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Some Cultural Studies with Holotrich Ciliate Protozoa of the Sheep's Rumen

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SUMMARY: In a suitable buffer at 39° the life of all three species of holotrich ciliates of the sheep's rumen can be extended for one or more days by addition of glucose, fructose, galactose, sucrose, cellobiose, raffinose, inulin, bacterial levan, salicin or melibiose (the least effective). Mannose, glucosamine or galactosamine are definitely toxic in that they greatly shorten protozoan life. This toxic effect is observed even in the presence of glucose. *Isotricha prostoma* and *I. intestinalis*, but not *Dasytricha ruminantium*, will ingest vegetable starch of small grain size, e.g. rice starch, thereby prolonging protozoan life.

The nitrogenous requirements of these protozoa are best met by whole grass juice, which extends the life of the culture for several weeks; and even the ethanolic precipitate from boiled and cleared grass juice is a better nitrogenous supplement than cleared rumen liquor. The ash from this alcoholic precipitate will definitely extend protozoan life in absence of nitrogenous supplement. The effect is probably due to a 'trace' metal, which is not Zn, Fe, Sn, Sr, Mn, Cu or Ni, but may be Ti, Mo, Cr, Co or V.

The holotrich ciliates here studied do not ingest lactobacilli, either when starved or when in an actively motile state after addition of glucose to the medium.

Although certain of the more abundant oligotrich ciliates of the sheep's rumen have been kept alive for long periods *in vitro*, particularly by Hungate (1942, 1943) there is no record in the literature of any attempt to do this with the holotrich ciliates, of which there are usually three species to be found in that situation, viz. *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*. The latter species is much the smallest of the three, and occurs in the greatest numbers. All three species are of considerable biochemical interest since it has been shown that they are able, under anaerobic conditions, rapidly to convert certain soluble sugars into a granular starch-like storage polysaccharide which may indeed account for four-fifths of the dry weight of the cell (Oxford, 1951; Masson & Oxford, 1951). In order to study this process more fully we have endeavoured to find out the requirements of these protozoa for growth, multiplication and maintenance for long periods *in vitro*, and now present an account of our positive knowledge on three topics, viz. mineral requirements, carbohydrate requirements and differences in behaviour between the *Isotricha* species on the one hand, and *D. ruminantium* on the other. The nitrogenous requirements of these protozoa have proved to be complex and are not dealt with in any detail in this paper.

METHODS

Animal. Rumen contents were obtained as required from a Cheviot wether (sheep no. 879), aged 4 years, which had been fitted with a permanent rumen fistula in 1950 by Dr A. T. Phillipson of this Institute. During the experiment it was fed on good quality meadow hay and had access to a salt lick.

Basal salt medium for protozoan cultures. The bicarbonate-rich buffer previously used for brief fermentations (Oxford, 1951) was replaced by a more stable, osmotically equivalent liquid of the same pH value (6.8–7.0) based on the formula of Hungate (1942). It contained: NaCl, 0.5; $\text{CH}_3\text{CO}_2\text{Na}$, 0.15; NaHCO_3 , 0.1; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 %. It was freshly prepared and heated to 39° immediately before use. Later, when the trace element requirements of the protozoa were specifically under investigation, it was enriched by the addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 %. All salts used were of Analar quality.

Nitrogenous supplements. Routine experiments with carbohydrate substrates were carried out with an addition of 10 % (v/v) cleared and sterilized rumen liquor to provide nitrogenous nutrients including growth factors. The sterilization was performed by centrifuging strained rumen liquor at high speed (30,000 $\times g$) followed by Seitz filtration. Later, after abortive attempts to provide a chemically-defined and soluble nitrogenous substitute for rumen liquor, the latter was replaced with advantage by a 5 % (v/v) supplement of fresh grass juice, prepared as follows.

Nitrogenous and mineral supplements from lawn grass. Freshly cut summer grass, which was free from dicotyledonous weeds, was minced in a domestic meat mincer and strained by hand squeezing in a muslin bag. The juice was then cleared as far as possible by centrifuging, and the supernatant used as the supplement of fresh grass juice referred to above. To prepare an active fraction from this juice it was first boiled for 5 min. to coagulate protein. After recentrifuging and filtration through a Whatman no. 1 paper, the clear liquid, which contained about 1 mg. N (Kjeldahl)/ml., was found to be sufficiently free from bacteria for direct use as a supplement when it was desired to keep bacteria at a minimum. A solid and almost equally efficient fraction was prepared from this liquid by addition of 4 vols. of 95 % ethanol, collection of the precipitate by centrifuging, and drying in a vacuum desiccator, after thorough washing with ethanol on the centrifuge. It contained polysaccharide (levan) and some protein (*c.* 8 % calc. from Kjeldahl N) and was both an adequate carbohydrate source and a fairly complete nitrogenous supplement for the protozoa. A purely mineral and N-free supplement could be prepared from it by simple ignition, when the loss in weight was *c.* 70 %.

Bacterial supplement. Although carefully washed protozoa (see below) were used as inoculum, they were certainly not entirely free from rumen bacteria. After incubation for a day or more *in vitro* in presence of a single soluble carbohydrate, it is quite likely that a markedly different bacterial flora will develop from that which formed the natural environment of the protozoa. It is very difficult indeed to determine by microscopic examination whether or not these protozoa have appreciable numbers of small rumen bacteria inside themselves. This is mainly because of screening by the multitude of storage starch grains (of approximate size $10 \times 3 \mu$.) and also because of the presence of numerous mitochondria. It was thought that if a suspension of a large, rod-like bacterium, both facultatively anaerobic and saccharolytic, and hence not likely to be affected unfavourably by rumen conditions, was presented to the

protozoa, the ingestion of bacteria by the latter, if it took place, would be plainly visible by vital staining, or even without any staining at all. For this purpose a strain of *Lactobacillus bulgaricus* (NCIB 76) was obtained from the Department of Scientific and Industrial Research, Teddington, Middlesex. Before use it was grown anaerobically for 2 days at 40° on slopes of tomato-glucose agar, and the growth scraped off and suspended in the basal salt medium.

Carbohydrate substrates. All carbohydrates used, save those specifically mentioned below, were of best commercial quality, obtained either from L. Light and Co. or from T. Kerfoot and Co. (Biochemical Reagent quality). The galactose used was glucose-free. Mannose (L. Light) was obtained absolutely ashless by recrystallizing first from redistilled methanol and then from redistilled ethanol. Inulin was a gift from the Edinburgh University Chemical Department, galactosamine hydrochloride was a gift from Prof. M. Stacey, University of Birmingham, and bacterial levan was the freshly prepared specimen used by Masson & Oxford (1951). Glucose-1-phosphate was given by Prof. S. Peat of the University College of North Wales.

Preparation of inoculum. This was done as described by Masson & Oxford (1951, p. 665) under 'carbohydrate fermentations without appreciable bacterial competition'.

Methods of cultivation. These were essentially those described by Masson & Oxford (1951); 100 ml. Erlenmeyer flasks were used, and care was taken to fill them completely with warm buffer, before finally replacing the cork bearing the Bunsen valve. In point of fact, the sheep holotrichs are not nearly as sensitive to atmospheric oxygen as the large rumen oligotrichs, so that the addition of Na₂S, or similar reducing agent, is not only unnecessary but is really disadvantageous in that it tends to prolong the life of the latter. The initial concentration of carbohydrate or other substrate was always about 0.1 %; higher concentrations are liable to cause bursting of cells through excessive starch storage. Cellobiose was used for the positive control rather than glucose because it was used at a more moderate rate by the Protozoa and doubtless by extraneous bacteria also, if any were present. A no-substrate control was also set up for each batch of washed protozoa. Each flask was given a fairly heavy inoculum, representing one-fifth of the total holotrichs separated from 1 l. of rumen contents. Incubation was at 39°.

The culture medium was renewed every 24 hr. after careful decantation of the supernatant. This had the dual purpose of restoring a neutral pH value and of controlling the free bacterial population. On each day microscopic examination was made of two separate small drops of deposit taken from the bottom of the flask, first at 1 hr. or earlier and then at 20 hr., after setting up the culture or replacing the medium, respectively. Starch storage was demonstrated by direct wet staining with Lugol's iodine, after examination for motility, dividing forms, etc. Occasionally counts were made by a simple dilution technique from samples of the well-shaken culture, Lugol's iodine again being used for fixing and staining the cells. Such counts are possible since dead holotrich protozoa

are so fragile that they do not remain intact for long. Hence it is safe to assume that nearly all the protozoa counted by this technique were alive before addition of the iodine.

RESULTS

Behaviour of sugars in prolonging protozoan life

With the no-substrate control the course of events was invariably as follows: the starch initially in the cells gradually decreased in amount; the cells never showed any division even during the first 24 hr. but gradually became more and more sluggish before death; the culture died out completely on the third day at the latest. Most of the sugars and other substrates tested did not

Table 1. *Carbohydrates which extended the life of sheep holotrichs*

Sugar	Increase in length of life of culture (days)
Glucose	4-5
Fructose	2-3
Galactose	2-3
Sucrose	2
Cellobiose	4-5
Raffinose	2
Inulin	2
Bacterial levan	2
Salicin	3
Melibiose	1

Nitrogenous supplement supplied by addition of 10 % (v/v) cleared and sterilized rumen liquor.

appreciably affect this behaviour. Such 'neutral' substrates included xylose, arabinose, rhamnose, ribose, fucose, sorbose, maltose, lactose, turanose, trehalose, melezitose, all hexitols, glucuronic and galacturonic acids, glucose-1-phosphate, hexose-diphosphate, dihydroxyacetone, malic, lactic and all lower fatty acids, ethanol, glycogen, soluble starch (as paste), ascorbic acid. Cellulose (in the form of finely divided cotton-wool) was also ineffective.

Certain carbohydrates did, however, definitely extend the life of the culture. The course of events was then as follows: increased activity, starch storage and cell division on the first day; little change for the next few days; finally, gradual death of all the cells presumably because no further cell division had occurred.

Table 1 lists the soluble sugars which aided survival of the protozoa.

The different effects of utilizable and non-utilizable substrates are shown clearly by counts made of the population of protozoa 3 days after setting up the experiments (see Table 2).

The 'toxic' sugars. Mannose, glucosamine hydrochloride and galactosamine hydrochloride definitely shortened the life of the culture. With mannose, most cells were abnormally sluggish after only 1 hr. exposure and *D. ruminantium* had completely died within 1 day. There was also a reduced population of the

Isotricha species. Galactosamine, on the other hand, killed all the cells in 1 day. The effect of glucosamine (see also Masson & Oxford, 1951) was greater on the *Isotricha* spp. than on *D. ruminantium*. It killed all the first-named ciliates of both species in 1 hr., whereas some *D. ruminantium* survived overnight. The effect of the amino-sugars was probably not primarily due to the amino group; ethanolamine and isoamylamine hydrochlorides had no effect on the protozoa at comparable concentrations. Ammonium chloride was also inert. Essentially the same result was obtained with mannose or glucosamine when an equivalent amount of glucose was added at the same time as the 'toxic' sugar.

Table 2. *Population density of rumen holotrichs after 3 days on various substrates*

Substrate	No. cells/ml.	
	<i>Isotricha</i> spp.	<i>D. ruminantium</i>
Cellobiose	760	2040
Galactose	360	1960
Lactose	40	0
Lactate	0	0
None	0	0

Utilization of granular starch by the Protozoa

Both species of *Isotricha* were found to ingest small vegetable starch grains when the latter were added to the rumen liquor basal without soluble sugar. Rice starch was particularly suitable for this purpose. Under these conditions the *Isotricha* spp. lived for 8 days whilst *D. ruminantium* did not ingest any starch grains and died of starvation within 3 days just as if no carbohydrate had been provided.

Neither the *Isotricha* spp. nor *D. ruminantium* were able to utilize purified suspensions of their own storage starch granules, although these granules are on the average much smaller than rice starch grains.

The rates of ingestion and utilization of rice starch grains were determined for protozoa which had been starved for 3 days, as in a no-substrate control, and which were on the point of dying. They were clearly much depleted in storage starch at this point. After addition of the rice starch, samples were withdrawn in 5, 10, 20, 60 and 120 min. and immediately stained with very dilute iodine; the vegetable starch grains then became dark bluish purple and the protozoan storage granules reddish brown. Ingestion of rice starch grains began immediately (see Pl. 1, fig. 1), and within 5 min. an increase in storage granules appeared in the motile cells of the *Isotricha* spp. After 2 hr. these cells contained such a solid mass of iodine-staining material that it was impossible to distinguish between ingested starch and storage starch.

Rate of storage starch deposition with cellobiose as substrate

By means of the technique described above, it was shown that protozoan starch storage with a soluble sugar was a very rapid process also. Within 5 min. some starch had already been laid down, and in less than 1 hr. nearly all the

motile cells appeared to be completely filled with starch. With glucose as substrate the effect was even more spectacular.

Grass juice and fractions prepared therefrom as nitrogenous supplements

It is obvious that rumen liquor is not a very satisfactory nitrogenous supplement, since even in presence of a utilizable sugar the cultures never lived more than 8 days. No better results were obtained with any of the usual nitrogenous sources effective for bacteria, such as yeast autolysate, Bacto-peptone, Bacto-tryptone, casein, casein acid hydrolysate, and mixtures of synthetic amino-acids. In fact such supplements, by encouraging bacterial growth, often proved detrimental to the protozoa. On the other hand, centrifuged but unboiled grass juice, added to the extent of 5 % of the volume of the basal medium, gave a most encouraging result in that the culture survived for 1 month, during which time a steady population of organisms of both genera was maintained as frequent divisions kept pace with mortality. When a higher concentration of grass juice was employed (e.g. 10 or 20 %, v/v), the medium was found to contain too much available carbohydrate since bursting of the cells through too rapid starch storage was encountered (see Pl. 1, fig. 2).

A 5 % supplement of boiled juice kept the organisms of all these species alive for 14 days. An advantage of the boiled grass juice was that fewer bacteria developed in the cultures. It was definitely disadvantageous to sterilize the boiled grass juice by Seitz filtration. The results with the boiled juice were regarded as sufficiently encouraging to warrant a further fractionation. The dried ethanol precipitate obtained from it, when added to the medium in 0.3 % concentration, was definitely superior to rumen liquor + cellobiose in that it kept all three species alive for 11 days instead of 7 or 8 days. The result is perhaps even better than it looks since the carbohydrate of the grass juice fraction (a mixture of levans) is probably not as suitable as cellobiose for prolonging protozoan life (see Table 1).

Observations concerning the mineral requirements of the protozoa

Since the ethanol precipitate from grass juice contained carbohydrate rather than protein as the chief organic constituent it readily yielded a clean ash on ignition. This ash, at 0.08 % concentration, was used as a supplement in conjunction with 0.1 % cellobiose. In order to neutralize the alkaline ash it was suspended in water and a little very dilute HCl added, before including in the basal medium. This medium, without any nitrogenous supplement at all, kept all three species alive and active for 8 days, i.e. 4 days longer than in absence of the ash. Very few bacteria developed in these cultures, and the supernatant liquid above the protozoan bottom layer was always quite clear. Since this calcium-rich ash is much more easily obtained than a clean sodium-rich ash from rumen liquor, its composition was regarded as throwing more light on the mineral requirements of the protozoa than did the rumen-liquor ash. Apart from silica, Ca^{++} , Na^+ and K^+ , the other metallic constituents, as determined spectrophotometrically at the Macaulay Institute, are listed in Table 3. In the opinion of Dr R. L. Mitchell of that Institute, the tin content

is much higher than would be expected for any grass fraction, and most of it has probably been added as a contaminant at some point in the fractionation procedure. The relative concentrations of the other elements are probably normal. A synthetic 'ash', representing a mixture of the first 9 metals in Table 3 with the exception of Ba and Pb, of the following composition was prepared (mg./l. basal medium): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 6.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.1; $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0; $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 0.95; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.6; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2; $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. This was tested in conjunction with cellobiose, but brought about no extension of life of the protozoa, whereas in the control experiment 'natural' ash did in fact extend protozoan life for several days. It is clear, therefore, that a real trace element effect is operative. Any further experiments along these lines would obviously require the rigorous purification of the salts of Na, K, Ca and Mg added to the basal medium.

Table 3. *Trace elements in ash from ethanol precipitate from boiled grass juice*

Element	Concentration in dried ethanol precipitate (p.p.m.)
Zn	460
Fe	340
Sn	180
Sr	104
Mn	52
Ba	40
Pb	40
Cu	20
Ni	15
Ti	6
Mo	5
Cr	2.4
Co	< 0.4
V	< 0.4

To obtain p.p.m. in the actual culture medium, divide by 333.

Possible ingestion of bacteria by the protozoa

Washed holotrichs which had been 'starved' for 3 days, i.e. kept anaerobically at 39° in the buffer without any addition of carbohydrate, showed no tendency whatever to ingest lactobacilli. The protozoa in this experiment were only sluggishly motile, but no different result was obtained on restoration of motility by addition of 0.01 % glucose together with a copious inoculum of *Lb. bulgaricus*. Apart from examination of the living culture by high dry objective, samples were removed at frequent intervals, the protozoa killed by osmic acid vapour and then examined under phase-contrast oil-immersion ($\times 95$) objective. In no instance were any rod-like bacteria seen in the cytoplasm or entering the gullet. This was also confirmed by Gram staining after heat fixation. The protozoan cell as a whole was Gram negative; any Gram positive rods inside it should have been clearly visible.

DISCUSSION

It will be seen that the list of carbohydrates which prolonged the life of the protozoa (Table 1) contains galactose, raffinose and melibiose, in addition to those compounds which Masson & Oxford (1951) found to be detectably converted into storage starch during a 3 hr. fermentation. It is probable that these three sugars are rather more slowly converted to starch than the others. The case of galactose, the only monosaccharide of the three, obviously merits further investigation. With regard to the mechanism of the conversion of soluble monosaccharides to storage starch, our results seem to contain contradictory implications. Thus if glucose-6-phosphate is an intermediate in the starch-forming process the inhibitory effects of glucosamine and mannose are explicable, since these two sugars, as well as glucose and fructose, are phosphorylated by the hexokinase system (see, for example, Long, 1952). On the other hand, the protozoa were not able to make use of glucose-1-phosphate, the assumed immediate precursor, for storage starch formation, when this sugar was dissolved in the medium. Further work is needed to resolve this apparent contradiction.

The uselessness of cellulose for prolonging holotrich life in presence of bacteria seems to dispose of the possibility mentioned by Masson & Oxford (1951) that the sheep holotrichs might utilize cellobiose derived from cellulose by bacterial action in the rumen, for storage starch formation. It now seems more likely, in view of the positive result with salicin, that the β -glucosidase enzyme system, which they undoubtedly possess, is brought into action in their natural environment only to attack the plant glycosides which may sometimes occur in the fodder.

From a biochemical point of view, *Dasytricha ruminantium* is the most bacterium-like of the three holotrich species studied, since it can apparently satisfy its carbohydrate requirements only through a soluble sugar. It is obvious that if the complex soluble nitrogenous requirements of this protozoan are to be worked out in detail, then a careful fractionation of the nitrogenous constituents of grass juice is a necessary preliminary. It is hoped to undertake this when fresh supplies of spring grass are available.

It seems clear that the inorganic requirements of these protozoa are also complex. All that can be said on this point at the moment is that although the ash from a grass juice fraction is beneficial, the effect is not to be ascribed to any of the more abundant metallic ions in this ash, but is probably due to a trace element, possibly Ti, Mo, Cr, Co, V or even to an element not yet detected. According to Bowen (1940) vanadium, as VOCl_2 , aids cell division of the flagellate *Chilomonas paramecium* when added at a concentration up to 10^{-5}M to an inorganic basal medium very similar to that which we have employed. As far as we are aware no work has yet been done on the effect of vanadium on ciliate protozoa.

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Fig. 1

Fig. 2