LXIX. STUDIES IN THE METABOLISM OF PROTOZOA.

I. THE NITROGENOUS METABOLISM AND RESPIRATION OF *BODO CAUDATUS*.

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I. NITROGENOUS METABOLISM.

THE nitrogenous metabolism of the protozoa has hitherto been much more extensively studied from the anabolic than from the catabolic aspect. This is probably due to the fact that information regarding nitrogenous food requirements is likely to facilitate culture of the protozoa, whilst the search for metabolic end-products is greatly hampered by the complexity of the cultural media which it is usually necessary to employ. A discussion of this difficulty and of the methods used in this work to minimise its effects is given later in this paper.

For a review of the nutritional requirements of the protozoa and many original contributions to this subject the reader is referred to the monograph by Lwoff [1932] and to the review of the rôle of bacteria in the nutrition of protozoa by Luck *et al.* [1931].

Griffiths [1888] stated that *Amoeba sphaerococcus*, *Vorticella* sp. and *Paramoecium bursaria* contained uric acid in their contractile vacuoles. His evidence consisted of positive murexide tests obtained upon the organisms in bulk and a flaky precipitate observable in their contractile vacuoles when the organisms were treated with alcohol. Adolph [1922] concluded that *Phagocata* and *Paramoecium* did not produce urea. Howland [1924] repeated the tests used by Griffiths with the organisms *Centropyxis* and *Amoeba verrucosa* and obtained negative results. Using the very sensitive Benedict's uric acid reagent, however, he obtained a positive reaction for uric acid in cultures of *Paramoecium* and *Amoeba*.

Weatherby [1927] concluded that *Paramoecium* produces urea, but his findings were not consistently positive using the urease test, nor was he able to demonstrate the presence of urea by the xanthhydrol test. Injection of Nessler's reagent failed to show the presence of ammonia in the contractile vacuole, but the limit of sensitivity of the test under these conditions is not made clear. The same worker [Weatherby, 1929] studied the production of ammonia in suspensions of protozoa washed by decantation and suspended in salt solution. His results are summarised as follows:

Organism	Ammonia	Urea	Uric acid	Creatine	Creatinine
<i>Paramoecium</i>	0	+	0	0	0
<i>Spirostomum</i>	0	+	0	0	0
<i>Didinium</i>	+	0	?	0	0

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He found that many culture media gave a positive test for uric acid with Benedict's reagent. In the case of the *Didinium* cultures the production of ammonia was shown to vary with the food supplied to the organism.

EXPERIMENTAL.

The types of cultures of protozoa available for metabolic studies may be considered under the following three headings:

Type A. Double cultures, containing one species of protozoan and one or more species of bacterium, both protozoa and bacteria growing and multiplying actively in a medium nutrient for the bacteria.

Type B. Cultures containing one species of protozoan feeding upon live bacteria suspended in some non-nutrient medium in which the bacterial growth and multiplication is minimum.

Type C. Bacteriologically sterile cultures of protozoa.

A culture of type A would give a large metabolic turnover corresponding to very active growth and division, but on account of the extreme difficulty of separating bacterial from protozoal effects in such a culture it was not considered advisable to attempt any metabolic studies upon a culture of this type.

A culture of type B represents a compromise between types A and C. The cultures of *Bodo* used in the experiments described below belong to this type. They have the advantage of providing high rates of metabolic activity, of growth and of division, whilst the effects due to the bacteria alone can be determined with sufficient accuracy for many studies.

A culture of type C is by far the most satisfactory for metabolic studies provided sufficiently active growth and division can be obtained. Metabolic studies of cultures of this type will be presented in future communications. In the case of a very few protozoa growth takes place upon killed bacteria, and such cultures belong to this type C.

The culture of Bodo caudatus.

The culture of *Bodo caudatus* was carried out by methods almost identical with those used by Robertson [1932]. The method may be outlined as follows.

The food supply consists of a suspension of bacteria in either sterile distilled water or a simple inorganic salt solution according to the particular experiment in hand. The salt solutions were sterilised by autoclaving at 110° for 20 minutes.

The bacterium was the predominant organism in the primary cultures which were isolated from a macerating tank. It is Gram-negative, asporogenous, motile, and rod-shaped, slightly pleomorphic and produces a slight yellow colour when grown on agar slopes. The bacterium is almost inert towards the sugars and slightly proteolytic. It has been repeatedly purified by replating.

The density of the bacterial suspension is so chosen that the final concentration of bacteria in the inoculated culture shall give an opacity matching the "opacity 3" of the "Wellcome" standard opacity tubes.

This food supply is then inoculated with a measured quantity of a previous culture, the size of the inoculum being so chosen that the new culture starts with approximately 300 bodos per 1 μ l. Measured samples, usually 6 ml., of cultures prepared in this way are transferred to sterile Petri dishes and kept in an incubator at 25°.

In order to estimate the number of bodos in a culture dish the contents are transferred to a test-tube and thoroughly mixed. 0.5 ml. is then transferred

into 2 ml. of a saturated solution of mercuric chloride in 1.5 % aqueous acetic acid. This fixed and diluted suspension is then counted in Thoma-Zeiss or Fuchs-Rosenthal counting chambers in the usual way. Before all volumetric measurements involved in the above procedure the suspension of organisms is very thoroughly mixed by means of Pasteur pipettes.

All glassware which is used in the manipulation of these cultures is sterilised by baking at 120° for 30 minutes.

BIOCHEMICAL FINDINGS.

The total nitrogen present in a culture prepared as above with a bacterial suspension of "opacity 3" density was found to be approximately 10 mg. of ammonia-nitrogen per 100 ml. of culture. In order therefore to study fluctuations in a fraction of this total nitrogen it was essential to use a delicate method of estimation. This consideration largely determined the use of the following methods.

Estimation of the ammonia content of the cultures.

The culture was first deproteinised with "colloidal iron" (B.D.H.). To 4 ml. of the culture in a 15 ml. centrifuge-tube was added 0.3 ml. of "colloidal iron." After mixing the contents of the tube thoroughly one drop of a saturated solution of potassium sulphate was added to complete the precipitation of the iron. The preparation could now stand for 24 hours if necessary without alteration of its ammonia content. Otherwise, the preparation was centrifuged at once, for 10 minutes at 3000 r.p.m. Alternatively, the precipitate could be filtered off. 2 ml. of the perfectly clear supernatant fluid were then transferred to a test-tube, and 5 ml. of a 0.1 % solution of gum ghatti in water saturated with thymol was added. After mixing by inversion, 1 ml. of Nessler's reagent was added. A series of standards was simultaneously prepared and nesslerised. The tubes were matched, after standing for 20 minutes, in a Klett-Bio or Bausch and Lomb colorimeter, using a heavy blue screen over the eyepiece. The value for the ammonia-nitrogen per 100 ml. of culture thus obtained was multiplied by 1.08 to correct for the dilution with "colloidal iron." Duplicate estimations carried out by this method agreed within 5 % and usually within 3 %. Recovery of ammonia added as ammonium sulphate was satisfactory as the following example shows:

Bacterial suspension washed free from ammonia and ammonium sulphate added to give a concentration of	2.00 mg. ammonia-N/100 ml.
Ammonia found by estimation	1.97 ,,
	Loss 1.5 %

Total nitrogen was estimated by a micro-Kjeldahl incineration and aeration of the ammonia formed into standard acid. This receiving acid was then neutralised and nesslerised. Standard ammonium sulphate solutions were aerated in exactly the same way.

Nitrogen partition in the cultures.

It was necessary at the outset of these experiments to determine to what extent ammonia formation accounted for the whole of the end-products of the nitrogenous metabolism of *Bodo*. The fraction "non-protein-nitrogen other than ammonia-nitrogen" was therefore estimated at the time of inoculation and after

24 hours' growth of two strains of *Bodo*, cultured simultaneously and fed upon the same stock of bacterial suspension. The results are tabulated below.

	Non-protein-nitrogen other than ammonia-nitrogen mg. per 100 ml. of culture	
	(a) At the start of the culture	(b) After 24 hours' growth
Bacterial suspension 58	0.65	0.36
<i>Bodo</i> culture N 58	0.72	0.83
<i>Bodo</i> culture R 56	0.82	0.62
Bacterial suspension 59	0.57	0.42
<i>Bodo</i> culture N 59	0.96	0.89
<i>Bodo</i> culture R 57	1.02	0.80
Bacterial suspension 60	0.49	0.37
<i>Bodo</i> culture N 60	0.68	0.58
<i>Bodo</i> culture R 58	0.51	1.09

A consideration of these figures led to the conclusion that the non-protein-nitrogen other than ammonia-nitrogen was probably largely derived from the food supply and did not show variations which could be correlated with the growth and multiplication of *Bodo*.

No urea could be demonstrated in the deproteinised filtrates from cultures by the xanthidrol test or by treatment with urease preparations. No uric acid could be demonstrated in these filtrates by means of Benedict's uric acid reagent [Benedict and Franke, 1922]. Neither urea-nitrogen nor uric acid-nitrogen can then be present in these filtrates to a greater extent than 3 % of the total non-protein-nitrogen formed. The cultures showed no urease activity.

Production of ammonia during the development of the cultures.

In order to follow the production of ammonia throughout the life of the culture a stock of bacterial suspension of standard opacity 3 containing about 300 bodos per μ l. was prepared in a large boiling-tube. After thorough mixing, this culture was distributed throughout a series of Petri dishes, 6 ml. of culture being measured into each dish. The cultures were incubated at 25° and representative dishes removed at appropriate intervals for counting of bodos and estimation of ammonia.

The bacterial suspension itself produces ammonia at a slow and approximately constant rate. It was only considered necessary therefore to measure the ammonia contents of the controls in these experiments at the start and end of the period of observation.

The relation of ammonia production to the development of the cultures was then studied by means of a series of daily subcultures carried out under conditions as nearly constant as practicable. Thus the transfer was so calculated each day as to produce a population of 300 bodos per μ l. in the new culture. Each culture was allowed to grow for the same length of time, 22 hours, before being subcultured. The handling of the cultures and exposure to room temperature which this involved were kept as constant as possible, and 22 hours of each culture's life were spent in the incubator at 25°. The bacterial food supply was prepared freshly each day from 24-hour old cultures on agar slopes, and it contained approximately 10 mg. of total nitrogen per 100 ml. After a few days' subculture under these conditions the cultures settled down to a steady rate of development, so that the extent to which they had to be diluted in making

a new subculture tended to a constant figure (about $\times 20$). Several Petri dishes were then set up from a common stock and dishes withdrawn for counting of bodos and estimation of ammonia at suitable intervals. The results of four such successive subcultures are shown in Fig. 1, in which the ammonia production of cultures and controls is shown. The dilution figures used in preparing cultures N 50 to N 53 were 23, 21, 20 and 23 respectively. These figures are indicative of the degree of reproducibility of the rate of multiplication of the bodos.

Fig. 1.

The following are the main features brought out by this figure:

(a) The contribution of the bacterial suspension alone is only a small fraction of the total ammonia produced.

(b) The total ammonia-nitrogen produced by the cultures less that produced by the corresponding controls was 3.9, 4.0, 3.7 and 3.0 mg. per 100 ml., *i.e.* an average of 3.7 mg. or 37 % of the total nitrogen in the culture.

(c) The bodos were multiplying rapidly until about the 24th hour, when their rate of multiplication fell off. About this time too encystation often appeared in the cultures. Thus the rapid formation of ammonia is associated with the period of rapid multiplication of the bodos rather than with the period just before the onset of encystation.

A similar and more complete set of curves was then obtained during experiments carried out in collaboration with Dr Robertson. Different strains of *Bodo* were used, grown, however, under conditions almost identical with those described above. The controls were obtained by a different procedure. A portion of the freshly prepared culture was set aside at 40° for a constant period of 2 hours. This treatment sufficed to kill all the bodos but left the bacteria alive. This killed bodo culture was then set up in Petri dishes in exactly the same way as the live culture. It is to be noted that every element in the culture is here represented in the control with the exception of the live bodos. This control is of course somewhat exaggerating the bacterial effect as all the bodos in the new control are here available for food supply for the bacteria, whilst the bacteria are not themselves being consumed as in the live bodo cultures.

In interpreting the figures for the ammonia contents of the cultures the ammonia gained by the control was deducted from the total ammonia present in the live culture to get a corrected curve of ammonia production for the live culture. From this corrected curve of ammonia production the rate of ammonia production was calculated for each period between two observations. Fig. 2 shows how this rate of ammonia production and the number of bodos present vary with age in the case of three representative cultures.

Fig. 2. Rate of ammonia production by: culture HS(2) $\circ$ — $\circ$; culture JN(1) $\bullet$ — $\bullet$; culture JN(3) $\triangle$ — $\triangle$. Number of bodos: culture HS(2) $\times$ — $\times$; culture JN(1) ∇ — ∇ ; culture JN(3) $\square$ — $\square$.

Considering first the curves of rates of ammonia production, it is seen that the initial rate of ammonia production is more than doubled by the time the culture is 8–10 hours old and thereafter remains steady or increases relatively slowly till the end of the period studied. It is at just this period when the rate of ammonia production ceases to increase rapidly that the bodos pass out of their period of lag in multiplication and enter upon a period of very rapid increase in numbers which is maintained to the end of the period studied. Thus from about the 8th to 10th hour onwards a roughly steady rate of ammonia production is being maintained by a rapidly increasing number of bodos, or in other words the rate of ammonia production per bodo falls steadily and rapidly from the 8th to 10th hours onwards until about the 26th hour of growth.

In the case of measurements of ammonia production made during the early hours of the culture's development the figures are small differences between relatively large quantities, and it would not therefore be justifiable to attempt to draw any more precise conclusion than that during this period the rate of ammonia production is very markedly low in comparison with the steady rate which is established later.

The possible significance of this feature of nitrogenous metabolism will be discussed at the end of this paper in relation with curves of oxygen uptake and certain morphological data.

II. RESPIRATION.

A number of more or less quantitative experiments have been carried out to determine what oxygen tension is most favourable for the development of various protozoa. Such experiments involve observation of the migration of protozoa in cultures into oxygen-rich or oxygen-poor localities and of the influence of altered oxygen tension upon the behaviour and viability of cultures. These experiments have indicated a pronounced sensitivity of protozoa to the oxygen tension of their environment. The work of Fox [1920-21] is particularly to be noted in this connection. Fox worked with a culture of *Bodo sulcatus* obtained from 6-day old infusions of grass. Square cover glasses were mounted over microscope slides and samples of the cultures run in between the cover glasses and slides. The preparations were kept in moist chambers and periodically examined under the microscope. At first the bodos were uniformly distributed, but after a time, varying from a few minutes to a day, they collected in the centre of the cell. Subsequently an area free from bodos appeared in the middle of the clump, and the ring of bodos thus formed expanded till it took up a position at a uniform distance from the edge of the cover slip. Finally these migrations were reversed and the bodos again clumped in the centre of the cell. The influence of bubbles of air and other gases, of solid particles, of de-oxygenating reagents and increased oxygen tension upon their behaviour was studied. The conclusion was drawn that *B. sulcatus* is very sensitive to changes in the oxygen tension around the optimum oxygen tension for its development and migrates to the region of optimum oxygen tension.

In connection with experiments to be described in this paper it is of particular interest to note that Fox has occasion to refer to the possibility of a change in the optimum oxygen tension occurring during development of the culture. Such a change might explain the migration of the bodos from the edge of the cell to the centre on allowing the cultures to stand in the moist chamber, and also, to a certain degree, the shape of the ring of bodos as viewed in vertical section.

Soule [1924] studied the respiration of *Trypanosoma lewisi* and *Leishmania tropica* grown on a blood-broth-agar medium. When his cultures were supplied with 100 ml. of air *Leishmania* used all the oxygen in 144 hours, whilst the trypanosomes required twice that time to consume all the oxygen. When the cultures were supplied with 2 litres of air the rates of oxygen consumption and carbon dioxide output were approximately equal for both species of protozoa. Excess of oxygen or anaerobiosis inhibited growth. Carbon dioxide was toxic in concentrations of over 20 %. The respiratory quotient for *Leishmania* measured from the time of inoculation to the seventh day of growth was 0.84, and that for the trypanosomes 0.74. It was suggested that much base was produced to account for the carbon dioxide retained in the culture. No counts were made. Degeneration was always present at the end of the experiments.

Fenyvessy and Reiner [1924] showed that cultures of trypanosomes gave a vigorous oxygen uptake and carbon dioxide output when studied by means of the Barcroft differential manometer. Their work was continued [1928] with the Warburg respirometer, and a washed suspension of trypanosomes in Ringer's

solution containing glucose was used. The gaseous exchange was observed for periods of about 20 minutes. The glucose was decomposed by both oxidation and "glycolysis"; the latter being greatest during anaerobiosis. The very interesting observation was made that neither germanin nor potassium cyanide inhibited either process when added to the suspension in any concentration up to 0.1 %. This finding is the more remarkable in that germanin is toxic to trypanosomes in their vertebrate host. If the p_H of the system is lowered "glycolysis" is increased at the expense of oxidation and *vice versa*. These workers found aerobic and anaerobic "glycolysis" to proceed at the same rate. The rate of oxygen uptake per 10^8 organisms per hour varied from 3.7 to 7.5 μ l., and it is very interesting to note that in experiments to be described immediately the same number of the very much smaller organism *Bodo caudatus* consumed 350 μ l. of oxygen in the same time. In the case of the trypanosomes the organisms were in a medium which was non-nutrient for them, whilst the bodos were metabolising foodstuffs, growing and multiplying very actively.

EXPERIMENTAL.

The oxygen uptake of cultures of *B. caudatus* was measured by means of the Barcroft differential manometer. The cultures were prepared in bulk in

Fig. 3. Rate of oxygen uptake by: culture JN(1) $\bullet$ — $\bullet$; culture JN(3) $\triangle$ — $\triangle$; culture JN(7) $\circ$ — $\circ$; Number of bodos: culture JN(1) ∇ — ∇ ; culture JN(3) $\square$ — $\square$; culture JN(7) $\times$ — $\times$.

boiling-tubes as described earlier in this paper. Control material was prepared by heating a portion of these newly inoculated cultures to 40° for 2 hours, which process kills the bodos but not the bacteria. 5 ml. of live culture were measured

into the right-hand cups of four differential manometers and 5 ml. of killed bodo control material into the left-hand cups. The water-bath was maintained at a temperature of 25°. At intervals throughout the next 24 hours cups were removed, the contents analysed for ammonia and the numbers of bodos per μ l. counted. The manometer cups were cleaned particularly scrupulously but were not sterilised, since the bodo culture is not likely to provide a medium which would allow of significant bacterial growth within the period of these experiments. The oxygen uptake of the control was of course automatically allowed for by the differential character of the apparatus. The results of three experiments of this type are shown graphically in Fig. 3. In this figure the rate of oxygen uptake per hour for the whole culture and the number of bodos in the culture are plotted against the age of the culture in hours.

It is seen that a most pronounced change occurs in the relationship between the rate of oxygen uptake and numbers of bodos present about the 16th to 18th hours. Up to this time the two curves have run almost parallel, implying that the rate of oxygen uptake is directly proportional to the number of bodos present throughout this period. Between the 16th and 18th hours the rate of oxygen uptake reaches a sharp peak value and then starts to fall rapidly. This occurs despite the fact that the number of bodos in the culture is still increasing. After the 16th to 18th hour, however, the generation time is always increasing; its minimum value having occurred between the 8th and 16th hours of development of the culture. By the 22nd to 24th hours the rate of oxygen uptake has fallen to about one-half of its peak value, although there are then nearly twice as many bodos in the culture as there were at the time of most rapid oxygen uptake. Very good confirmation of the earlier parts of these curves, up to the peak value, was obtained in the study of another strain of *Bodo*. With this strain, however, the experiments were not carried on sufficiently long to follow the curve after the 18th hour.

Variations in size of Bodo caudatus.

It is to be noted that the size of the bodos varies greatly at different ages of the culture, so that the curves of the numbers present give only a limited picture of the morphological development of the culture. Dr Robertson has kindly placed at my disposal a large series of unpublished measurements of individual bodos at various ages of the cultures. In Fig. 4 the data giving the area distribution curves for the typical cultures HN (2) and HN (13) are summarised. In these curves a decrease in the proportion of large bodos present is indicated by a flattening of the curve, and a decrease in the average size of the bodos present is indicated by a shift of the curve as a whole towards the left. As a matter of convenience $\text{area} \times 4$ is plotted instead of the actual area. These measurements were obtained by tracing the outline of bodos by means of the camera lucida. Hence the area measured is that projected by the bodo upon the plane surface upon which it is lying and not the actual surface area. It is clear from this figure that the proportion of small bodos increases very greatly between the 11th or 12th and the 22nd hours. Thus there are from three to four times as many bodos of 50 units size at 22 hours' growth as there were at any previously measured period. Moreover, there are hardly any bodos bigger than 100 units at 22 hours although at previously measured periods such large individuals were numerous.

It is during this period when multiplication is outpacing individual growth that the marked peak in the rate of oxygen uptake by the culture occurs (Fig. 3). It must be borne in mind that during this period (the 12th to 22nd hours) no

dead bodos are appearing in the cultures, encystation is not occurring and motility does not appear to be in the least diminished. It would appear then that during this period some form of anaerobiosis is supplying the energy requirements of the organisms to an increasing extent. The occurrence of this biochemical change during a period of pronounced morphological change must

Fig. 4.

be noted, although any correlation between these two types of change is not at present apparent. If anaerobiosis did indeed play an important part in the metabolism of cultures of *B. caudatus* during their later phases, it would be reasonable to expect their optimum oxygen tension to be lower in these later phases. The work of Fox [1920-21], quoted above, may indicate such an effect in the case of *B. sulcatus*, although it must be emphasised that Fox was able to account for his results without assuming any change in the optimum oxygen tension for *B. sulcatus*.

The development of the culture may be divided into two phases with respect to the three aspects for which data are here presented. In the case of nitro-

genous metabolism, as studied by ammonia production, there is an early period of relatively great activity, when the rate of ammonia production runs roughly parallel with the increase in the numbers of bodos in the culture. This period lasts till about the 8th hour of growth. The second phase then commences, and during this period the rapid increase in numbers of bodos is no longer paralleled by an increase in the rate of ammonia production, which becomes steady or shows only relatively slow increase. The motility of the organisms is maintained during this second phase and no death or encystation is seen. A probable inference from these observations would be that during the early development of the culture, taking place in the presence of abundant food supply and in a low concentration of growth products, nitrogenous foodstuffs are preferentially metabolised, whilst during the later life of the culture, when metabolic products are accumulating and food supplies decreasing, the food requirements of the organism are met by the preferential metabolism of non-nitrogenous food materials.

The two phases brought out by the curve of rate of oxygen uptake have already been discussed and their time relationship to the morphological changes noted.

SUMMARY.

Data are presented for the production of ammonia, consumption of oxygen, rate of multiplication and growth of the protozoan *Bodo caudatus*. The metabolic significance of these findings is discussed.

I wish to express my thanks to Sir F. G. Hopkins for his interest in this work and to Dr M. Robertson both for her generous advice and hospitality at the Lister Institute of Preventive Medicine and for providing me with the strains of *Bodo caudatus* used in this work. I have also to thank Dr Robertson for permission to quote certain of her unpublished data.

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