the green plants to holozoic or saprozoic nutrition as found in animals. As a matter of fact such a situation is observed among the Phytomastigina where chlorophyll-bearing forms such as *Chlorogonium, Haematococcus* and *Chlamydomonas* are entirely autotropic. Within this group also it is noted that *Euglena* functions autotrophically when conditions are favorable but if deprived of light it continues normally, providing certain organic substances are present in its environment (Mainx, 1928).[1] *Polytoma* and *Chilomonas,* colorless flagellates, are members of this group also and their nutrition definitely approaches that of animals. (Pringsheim, 1921;[2] Loefer, 1934[3]). Within one group of protozoa then we see transitional forms ranging from those entirely autotrophic to those well advanced toward the animal type of nutrition.

Considerable mixed culture work has been done among the protozoa — that is, cultures containing several protozoa other than the one in question as well as bacteria and yeasts. In the present discussion the author is concerned only with protozoa grown in pure culture, and by that is meant protozoa of one species grown in artificial media separated from all other living microorganisms. It is the opinion of the writer that problems in nutrition of protozoa must be approached from this angle if they are to succeed. To attempt to

[1] Mainx, F. (1928). Arch. f. Protist. 60:355–415.
[2] Pringsheim, E. G. (1921). Beitr. z. Allgem. Bot. 2:88–138.
[3] Loefer, J. B. (1924). Biol. Bull. 66:1–6.

study the nutrition of protozoa in an environment with other microorganisms invalidates the results to a large extent. Under such conditions one can never be certain whether the effect is due to direct action on the protozoan in question or indirectly through its food supply.

Efforts to grow amoebae in pure cultures have never been completely satisfactory, although some of the first work in pure cultures was done with this group of protozoa. Frosch (1897)[4] succeeded in culturing *Amoeba nitrophila,* a soil rhizopod, on one strain of living bacteria. Oehler (1916)[5] made some interesting observations on several different rhizopods and was able to demonstrate the specific types of bacteria that would fulfill the complete food requirements of certain amoebae. The pathogenic *Endamoeba histolytica* has been successfully cultured in vitro by Cleveland and Sanders (1930)[6]; their cultures were not bacteria-free, however.

The problem of culturing ciliates has been a difficult one and complete success has been achieved only recently. Oehler (1919)[7] was the first investigator to obtain cultures of ciliates growing on dead microorganisms but he was unable to force these infusoria to maintain themselves saprozooically. However, in 1924 he succeeded in isolating cultures of *Colpoda steinii* and *Colpoda cucullus* in spinach broth.[8] In the same year Lwoff isolated *Glaucoma piriformis,*[9] another ciliate. Since that time several others have been isolated including two species of *Paramecium* by Glaser and Coria (1935).[10] The writer succeeded in growing a pure strain of *Colpidium striatum* in 1933,[11] the nutrition of which has been his concern for some time and which will be the subject for the remaining portion of this discussion.

There have been several methods employed in isolating ciliates in obtaining pure cultures. The method here described is one employed by the author and which is a modification of the one used by Parpart (1928).[12] Sterile petri dishes and depression slides containing diluted broth were used to maintain the protozoa until they were almost bacteria-free. With the aid of sterile micropipettes several organisms were washed by allowing them to swim about for several minutes in a dozen or more changes of media. It was a simple matter to continue this process until all the bacteria were removed both from the external portion of the animal and from the food vacuoles. However, in the case of *C. striatum* if all the bacteria were removed the organisms promptly died; it was necessary then

[4] Frosch, P. (1897). Centralb. f. Bakt. 24:926.
[5] Oehler, R. (1916). Arch. f. Protistenk. 37:175.
[6] Cleveland, L. R. and E. P. Sanders (1930). Science. 72:149.
[7] Oehler, R. (1919). Arch. f. Protistenk. 40:16–46.
[8] Oehler, R. (1924). Arch. f. Protistenk. 49:287–296.
[9] Lwoff, A. (1924). C. R. Soc. Biol. 91:344–345.
[10] Glaser, R. W. and N. A. Coria (1935). Am. Journ. Hygiene. 21:111–120.
[11] Elliott, A. M. (1933). Biol. Bull. 65:45–56.
[12] Parpart, A. K. (1928). Biol. Bull. 55:113.

to leave behind a small number of bacteria which were constantly reduced in numbers through subsequent subinoculations. After three months of such culturing the bacterial count was practically nil; by plating and picking off the isolated colonies which were inoculated into tryptone broth tubes, pure cultures were obtained. They have been maintained on Difco tryptone broth media for a period of over four years and are in excellent condition at the present time. As a matter of fact they grow better in the artificial medium free from bacteria than in any bacterized culture which it has been possible to prepare. Cultures of over 200,000 per cubic centimeter are not uncommon.

Once the culture was isolated and growing well it became necessary first to learn something about how environmental factors influence its growth rate. This had to be done before experiments on nutrition could be attempted. It was necessary, for instance, to know over what pH range the organisms grow and at what point or points in the range optimum growth occurs; also it was necessary to know at what temperatures it grows best and what concentration of medium is conducive to optimum growth. These experiments were performed and it was found that *C. striatum* grew best at a pH of 5.8 and 7.4 with low points at the two extremes of the range (4.0 and 8.6) and one at neutrality. This bimaximal growth curve was typical when the ciliates were grown in tryptone broth but became a unimaximal curve in other protein extracts. The optimum temperature for growth was 25 degrees Centigrade, and the best concentration of dessicated media was found to be 1.5%. With these preliminary experiments concluded it was possible to study the effects of certain proteins and their derivatives, fatty acids, and carbohydrates, in an effort to determine the food requirements of these organisms.

The method of determining the effect of various substances on growth was counting samples from culture tubes after a certain time had been allowed for growth. A Sedgwick-Rafter counting chamber and a Whipple ocular micrometer was employed in making the counts. This method proved very satisfactory and far surpassed simple microscopic examination.

In attempting to determine the carbohydrates used in the nutrition of this ciliate it was at first decided to note the sugars which were fermented with acid production.[13] In doing this the basic medium was 1.5% tryptone solution to which sugars were added in 0.5% concentration. The organisms were inoculated and incubated at room temperature for a period of 72 hours. The pH in all cases was set at 6.6 before growth occurred; when fermentation occurred the pH dropped to 5.0 in 36 hours. If there was no acid formation in the tube the pH rose to 7.0 in the same period of time. The results of these experiments are recorded in Table 1 together with those of other investigators included for comparison.

[13] Elliott, A. M. (1935). Arch. f. Protistenk. 84:156–174.

It is obvious that there is a wide range in the ability these organisms possess in powers of fermentation. This is a decided diagnostic characteristic in cases where two animals very closely resemble each other morphologically. For instance, in the case of *Glaucoma piriformis* and the two species of *Colpidium*, where morphological resemblances are very close, one sees marked physiological differences. *Glaucoma* ferments galactose but not starch while *Colpidium* ferments the latter but not the former.

TABLE I.

	Starch	Dextrin	Inulin	Salicin	Melizitose	Sucrose	Lactose	Maltose	Mannite	Levulose	Mannose	Galactose	Dextrose	Rhamnose	Xylose	Arabinose	Cellulose
Colpidium striatum (Elliott, 1935)	+	±	−	−	−	−	−	±	−	+	+	−	+	−	−	−	..
Colpidium campylum (Elliott, 1935)	+	±	−	−	−	−	−	+	−	+	+	−	+	−	−	−	..
Glaucoma piriformis (Colas-Belcour and Lwoff, 1925)	−	..	−	..	..	−	−	+	−	+	..	+	+	..	−	−	..
I Saprophilus oviformis (Glaser and Coria, 1935)	+	..	..	..	..	−	−	+	..	..	..	..	+	..	..	..	+
Paramecium caudatum (Glaser and Coria, 1935)	+	..	..	..	..	−	−	−	..	..	..	..	−	..	..	..	+

+ = Acid formation ± = Slow acid formation − = No acid formation

Concerning nitrogen metabolism it is advisable to learn if possible in what form nitrogen is utilized in this ciliate. Can it use the element in simple form such as that found in inorganic salts or even in single isolated amino acids, or must it have nitrogen supplied in some complex form such as peptones or proteins? Experiments were devised to determine this point.[14] An adequate source of carbohydrates was supplied in the form of a starch in a buffered salt medium; to this was added the nitrogen in several different forms ranging from complete proteins to ammonium salts. The results of this experiment are given in Table 2.

From these results it is obvious that *Colpidium* is unable to utilize nitrogen in the form of inorganic salts and single isolated amino acids, but requires the element in the form of complete proteins, or particularly hydrolized products of certain complete proteins. Incomplete proteins such as haemoglobin, gelatin, zein and gliadin are not utilizable. It is interesting to note that the protein, casein, is not used by the ciliate while the partially hydrolized casein, tryptone, is the best source of nitrogen that has been found. The yeast extract also supplies sufficient nitrogen for abundant growth.

[14] Elliott, A. M. (1935). Arch. f. Protistenk. 84:472–494.

In testing the effect of fats in the nutrition of *Colpidium* it was decided, after several preliminary experiments, to test the effect of two lower fatty acids over a wide pH range. The reason for this decision was that it was suspected that the ionization of the acids might have something to do with their utilization as a carbon source. Acetic and butyric acids were employed and added to the tryptone base in a 0.5% solution.

TABLE II.

	NH_4NO_3	Glycine	dl-Valine	dl-Leucine	dl-B-Phenylalanine	l-Tyrosine	Asparagin	Bacto-peptone	Neopeptone	Proteose-peptone	Bacto-tryptone	Yeast Extract	Beef Extract	Bacto-liver	Bacto-veal	Casein	Bacto-hemoglobin	Bacto-gelatine	Zein	Gliadin
Colpidium striatum	−	−	−	−	−	−	−	+	+	+	++	++	+	+	+	−	−	−	−	−
Colpidium campylum	−	−	−	−	−	−	−	+	++	++	++	+	+	++	+	−	−	−	−	−

+ = Good growth ++ = Excellent growth − = No or very poor growth

It was found that the acids were decidedly toxic below pH 6.0 but above this point they were non-toxic. in fact, in the case of butyric acid actual acceleration occurred. This would indicate that the acids in molecular form are toxic but in the ionized condition are not only non-toxic but may even be utilized as a carbon source for *Colpidium*.

The question of accessory food substances has only been touched upon. Due to the fact that these ciliates have been living on autoclaved media for several years, it is obvious that any substances, vitamins or whatnot, essential for their continued growth, must be heat stable. For that reason the writer has utilized one substance, pantothenic acid, which has certain characteristics similar to vitamin B_2 (G).[15] It was necessary to add the substance in very small quantities to a tryptone base and note the accelerating effect on growth rate of the ciliate. Furthermore, since this growth-promoting substance is an acid it was necessary to observe its effect over a wide pH range in order to determine its influence in an ionized and molecular state.

The growth was doubled in cultures containing pantothenic acid which indicates that this substance has a growth stimulating effect on *Colpidium*. The great dilution of the acid (1 part in 250,000) precludes the possibility that it could be utilized as a source of food. It would appear then that the effect simulates that of a vitamin.

In this discussion the nutrition of protozoa has only been touched upon and what has been learned serves to give us a glimpse of what

[15] Elliott, A. M. (1935). Biol. Bull. 68:82–92.

still remains to be investigated. It is hoped that in the future more protozoa will be grown in pure cultures and that their physiological reactions will be carefully investigated. When this is done protozoology may be said to be on an equal footing with bacteriology. It is the belief of the writer that most, if not all, of the protozoa may eventually be grown in pure cultures and that, in questionable cases of identification, physiological reactions will be relied upon just as in modern bacteriology. As to the practical application of such work it is difficult to predict anything. We are quite certain, however, that it is essential to understand the nutrition of any microorganism, particularly parasitic species, if we are to know the role played in the host. Perhaps such investigations as these with free living forms may add to the solution of problems in diseases caused by parasitic protozoa.

WILL PINE OR ASPEN DOMINATE MINNESOTA FORESTS?

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Aspen originally occupied only 3.5 per cent of the present forest land in Minnesota. It formed a dominant type only along the prairie-forest transition zone in the north, whence in association with oak it invaded the grassland in narrow fingers along the water courses, here and there establishing itself on the more favorable upland soils. In the forest land proper aspen and paper birch were confined chiefly to recent burns and the borders of lakes and streams. Early travelers and surveyors also described an aspen-birch-coniferous type and an aspen-birch hardwood type. These types may still be found today on pine or hardwood lands that have been subjected to frequent fires which resulted in killing their own reproduction as well as thinning out the older trees. Under such conditions aspen was able to invade original white and Norway pine forests. On burned over areas aspen and birch often seeded in contemporaneously with spruce and balsam giving rise to a mixed aspen-conifer type which if left undisturbed tends eventually to revert to conifers.

The advent of man has produced a profound change in the relative dominance of aspen and scrub oak. From an original area of 681,000 acres it has increased to 7,418,000 acres and now occupies 38 per cent of the forest land, an increase of over ten-fold. These figures are no longer a guess but are based upon field notes of the original surveyors and an accurate forest survey covering the entire State of Minnesota which was completed last year.