Factors Involved in the Production of a Novel Kind of Derangement of Storage Mechanism in Living Holotrich Ciliate Protozoa from Sheep Rumen

BY J. MARGARET EADIE AND A. E. OXFORD

Rowett Research Institute, Bucksburn, Aberdeenshire

SUMMARY: When metabolizing glucose or other fermentable sugar *in vitro* at a temperature 8-12° below the normal rumen temperature, three species of rumen holotrich ciliates were liable to exhibit highly abnormal appearances which were not seen at 35-38°. These abnormal appearances consisted essentially in a contraction and fusing together of the bulk of the storage polysaccharide (amylopectin) granules into a narrow central zone in the endoplasm. The outer clearer endoplasmic region, however, still contained numerous discrete granules in *Isotricha prostoma* and *I. intestinalis* but not in *Dasytricha ruminantium*. The abnormality was not quickly lethal to the organisms and appeared not to involve any alteration in shape or position of the macro-nucleus. The presence of rumen liquor, with all its soluble constituents but not necessarily its bacteria, is required for a high incidence of abnormality and the organisms should also initially contain but little storage polysaccharide. Evidence is presented in favour of the view that the abnormalities are connected with the utilization (auto-fermentation) of storage polysaccharide rather than with its synthesis. Nevertheless, the abnormalities cannot be induced in *Isotricha* when starch grains (vegetable or protozoan) have first been ingested.

When harvesting in quantity from sheep rumen contents the holotrich ciliates (i.e. a mixture of *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*) by a labour-saving and apparently quite reproducible modification of the glucose-fermentation methods of Masson & Oxford (1951) and Heald & Oxford (1958), we noted in our washed suspensions what seemed to be the sporadic and unpredictable appearance of a relatively few highly abnormal but still living ciliates. Furthermore, the kind of abnormal appearance in the *Isotricha* organisms was not exactly the same as in the *Dasytricha* organisms, although a marked contraction of internal contents was a feature in each. This paper records our observations on: (a) how to prevent these abnormalities from occurring; (b) how to ensure their appearance in a large percentage of living ciliates in the culture; (c) the nature of the functional derangement which can bring about such abnormal appearances in still living organisms.

METHODS

Rumen contents of sheep on various diets as source of holotrich ciliate protozoa. For a reason to be stated later, it was important to know whether the experimental animal was being fed an appreciable amount of vegetable starch and if so whether this consisted in large part of grains small enough to be swallowed by the *Isotricha* spp. (cf. Sugden & Oxford, 1952). Table 1 lists the rumen-

fistulated sheep used at various times in this study, together with relevant details concerning their respective rations. By microscopic examination of the concentrates fed (for composition see Table 1) it was established that a high proportion of the constituent starch grains were in fact small enough to be swallowed by the *Isotricha* spp. (This would not have been so had potato starch been fed.) The ration of sheep no. 1004 was changed during the experiment because the population of holotrichs in its rumen had dwindled almost to zero. Supplementation of hay by fodder beet, which contains relatively much soluble sugar and little starch, did in fact soon cause the holotrich population to increase. Unfortunately a similar dwindling of the holotrich population also took place in another sheep (no. 272) maintained on a starch-free diet, but on account of its age it was not thought advisable to change the ration in this instance.

Table 1. *List of the rumen-fistulated sheep used and their rations*

Ref. no. of sheep	Age (years)	Ration (g./day)	Vegetable starch in ration
272, Suffolk cross	7	Hay: 1200 Yeast: 40	0
879, Cheviot	4	Hay: 750 Concentrates:* 450	+ + + (small grains)
997, Grey face	4	Hay: 500 Concentrates: 450	+ + + (small grains)
1004, Cheviot	5	Hay only at first Later: Hay: 1500 Fodder beet: 800 }	0 +, but grains mostly too large to be swallowed by <i>Isotricha</i> spp.
8060, Cheviot	3	Hay: 800 Grass cubes: 400	Almost 0

* Concentrates: 4 parts maize; 1 part oats; 1 part bran; $\frac{1}{2}$ part linseed; $\frac{1}{2}$ part white fish meal.

Method of separation of holotrich ciliates from strained rumen contents. The soluble carbohydrate fermentation leading to deposition of holotrichs as a bottom layer after polysaccharide storage had taken place was carried out as described by Heald & Oxford (1953), the strained rumen liquor being allowed to stand for 1 hr. at 38° before the removal of scum. The removal of scum and the subsequent transfer to funnels of 250 ml. capacity was carried out at about 15° (i.e. room temperature). Glucose or other sugar was added to give a final concentration of usually not more than 0.75 % (w/v), after which the funnels were placed in a 38° incubator. It was later found that the temperature of the rumen liquor at this point was seldom above 33°. When it was realized that this method sometimes led to the deposition of abnormal organisms, many modifications in procedure were made, some of which are described later under 'Results'. Temperature was one factor which was brought more closely under control, and when it was desired quickly to cool or warm the contents of a separating funnel to a desired temperature, a current of cold or hot water from an Ascot heater was directed on to the wide part of the funnel while the

liquid within was kept swirling by rotation of the funnel by hand. At suitable intervals of time the funnel was brought to rest and the temperature of its contents taken. The procedure was repeated until the desired temperature was reached.

Preparation of washed and starved suspensions of living holotrichs for in vitro experiments. The white bottom layer which settled during glucose fermentation was withdrawn after various periods of time into boiling- or test-tubes nearly filled with phosphate-acetate buffer of the following composition (% w/v): NaCl, 0.5; anhydrous sodium acetate, 0.13; KH_2PO_4 , 0.03; K_2HPO_4 , 0.10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; and sometimes $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01. Any necessary adjustment of pH required to reach the value 7.2 (6.5 when the buffer contained Ca) was made with dilute caustic soda or acetic acid. After washing by decantation the protozoan suspensions were kept overnight at 38° and examined next morning for living and relatively empty organisms. Sometimes the starvation period had to be extended for a day, with one or more changes of buffer, in order to obtain organisms sufficiently depleted of storage polysaccharide to give a visible response to added glucose. When the protozoan suspensions contained much debris and dead protozoa these could mostly be removed by allowing the motile organisms to swim through a layer of fine-meshed St Martin's 25N nylon bolting cloth attached to the mouth of a boiling tube filled with buffer. The above stable buffer, which contains no bicarbonate, is the simplest possible for maintenance of the holotrichs, although of course it bears no close resemblance to the salt content of rumen liquor, save perhaps with respect to total Na + K content.

Preparation of 'partly' and 'totally' cleared rumen liquor for use as suspending media. It was important to find whether rumen bacteria or their products were responsible for the observed abnormalities. To obtain rumen liquor free from ciliate protozoa but still containing most of its bacteria, i.e. 'partly' cleared, the top layer of strained rumen liquor from which scum had been removed was centrifuged 2 or 3 times for 3 min. at 2200 g and the highly turbid supernatant fluid used. Totally cleared rumen liquor (i.e. free from both protozoa and bacteria) was this supernatant fluid from which all or nearly all particulate matter had been removed by centrifuging twice at 11,000 g for 20 min.

Examination of holotrich suspensions for abnormal organisms

(a) *Direct microscopy of living ciliates in wet preparations.* When the organisms were moving too rapidly, a brief cooling of the slide preparation in the refrigerator soon slowed them down sufficiently for observation with the high dry objective. It is fortunately not at all easy, even by prolonged cooling, to stop all ciliary motion in these anaerobic ciliates. The living ones always regain motility on warming up again.

(b) *Staining with Lugol's iodine.* The holotrichs, even when relatively empty, still stain solidly brownish purple with excess of iodine because of the highly iodophilic granules of storage polysaccharide which still remain. In order to ensure that a few organisms at least would take the stain only in part it was

best to add a very little Lugol's iodine to a living suspension on a slide and then to add a further small drop of the same suspension of organisms, mixing well.

(c) *Rapid staining with acetocarmine for protozoan macronuclei.* When the organisms are filled with storage starch it is difficult to determine the exact position of the oral region and in this condition overall shape is of little diagnostic value. The shape and situation of the macronuclei are however characteristic. When a drop or two of really saturated acetocarmine (Schneider; see Gurr, 1953, p. 296) is added to a drop of living suspension on a slide, the organisms are immediately killed and fixed by the acetic acid in the stain and their macronuclei become stained discernibly pink, after various periods up to 1 hr. at room temperature. The time required is proportional to the density of the storage polysaccharide. It sometimes helped to starve the suspension overnight before using the stain.

Permanent preparations

The difficulty here was to devise a method of fixing the ciliates to a coverslip which, without distorting the organisms, ensured that they remained attached throughout the subsequent changes of solution. This was best achieved by drying as much as possible in air without killing the ciliates in the drop and fixing rapidly by the flotation method on Bouin or Schaudinn fluids. The stains used were iron-haematoxylin or picro-carmine, whereby the large macronucleus was made clearly visible. *Isotricha prostoma* has a slightly curved and elongated macronucleus not centrally situated while *I. intestinalis* has a more egg-shaped macronucleus in the centre of the organism. The macronucleus of *Dasytricha ruminantium* is more or less spherical and central in position (see Kudo 1950, p. 606; but his fig. 287 c is clearly *I. intestinalis* and fig. 287 d *I. prostoma*, not vice versa as stated).

The micronucleus is only seen with difficulty in Feulgen preparations and appeared to be of no diagnostic value in this investigation. The use of horse serum or glycerol albumin for fixing the ciliates to the coverslip did not give successful results nor did the use of eosin or light green as counterstains greatly improve the preparations.

RESULTS

Nature of the abnormalities in the three species of holotrichs

As previously shown (Oxford, 1951) the metabolism of glucose by these ciliates very rapidly leads to a great increase in the numbers of storage polysaccharide (amylopectin) granules of quite uniform size resembling yeast cells. These granules are evenly distributed throughout the endoplasm (see Oxford & Sugden, 1953, for a photomicrograph of a suspension of these granules). Pl. 1, fig. 1, of the present paper shows five completely filled normal organisms, the smallest of which is *Dasytricha ruminantium* (this figure also shows one quite abnormal representative of this latter organism). When such normal

organisms are ruptured, the discrete grains stream out; they never seem to be stuck together in clumps. The abnormality here discussed, which was at times shown by holotrichs from all five sheep, is a markedly uneven distribution of storage granules in living and actively motile organisms. By direct microscopy of living ciliates, the granules are seen to be mostly condensed together into the inner region of the endoplasm as shown in Pl. 1, fig. 2, which exhibits all stages of 'compression' of the storage granules save the most extreme. In the middle stage of the process it might almost seem that a membrane had developed to enclose this differentiated inner zone (Pl. 1, fig. 3), but for the following reasons this is held to be unlikely. (1) Careful examination by both ordinary and phase microscopy with the high dry objective did not reveal a true membrane but only a slightly irregular boundary. (2) At a later stage in the life of the abnormal *Isotricha* organisms, a jagged fracture of the inner zone may occur (Pl. 1, fig. 4). It seemed as if the inner zone itself was rigidly solid, rather than a collection of a large number of discrete grains enclosed by a membrane. (3) This conclusion was confirmed by rupturing the organisms mechanically and then staining with iodine. The rupture could be achieved very simply by forcing the coverslip over the slide, by use of the thumb, with a simultaneous squeezing and shearing motion. When this operation was carried out with a drop containing only normal organisms an even distribution of discrete grains was obtained on rupture. A suspension which contained abnormal organisms, however, yielded numerous iodophilic 'lumps' of various sizes and shapes as well as discrete grains. Further examination of these lumps showed that they did not have a smooth margin, but parts of individual granules could be seen projecting. It could be clearly ascertained that the smallest lumps did in fact consist of only a few granules stuck together.

It will be seen from Pl. 1, fig. 2, 4 and 5 particularly, that the highly abnormal *Isotricha* organisms still contained some storage granules in the outer region of the endoplasm, whereas the *Dasytricha* organisms seemed to have none or very few indeed in this region. This conclusion was confirmed by overstaining with Lugol's iodine when nearly all the abnormal *Isotricha* organisms stained solidly, while the clear outer zone in abnormal *Dasytricha* organisms remained uncoloured or at most showed a faint diffuse reddish brown. When, however, the preparation was incompletely stained with iodine, as described under 'Methods', the outer zone in the endoplasm of the *Isotricha* organisms could be discerned in certain instances, and appeared to contain a soluble starch-like material as well as storage starch in granular form, since a distinct brownish purple hue, more or less evenly diffused, was perceptible in that region. The rapid acetocarmine stain also disclosed a clear outer region in the *Dasytricha* organisms, but the endoplasmic picture with this stain was complicated by the swelling of the organism as a whole, and particularly of the ectoplasm, because of the acetic acid.

A very clear indication of abnormality was shown in the permanent, fixed and nuclear stained preparations. The normal holotrich organism had an entire outline, while the abnormal organism had an extremely battered or crenated outline, as if the outer endoplasmic region had not withstood the

drying and fixing process, although both types of organism were on the same slide. The macronucleus showed no abnormality.

The nuclear stains revealed also that both species of *Isotricha* showed the characteristic abnormality, identical in each organism in the later stages, but perhaps not quite beginning in the same way in the two species. Thus, the early stage of 'compression' of the storage grains seemed to encompass the whole mass in *I. intestinalis* but sometimes occurred at one end only in *I. prostoma*.

Prevention of the abnormality

To avoid the possibly harmful fall in temperature when the scum was removed and the liquor was transferred to funnels (mentioned on p. 299), the procedure was modified so that the straining through gauze was carried out in the incubator. The filtrate, after removal of scum, was warmed to 38° by placing the beaker containing it in a bath of warm water, and finally transferred to warm funnels in an incubator at 42–45°. These simple expedients, which ensured that the rumen liquor remained continuously at rumen temperature or slightly above it, did in fact eliminate the abnormalities entirely, and showed that a slightly lowered temperature was an important factor in causing them.

Factors which favour the development of abnormal organisms

(a) *Lowered temperature at or near the time of adding glucose.* The following does not apply to rumen liquor obtained from concentrate-fed sheep (see later). After various trials it was found that rapid cooling of the rumen liquor in the funnel to 25°, then addition of glucose preferably dissolved in the minimum volume of cold distilled water, and replacement of the funnel in the 38° incubator, always gave a final yield of holotrich ciliates, after 2–3 hr., not far short of that expected; all the *Dasytricha* organisms then showed abnormality and so did a considerable proportion (up to 50 %) of the *Isotricha* organisms. It was never possible to bring down all the *Isotricha* organisms in an abnormal state. When the temperature was maintained at 25° throughout, before and after addition of glucose, the yield of organisms was much lower and the incidence of abnormality not significantly greater. The temperature of the funnel after replacement in the incubator of course remained below 30° for a considerable time, at least 45 min. It was not necessary to cool to 25° before adding glucose; the cooling could be carried out somewhat later. Thus, when glucose was added to strained rumen liquor at 32° the whole incubated for 20–60 min. (not longer) then rapidly cooled to 25° and finally re-incubated, quite as high an incidence of extreme abnormality was observed as with cooling immediately before the addition of glucose. Examination of the protozoa in the bottom layer withdrawn after 30 min. re-incubation following addition of glucose at 25° revealed that the organisms at this early stage were well filled with storage starch and only the initial stage of the abnormality was in evidence, viz. a slight withdrawal of the internal granular contents

away from the ectoplasm as in Pl. 1, fig. 2, organisms. It was repeatedly noticed that the holotrichs become more abnormal after they had settled at the bottom of the funnel, when allowed to remain there. Although fresh organisms were continually being deposited for several hours after addition of glucose, an examination of the holotrichs in the rumen liquor supernatant fluid at any given time (the organisms being collected by light centrifugation) revealed no more striking an incidence of abnormality than in the deposit which had collected by this time. It seemed unlikely, therefore, that there could be a preferential deposition of only slightly abnormal organisms in the early stages of incubation. Furthermore, motile abnormal organisms in a wet preparation examined at room temperature seemed consistently to appear more abnormal the longer they were examined.

Careful microscopic examination of freshly withdrawn rumen contents from hay-fed and concentrate-fed sheep did not reveal any holotrichs with the particular abnormality being investigated. This is consistent with the fact that the rumen temperature in a healthy animal is usually above rather than below body temperature (Krzywanek, 1929). It was found that the holotrichs *in vitro* required to be maintained at 25° or thereabouts for an appreciable time before abnormalities occurred, for when strained rumen liquor was rapidly cooled to 25°, glucose added, and the whole quickly warmed again to 37°, only a very small proportion of abnormal organisms was deposited on further incubation.

(b) *Necessity for the presence of a soluble metabolizable sugar.* Substitution of fructose or sucrose for glucose in the procedure outlined above under (a) gave a comparable deposition of abnormal organisms. Cellobiose, however, gave only abnormal *Dasytricha* organisms. The four sugars just mentioned were those found by Masson & Oxford (1951) to be convertible into storage starch by the holotrichs. The final concentration of glucose for production of abnormal organisms could be considerably less than 0.75%; even 0.1% yielded some completely abnormal forms.

Among the sugars found by Masson & Oxford (1951) to be not quickly convertible into storage starch, galactose, maltose, lactose and xylose were likewise found not to yield abnormal organisms of both genera. Because of the fermentability of these sugars by rumen bacteria with production of a scum of debris which would otherwise have sunk to the bottom, their use often facilitated the deposition of a clean layer of incompletely filled normal holotrichs. Galactose (1% or more) gave particularly good results in this respect and was used for obtaining holotrich suspensions for *in vitro* studies, the organisms in which could sometimes be rendered quite empty without undue mortality by overnight starvation in buffer. The holotrichs in rumen liquor were not harmed by long contact with any of these sugars at 25–32°. In one experiment with maltose the addition of glucose after 1 hr., by which time the temperature had risen to 32°, resulted in the later deposition of normal cells only.

The abnormalities could not be induced when an apparently insoluble metabolizable constituent (e.g. granular vegetable starch; see Sugden &

Oxford, 1952) was substituted for glucose. Rice starch, the grains of which are relatively small (see Kerr, 1950, p. 21, fig. 15), was swallowed with avidity by *Isotricha intestinalis*, and rather less readily by *I. prostoma* where the distribution at first was uneven, the grains being collected in that part of the endoplasm near the mouth. In neither species was any contraction of internal contents observed even when the strained rumen liquor (from sheep 8060 or sheep 1004) + starch in the funnel was kept at 35° for 45 min. before cooling (see section (a) above for corresponding experiment with glucose). The ciliates were more actively motile after ingesting rice starch than after metabolizing glucose. Since rice starch consists of a mixture of amylose and amylopectin, it is of course possible that amylose, the more readily soluble constituent, was preferentially attacked. The experiment was therefore repeated with waxy maize starch which is chemically similar to the protozoan storage starch, being purely amylopectin. Unfortunately the grains in this instance were mostly too large to be swallowed by *I. prostoma* (see Kerr, 1950, p. 19, fig. 9), but they were readily ingested by *I. intestinalis* without giving rise to any abnormality. Sugden & Oxford (1952) found that these holotrichs were apparently unable to utilize their own storage starch granules when the latter were supplied to them in suspension in a buffer. The holotrich starch preparation used had however been obtained from cells disintegrated by a detergent, and therefore might have contained traces of the latter. By using a more satisfactory preparation, obtained by mechanical disintegration, followed by purification by differential centrifugation, washing by decantation and finally dialysis, we now find that *Isotricha* sp. undoubtedly swallow the granules, becoming appreciably denser in appearance, although the distribution of the ingested granules is sometimes uneven. No well-marked contraction of the granules in a central zone was, however, observed.

(c) *Necessity for only a small initial content of storage polysaccharide.* Reproducible results with respect to abnormality in *Isotricha* organisms were not obtained with rumen liquors from concentrate-fed sheep no. 879 and 997, although *Dasytricha* organisms always became visibly abnormal in these instances after cooling and addition of glucose. This behaviour seemed to be due to the fact that only the *Isotricha* organisms can ingest and metabolize small vegetable starch grains (Sugden & Oxford, 1952). It was observed that many of the isotrichs deposited from such rumen liquors did in fact contain iodophilic granules of much larger size than their own storage grains. These larger granules were absent from the cells deposited from the rumen liquors of hay-fed sheep nos. 272 and 8060. Furthermore, the content of storage polysaccharide, with galactose-deposited cells, was visibly greater, on the whole, with isotrichs from concentrate-fed sheep than with those from sheep which had no, or very little, starch in the ration. *Dasytricha ruminantium*, on the other hand, occurred in much the same relatively empty state in the rumen contents of all five sheep and was relatively less abundant in the concentrate-fed sheep. These observations help to explain the further fact that although it was always possible to harvest a good yield of holotrichs by addition of galactose to rumen contents of sheep 879, these preparations could not always be

satisfactorily starved *in vitro*, to yield cells sufficiently empty to give the abnormality by the method described in the next section.

An observation of possible significance was that the dominant *Isotricha* species in the concentrate-fed sheep was *I. intestinalis* which, as previously shown, ingests vegetable starch more readily than *I. prostoma*. This may account for the difficulty of obtaining from the concentrate-fed sheep rumen a suspension of *Isotricha* organisms, in which the majority of the cells showed a well-marked abnormality.

Attempts to reproduce the abnormal organisms in vitro: necessity for rumen liquor but not necessarily rumen bacteria

Reasonably empty holotrichs are obviously required for these experiments. Unfortunately 3 days of incubation in buffer were necessary before the organisms brought down by glucose were suitable, and by this time they were mostly moribund. Furthermore, when galactose was added to rumen contents from the sheep not fed concentrate the yields of holotrichs were liable to be excessively small. Sheep 8060 was the most useful one here. Fortunately it was occasionally possible to produce a marked occurrence of abnormal organisms particularly in *Dasytricha ruminantium*, in a bottom layer of buffer-starved normal holotrichs which had been initially obtained from the rumen contents of a concentrate-fed sheep by use of galactose. This was achieved by resuspending the organisms in partly or totally cleared rumen liquor freshly taken from a hay-fed sheep, cooling to 25°, adding glucose and re-incubating as previously described. In some instances the abnormal forms were observed even when the partly cleared rumen liquor had been heated at 90–100° for 2½ hr. in order to kill or at least inactivate most of its bacteria. On the other hand, when artificial buffer was used throughout as the suspending medium for the starved organisms, and when debris had been removed from the protozoan suspension by use of Nylon bolting cloth as previously described, numerous trials showed that it was practically impossible to produce the abnormality with starved suspensions, even in *Dasytricha ruminantium*. The presence or absence of calcium in the buffer seemed immaterial.

Survival of abnormal cells

Long-continued microscopic examination of living abnormal organisms never disclosed any tendency for spontaneous healing; the abnormality tended rather to get worse, as already stated. When washed suspensions containing a large proportion of abnormal organisms were kept in buffer overnight at 38°, the mortality of the abnormal forms was almost always apparently 100 %, even when the normal organisms survived satisfactorily. It was concluded that the abnormality is definitely harmful in the long run.

*Agencies which play no important part in the production of
these abnormalities*

At various times the factors listed below were considered as being involved in producing abnormalities of this kind but rejected for the reasons given:

Possible cause of abnormality	Reasons for rejection
Ingested rumen bacteria might be toxic	A washed suspension of mixed total rumen bacteria had no obvious effect when re-suspended in buffer, or in totally cleared rumen liquor
Ingested amylolytic bacteria might become toxic by attacking the holotrich storage starch	A pure culture of a saccharolytic and amylolytic rumen streptococcus (MacPherson, 1953) likewise had no obvious effect
Hyper- or hypotonicity in buffer, or unfavourable acidic pH value due to glucose fermentation	Abnormalities due to such causes do not in the least resemble those figured in Pl. 1.
Presence of pyrophosphate in the buffer used to dissolve the glucose, AnalaR K_2HPO_4 being unobtainable	Making up the buffer with AnalaR KH_2PO_4 and AnalaR KOH caused no change. The ciliates also tolerated added pyrophosphate quite well
Slower mutarotation of α -glucose (the ordinary crystalline form) at 25° as compared with 38°	Exactly the same result was obtained starting with crystalline β -glucose as with the ordinary α -variety
It is known that mannose is toxic to the holotrichs (Sugden & Oxford, 1952). Lowering of temperature might cause a slowing down of glucose fermentation to such an extent that a Lobry de Bruin transformation of glucose to fructose and mannose might take place within the cell	A careful microscopical examination of the early stages of the 'mannose' effect showed no close resemblance to the abnormalities due to glucose although an irregular distribution of cell contents was observed
Presence of traces of soap in the funnels left from previous scouring	A little household soap added to rumen liquor aided, rather than hindered, the deposition of normal cells, and did not cause any abnormalities to appear
Presence of bacterial fermentation products derived from glucose, but not usually present in rumen liquor as withdrawn from the rumen	Up to 0.01 N-lactic acid, succinic acid, ethanol, <i>n</i> -butanol, or acetone had no particular effect at pH 5.8.
Traces of possible products from bacterial amino acid degradation might be toxic	Effect is sometimes drastic (cf. Hogg & Elliott, 1951, on effect of indole on <i>Tetrahymena</i> sp.) but visible abnormalities in holotrichs did not in the least resemble those here studied.
Autolytic or other products from 'abnormal' ciliates might induce abnormality in others.	A finely ground suspension from a particularly abnormal holotrich culture had no effect in inducing abnormality when added to a suspension of normal cells.
Aeration of rumen liquor (during straining) may predispose the holotrichs to abnormality when subsequently fermenting glucose.	Quite vigorous pre-aeration of rumen liquor for 15 min. had surprisingly little effect when temperature control was maintained.

DISCUSSION

Five factors seem concerned in the production of these abnormalities and all must be acting in conjunction: (a) protozoan metabolism (considered in its widest sense) of glucose or other readily utilizable sugar which (b) must be dissolved in a buffer containing the soluble constituents, at least, of rumen liquor, the action taking place for (c) 30 min. or more, at (d) a temperature (25–30°) rather lower than the rumen normal, by (e) organisms which although motile and quite healthy in appearance initially, contain relatively little granular storage polysaccharide. The abnormality consists simply in an apparent alteration of the usual mode of storing newly synthesized reserve polysaccharide (i.e. amylopectin, Forsyth & Hirst, 1953). This new mode of storage, although drastically different from the normal, is not quickly lethal to the organism.

It remains to be discussed whether the alteration concerns the storage granules before or after they are made; in other words, whether it concerns starch synthesis or starch utilization (by autofermentation). We think that the weight of evidence points clearly to the last-named alternative. The abnormality does in fact get more pronounced after the virtual cessation of granule synthesis; its initial stages are often seen in organisms otherwise normally filled with storage granules; and most conclusive of all, the greatest incidence of abnormalities appears when the cooling to 25° occurs some 20–60 min. after the addition of glucose, during which period the organisms, acting at well above 30°, have stored much starch. When the cooling takes place after 90 min., no abnormalities develop. There is thus no evidence that the storage starch tends to be produced in a lump, rather than as separate granules, in a relatively empty organism which is metabolizing glucose at a somewhat lower temperature than usual. It appears rather that the characteristic change occurs after the granules are formed. As a working hypothesis we suggest that the abnormality is due to a profound disorganization of the enzyme system concerned with the autofermentation of storage starch. Heald & Oxford (1953) showed that this process, which finally leads to production of lactic, acetic and butyric acids, CO₂ and hydrogen, is not in abeyance when these organisms are actively metabolizing glucose. At present nothing is known about the mechanisms of synthesis and degradation of starch by these protozoa. Whatever the initial stages of the degradation may be they probably result in the production of soluble intermediate products of high molecular weight i.e. 'gums' which if allowed to accumulate might cause the granules to adhere to form a coherent mass when tightly packed together. It is clear from our results that the rate of production of storage granules is not greatly altered by a lowering of temperature.

French, Knapp & Pazur (1950), dealing with plant amylases, have shown that not only the rate of starch degradation, but also the degree of accumulation of intermediate products of high molecular weight, are dependent upon various conditions, including temperature. Without making any assumptions as to the nature of the protozoan 'amylases' we suggest that an abnormally

low temperature leads to a similar result with these holotrichs, namely that although the rate of production of the complete degradative enzyme system no longer keeps roughly in pace with starch synthesis, as it would at 38°, nevertheless the initial stage, i.e. the 'gum' production, still takes place.

We have no explanation however for two important facts: (i) inability to reproduce the phenomenon *in vitro* except in the presence of rumen liquor; (ii) the fact that the abnormalities cannot be produced at all by cooling if the ciliate has first ingested starch grains supplied extraneously in place of soluble carbohydrate. This is the case even when these granules consist of the holotrich storage starch itself. A detailed biochemical study of the 'amylase' of these holotrichs is clearly required in order to gain further insight into the cause of the abnormalities. This is now being carried out.

Our best thanks are due to the following colleagues: Dr J. W. Porteous for a supply of purified holotrich starch obtained by mechanical disintegration; the Photographic Department of this Institute for the photomicrographs and Mr M. Great for much technical assistance. We are also indebted to Mr J. Smiles of the National Institute for Medical Research, Mill Hill, for the agar method of 'fixation' of ciliates before photography.

REFERENCES

- FORSYTH, G. & HIRST, E. L. (1953). Protozoal polysaccharides. Structure of the polysaccharide produced by the holotrich ciliates present in sheep's rumen. *J. Chem. Soc.* p. 2132.
- FRENCH, D., KNAPP, D. W. & PAZUR, J. H. (1950). Amylase action under conditions of unfavourable temperature or hydrogen ion concentration. *J. Amer. chem. Soc.* **72**, 1866.
- GURR, E. (1953). *A Practical Manual of Medical and Biological Staining Techniques*. London: Leonard Hill Ltd.
- HEALD, P. J. & OXFORD, A. E. (1953). Fermentation of soluble sugars by anaerobic holotrich ciliate protozoa of the genera *Isotricha* and *Dasytricha*. *Biochem. J.* **53**, 506.
- HOGG, J. F. & ELLIOTT, A. M. (1951). Comparative amino acid metabolism of *Tetrahymena geleii*. *J. biol. Chem.* **192**, 131.
- KERR, R. W. (1950). *Chemistry and Industry of Starch*, 2nd ed. New York: Academic Press Inc.
- KUDO, R. R. (1950). *Protozoology*, 3rd edn., Springfield, Ill.: Charles C. Thomas.
- KRZYWANIEK, F. W. (1929). Über die Temperatur in Pansen des Schafes. *Pflügers Arch.* **222**, 89.
- MACPHERSON, M. J. (1953). Isolation and identification of amylolytic streptococci from the rumen of the sheep. *J. Path. Bact.* **66**, 95.
- MASSON, F. M. & OXFORD, A. E. (1951). The action of the ciliates of the sheep's rumen upon various water-soluble carbohydrates, including polysaccharides. *J. gen. Microbiol.* **5**, 664.
- OXFORD, A. E. (1951). The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. *J. gen. Microbiol.* **5**, 83.
- OXFORD, A. E. & SUGDEN, B. (1953). A reconsideration of the role of ciliate protozoa in ruminant digestion. *Brit. agric. Bull.* **5**, 308.
- SUGDEN, B. & OXFORD, A. E. (1952). Some cultural studies with holotrich ciliate protozoa of the sheep's rumen. *J. gen. Microbiol.* **7**, 145.

EXPLANATION OF PLATE

All organisms photographed were alive and motile immediately before removal from the culture. They were rendered stationary by placing a coverslip with a small drop of culture on top of a smooth film of 0.2% agar which was just on the point of solidification. The cilia do not show clearly because they were still in motion.

- Fig. 1. Four normal organisms of *Isotricha* spp. and one of *Dasytricha ruminantium* (to right of them), after glucose metabolism and filling of the organisms uniformly with storage polysaccharide granules. One highly abnormal *D. ruminantium* at extreme top right. $\times 152$.
- Fig. 2. Various stages of the abnormality in *Isotricha* spp. and *Dasytricha ruminantium*, the earliest being in organisms A and B respectively. Note numerous discrete granules still present in outer clearer endoplasmic zone of *Isotricha* spp. only. $\times 105$.
- Fig. 3. Usual appearance of the abnormality in an *Isotricha* organism with appearance of a simulated inner membrane which encloses the bulk of the storage polysaccharide. $\times 385$.
- Fig. 4. Later stages of the abnormality in an *Isotricha* organism (centre) and in two *Dasytricha ruminantium* organisms. $\times 153$.
- Fig. 5. Four abnormal *Dasytricha ruminantium* organisms. $\times 168$.

(Received 13 October 1954)