OXFORD, A. E. (1951). *J. gen. Microbiol.* 5, 83-90.

The Conversion of Certain Soluble Sugars to a Glucosan by Holotrich Ciliates in the Rumen of Sheep

By A. E. OXFORD

The Rowett Research Institute, Bucksburn, Aberdeenshire

SUMMARY: When *in vitro* fermentations of glucose, fructose or sucrose are carried out under conditions comparable with those in the rumen by means of relatively small inocula of strained rumen contents from hay-fed sheep, the holotrichously ciliated protozoa which are present store immense numbers of microscopic granules consisting of a practically protein-free glucosan giving a purple colour with iodine. The granules can be liberated by bursting the protozoa by the action of the synthetic detergent Teepol XL under mild conditions. An exactly similar polysaccharide granule preparation can be made from protozoa present in the rumen itself if rumen contents are withdrawn 2-4 hr. after feeding. The yield of iodophilic polysaccharide so obtained may be equivalent to approximately 25% of the weight of water-soluble, yeast-fermentable sugars in a single feed of hay. No granule-filled protozoa are present 9 hr. after feeding.

Other simple sugars such as glucuronolactone, galactose, mannose, xylose, arabinose, sorbose, lactose, cellobiose and maltose, are not markedly converted into polysaccharide granules by rumen protozoa, during *in vitro* fermentation.

The most obvious group of micro-organisms ordinarily inhabiting the rumen of sheep are the large ciliated protozoa. Bulk for bulk, although of course not numerically, they usually exceed the bacterial population (Mangold, 1929). Nevertheless, they appear not to be entirely essential for the adequate functioning of at least some rumen fermentations, since defaunated sheep have been shown to continue to thrive (van der Wath & Myburgh, 1941; Johnson, Hamilton, Robinson & Garey, 1944). The current view is therefore, not unnaturally, that the protozoa, from the point of view of fermentation reactions, are of minor importance when compared with the rumen bacteria (see Elsden & Phillipson, 1948). A very simple experiment (recorded later) having disproved this extreme view *in one particular instance*, namely glucose fermentation by the rumen contents of hay-fed sheep, it became of interest to find out the action of rumen protozoa on fermentable sugars. No claim is here made that protozoa are important in all rumen fermentations. This paper is mainly concerned with the storage of polysaccharide granules during carbohydrate fermentation by one important group of protozoa—the holotrichously ciliated—usually present in the sheep's rumen.

METHODS

Animals. Rumen contents were obtained as required from two Suffolk-cross ewes, Sheep 272 and 337, aged 2½ years, fitted with permanent rumen fistulae by Dr J. Tosic (1949). They had been fed exclusively on good-quality starch-free meadow hay, containing about 14% of water-soluble constituents of which about one-seventh was yeast-fermentable carbohydrate (probably fructose), for several months before the experiments. Their weights had been well main-

tained during this period. Each was fed at 12 hr. intervals with 800–900 g. hay; nearly all the food was consumed within 1 hr. Sheep 272 was chiefly used but 337 gave similar results whenever its rumen contents were used in parallel experiments.

In vitro fermentation of simple sugars by rumen contents. The method was essentially that of Elsdén (1946) for glucose, with small Buchner flasks as fermentation vessels; in order to quicken the fermentation the inoculum of strained rumen-liquor was usually increased from 10 to 17 % (v/v), an extra buffering agent in the form of sodium acetate (0.5 %) was added (this is permissible because rumen contents always contain this salt), and the saturation with CO₂ from an external source was omitted. The same end was achieved more quickly by adding the requisite amount of glacial acetic acid, last of all to the buffer mixture containing bicarbonate, arranging matters so that after the addition of the inoculum the Buchner fermentation flask was full to the side-arm. The final concentration of sugar was always 0.9 % or less and the pH value at the beginning of the fermentation was 6.8–7.0. The fermentation flasks were shaken from time to time.

This simple fermentation system was used to demonstrate that the true fermentative action of protozoa was on occasion at least of the same order of magnitude as that of rumen bacteria. Muslin-strained rumen contents (500 ml.; see below) taken 3 hr. after feeding was allowed to stand for 1 hr. at 40° in a tall beaker covered with a clock glass. The supernatant liquor was then carefully decanted from the bottom protozoan layer, the latter was washed twice by decantation with the Elsdén buffer (50 ml.) to remove most of the free bacteria, and then made up to 20 ml. Fermentations with 10 ml. of the final protozoan suspension and 30 ml. of the first rumen supernatant (rich in bacteria but still containing some protozoa) were run side by side, each fermentative liquid containing initially 0.4 % glucose. The pH 7 buffer used in each case to make up the reaction volume to 100 ml. was prepared with sodium carbonate (not bicarbonate) and acetic acid; the medium was then not supersaturated with CO₂ at the start. The gases evolved during the fermentations were collected over warm water. After 4 hr. at 40° the protozoan fermentation had evolved two and a half times as much gas (22 ml.) as the (mainly) bacterial fermentation (9 ml.). It must be remembered that the protozoan fermentation was due to the large organisms from some 250 ml. of rumen liquor, as against 30 ml. for the bacterial fermentation; on the other hand, the so-called 'bacterial' fermentation was partly due to small protozoa which did not sediment readily. There was, in fact, a distinct layer of protozoa on the bottom after 4 hr.

Separation of the larger protozoa from rumen contents or from in vitro fermentation liquids. The method of straining through six layers of surgical gauze without squeezing allowed surprisingly little plant debris through into the filtrate, but in order to collect the greater part of the larger protozoa it was necessary to scrape the deposit from the gauze, resuspend it in distilled water and re-strain through clean gauze. The combined filtrates were then stood for at least 1 hr. in boiling-tubes filled to the top, when the larger, whiter, and heavier protozoa gradually sank to the bottom. The supernatant was carefully decanted, the

deposit washed several times by decantation with 0.003M phosphate buffer (pH 6.8) and finally with distilled water. The final deposits contained relatively very few free bacteria and consisted almost entirely of protozoa together with a very little plant material. As will be seen from the results reported, many fermentation liquids contained polysaccharide-filled protozoa which were very easily and quickly washed clean by the above simple method, because they settled rapidly under the influence of gravity, being much more dense than their unfilled fellows or than water or rumen liquor itself.

Liberation of the granules from washed protozoa. This was readily achieved with the holotrichs by incubation at 39° for 1–2 hr., with occasional shaking, in about one-tenth of the original rumen liquor volume of a solution of Teepol XL (0.2 %) in 0.003M phosphate buffer (pH 6.8). (Teepol XL; British Drug Houses Ltd. is sold as a powder giving an almost neutral solution in water and is said to be the sodium salt of a secondary alkyl sulphate.) The tube was then allowed to stand at room temperature for 1–2 hr. until most of the protozoan debris and unburst protozoa of other genera had settled to the bottom as a greyish deposit below a milky supernatant liquor. The latter, which on examination with the high dry objective was seen to consist almost entirely of a suspension of tiny granules, was carefully decanted and the granules therein collected by centrifuging. The deposit was twice resuspended in water, separated by decantation and finally collected and dried *in vacuo* over concentrated H₂SO₄ at 39° for several days. The dried preparations were analysed for total nitrogen (micro-Kjeldahl) and for reducing sugar by Neish's (1946) modification of the Shaffer-Somogyi method after hydrolysis with 0.55N-HCl at 100° for 2 hr., followed by neutralization with N-NaOH. In certain instances the alkaline iodine method of Hinton & Macara (1924), under conditions applicable to aldose sugar only, was also used. No attempt was made to determine the carbohydrate content of preparations containing more than 2 % N.

RESULTS

Storage of iodophilic granules in protozoa during in vitro fermentations with a small inoculum of rumen liquor

The only simple sugars with which a visible fermentation began within 1–3 hr., when the inoculum was derived from rumen liquor in its most active state (viz. 2–4 hr. after feeding of the sheep), were glucose, fructose, sucrose, galactose and xylose. With arabinose, mannose, cellobiose, lactose, maltose, glucuronolactone and soluble starch, there was a longer delay before gas evolution was observed. With sorbose, or with no added sugar at all, no obvious fermentation took place. In the case of the first three sugars listed a conspicuous, pure white, sandy layer of protozoa had settled to the bottom of the fermentation vessel within 3 hr., and this heavy white layer persisted apparently unchanged even after 24 hr. provided that the initial sugar concentration was not too great. It could then easily be collected, washed by decantation, and thus separated from a much less dense and more flocculent greyish green layer situated above it. The bottom white layer consisted mostly of large holotrichs, together with some equally

large oligotrichously ciliated protozoa (*Diplodinium*, etc.). Every one stained solidly brownish purple with iodine. The more flocculent greyish green layer contained representatives of both groups, sometimes individually iodophilic, together with clumps of bacteria and some plant debris. A similar bottom layer was obtained with the other fermentable sugars, but iodophilic protozoa were not especially in evidence therein, and indeed, with xylose, almost entirely absent. The action of Teepol XL upon the heavy white protozoan bottom layer was to cause nearly all the iodophilic holotrichs to burst, thereby liberating an uncountable number of discrete granules from each ruptured organism. These granules stained intensely purple-brown with iodine; leaving the protozoan cases and macro-nuclei, often distorted in shape, stained only a yellowish brown. The granules varied in size from that of large cocci to small yeasts, and were of an assortment of shapes, chiefly elliptical or roughly circular. A few much larger pseudo-crystalline particles were also seen, usually five or six-sided. These were apparently not derived from the holotrich ciliates but from the rare bursting of smaller oligotrich ciliates. If a wet preparation was made by mixing one drop of the milky granule suspension with one drop of Loeffler's methylene blue, the protozoan debris took the stain whereas the granules did not. Likewise, if a heat-fixed preparation was stained, the granules were found not to take any of the ordinary bacterial stains.

The inference that the granules were almost entirely polysaccharide in nature was confirmed by analysis of the various dried preparations made from *in vitro* fermentations. The results are shown in Table 1 which also includes some analyses carried out on the protozoan granule preparations made from strained rumen liquor itself, taken 2-3 hr. after feeding of the sheep. The purification by washing of the iodophilic protozoa was somewhat more difficult in these latter experiments, but relatively protein-free granule preparations could nevertheless be obtained. The results in Table 1 further show clearly that a synthesis of protozoan polysaccharide granules must have taken place in the *in vitro* sugar fermentations. This conclusion was supported by a comparative microscopic examination of iodine-stained wet preparations, when it was seen that the washed protozoa from *in vitro* fermentations were on the average much whiter, larger and stained more solidly, than those present in the rumen-liquor used as inoculum.

*Influence of 'age' of inoculum on the in vitro synthesis of
polysaccharide granules*

By 'age' of inoculum is meant here the time in hours between the feeding of the sheep and the withdrawal of the rumen-liquor through the fistula. Sheep 272 was used for an experiment in which the various 'ages' were 2, 4, 6.5, and 9 hr. Fermentations were carried out in each instance upon fructose *in vitro* using the customary 17% (v/v) inoculum of strained rumen-liquor. After 24 hr. all the fermentation liquors contained a copious white bottom layer of iodophilic protozoa which was easily washed, by decantation, with distilled water. Holotrichs predominated in all save in that from the 9 hr. inoculum where the iodophilic oligotrichs perhaps predominated. The nitrogen content of

Table 1. *Analysis of dried protozoan preparations obtained from rumen liquor and sugar fermentations therewith*

(All results are calculated for an inoculum of 100 ml. of rumen liquor; fermentation period with sugars, 20 hr.)

Sugar fermented (mg.)	Inoculum (%, v/v)	Protozoan debris after action of Teepol XL		Polysaccharide granule preparation by action of Teepol XL			
		Weight (mg.)	N content (%)	Weight (mg.)	N content (%)	Reducing sugar after hydrolysis, as glucose	
						By Shaffer- Somogyi (%)	By Hinton- Macara (%)
None. (Rumen liquor 2 hr. after feeding sheep 337*)	—	124	6.0	18	0.40	86	—
None. (Rumen liquor 2 hr. after feeding sheep 272)	—	54	7.5	41	1.84	—	—
None. (Rumen liquor 3 hr. after feeding sheep 272†)	—	17	3.0	43	0.39	77	80
As above, on another day	—	—	—	34	1.55	—	—
Glucose: 5300	17	—	—	125 (1.4% ash)	0.14	91	—
Fructose: 5300	17	—	—	164	0.18	90	90
Sucrose: 1330	86	—	—	100	1.96	78	77
Glucose: 10,000	10	—	—	110	0.135	—	—

* Yielded 220 mg. of dried whole protozoa with nitrogen, 5.1 %.

† This specimen of rumen liquor gave a much whiter bottom protozoan deposit than usual.

the respective dried preparations of whole protozoa so obtained are given in Table 2. It will be noted that the last preparation listed, which as mentioned above contained relatively more iodophilic oligotrichs than usual, had a much higher nitrogen content than the others. Attempts to burst the oligotrichs at a neutral pH by means of Teepol XL were unsuccessful.

Table 2. *Nitrogen contents of dried whole protozoa obtained after fructose fermentation with small inocula of strained rumen liquor of various ages*

Time elapsing between feeding of the sheep and removal of inoculum (hr.)	Type of protozoon: (H = holotrich, O = oligotrich)	Washed and dried protozoa/100 ml. of rumen-liquor inoculum	
		Weight (mg.)	N content (%)
2	H	50	1.61
4	H	60	1.44
6.5	H	73	1.13
9	H, O	27	3.51

The immediate working-up of samples of the original inocula for polysaccharide granule preparations gave results which by no means paralleled the above. Only the first two, for instance, gave heavy white bottom layers on

standing; the last (9 hr.) inoculum gave hardly any iodophilic protozoa at all, and the relatively insignificant granule preparation made from the greyish green deposit yielded by it consisted chiefly of non-iodophilic bacteria, as its nitrogen content likewise showed. These results are summarized in Table 3. It will be seen that only the granule preparations from the 2 and 4 hr. inocula contained less than 2% nitrogen. Microscopic examination showed that at 6 hr. after feeding the iodophilic protozoa in the rumen had clearly lost the greater part of their polysaccharide granules, and that these had entirely disappeared after 9 hr.; yet, as Table 2 shows, the holotrich protozoa themselves were still capable to some extent of resynthesizing polysaccharide granules *in vitro* if presented with a fermentable sugar, e.g. fructose.

Table 3. *Nitrogen contents of protozoan granule preparations obtained from rumen liquor (100 ml.) of various ages*

Time between feeding of the sheep and removal of rumen liquor for protozoa isolation (hr.)	Type of 'granule' (P=polysaccharide, B=bacteria)	Washed and dried granule preparations	
		Weight (mg.)	N content (%)
2	P	19	1.01
4	P	32	1.74
6.5	P, B	18	3.73
9	B	8	5.84

Properties of the granule preparation

The dried granule preparations of low nitrogen content (see Table 1) could easily be resuspended in water to give a milky liquid which, when gradually heated, cleared at 70–80° owing to the bursting of the granules. The now faintly turbid liquid was kept at 100° for 5 min., cooled, tested for reducing sugars (absent) and for bacteria by means of a wet preparation made with Loeffler's methylene blue (only a very few bacteria visible). It gave a purple colour with iodine reminiscent of that given by amylopectin, but quite unlike that usually given by glycogen. The chemical structure of this polysaccharide is now being investigated by Prof. E. L. Hirst and Dr E. G. V. Percival at Edinburgh.

There can be little doubt that the main simple sugar formed on hydrolysis, even when the preparation is derived from a fructose fermentation, is essentially glucose, since the reducing-sugar by alkaline-iodine oxidation was always equal to that determined by copper reduction (see Table 1). No hydrolysate gave the Seliwanoff reaction for fructose. Phenylglucosazone (m.p. and mixed m.p. 204°) was in one instance prepared from the hydrolysate of granules obtained from a fructose fermentation.

DISCUSSION

Although the holotrich ciliates (e.g. *Isotricha* and *Dasytricha*) are not usually regarded as the most abundant type of protozoon in the rumen of sheep (Mangold (1929) gives about 10% of the total numbers for representatives of

this group, as against 40 % each for the oligotrichs *Diplodinium* and *Entodinium*) they are clearly the most active in depositing polysaccharide granules inside themselves when glucose, fructose or sucrose is present. The process is so rapid, and the extent of polysaccharide synthesis so considerable, that there can be little doubt that the protozoa themselves directly convert these simple sugars into iodophilic polysaccharide granules. It seems probable that a considerable fraction of the water-soluble and yeast-fermentable carbohydrate (presumably fructose) in the hay fed to the sheep in these experiments is stored in this form, at least temporarily, in rumen protozoa. A feed of 800 g. hay would contain roughly 15 g. of simple yeast-fermentable sugars. Assuming a total rumen-liquor content of 10 l. and that average samples are in fact withdrawn through the fistula, it can be calculated, from the figures given in Table 1 for sugar fermentations, that 2–3 hr. after feeding, as much as 4 g. at least of protozoan polysaccharide granules have been synthesized and stored in holotrich ciliates alone. Owing to inevitable experimental losses the real amount stored must be considerably greater than this, even if no allowance be made for the lesser but obviously not inappreciable amount synthesized from simple sugars by oligotrich ciliates such as *Diplodinium* and *Entodinium*.

These granules cannot be micro-organisms since they contain far too little protein (less than 1 % in the best preparation) and far too much carbohydrate (up to 90 %). Buchanan & Fulmer (1928) listed eighteen species and strains of anaerobic and facultatively anaerobic bacteria with average nitrogen content of 9.5 % (limits, 4.0–14.2 %), and pointed out that even yeasts rarely contain more than 60 % of carbohydrate in their dried bodies. The synthesis of iodophilic polysaccharides by micro-organisms of the digestive tract is of course a well-known phenomenon, studied, for example, by Nasr & Baker (1949) who, however, pointed out that this process is incompletely understood, particularly as concerns yeasts and protozoa.

The storage of protozoan polysaccharide granules in the rumen is only temporary since no trace of them could be obtained on working-up protozoa 9 hr. after feeding the sheep. It remains to be shown whether protozoa auto-ferment their polysaccharide store, or whether bacteria play a part in this process.

Of the three yeast-fermentable hexoses having a common enolic form, namely glucose, fructose and mannose, only the first two seem to be utilized *in vitro* by the holotrichous protozoa for making polysaccharide granules. An analogous example was observed by Kluver & Hoppenbrouwer (1931) who showed that *Termobacterium mobile* Lindner did not ferment mannose, although it readily fermented glucose and fructose, and, more slowly, sucrose. The fact that the glucose-containing disaccharides maltose and cellobiose are apparently not readily utilized by *Isotricha* and other holotrichs for iodophilic polysaccharide synthesis shows further how strictly limited are their synthetical powers.

I am indebted to Miss Frances Masson for much technical assistance, to Mr F. Baker for many helpful discussions, and to Mr P. J. Heald for the care of the animals and for the determination of yeast-fermentable carbohydrate in hay.

REFERENCES

- BUCHANAN, R. E. & FULMER, E. I. (1928). *Physiology and Biochemistry of Bacteria*. London: Baillière, Tindall and Cox.
- ELSDEN, S. R. (1946). The fermentation of carbohydrates in the rumen of sheep. *J. exp. Biol.* **22**, 51.
- ELSDEN, S. R. & PHILLIPSON, A. T. (1948). Ruminant digestion. *Ann. Rev. Biochem.* **17**, 705.
- HINTON, C. L. & MACARA, T. (1924). Application of the iodometric method to the analysis of sugar products. *Analyst*, **49**, 2.
- JOHNSON, B. C., HAMILTON, T. S., ROBINSON, W. B. & GAREY, J. C. (1944). On the mechanism of non-protein nitrogen utilization by ruminants. *J. Anim. Sci.* **3**, 287.
- KLUYVER, A. J. & HOPPENBROUWER, W. J. (1931). Ein merkwürdiges Gärungs-bakterium Lindner's Termobakterium mobile. *Arch. Mikrobiol.* **2** (2), 245.
- MANGOLD, E. (1929). *Handbuch der Ernährung und des Stoffwechsels der Landwirtschaftlichen Nutztiere*. Berlin: J. Springer.
- NASR, H. & BAKER, F. (1949). Microbial synthesis of iodophile polysaccharide by a *Clostridium* from the caecum of the pig. *Nature, Lond.*, **164**, 745.
- NEISH, A. C. (1946). *Analytical methods for the Bacillus subtilis fermentation*. National Research Council of Canada, Rep. no. 46-8-3.
- TOSIC, J. (1949). Effect of small quantities of a yeast preparation on the recovery of appetite in sheep. *Brit. J. Nutrit.* **3**, 234.
- WATH, J. G. VAN DER & MYBURGH, S. J. (1941). Studies on the alimentary tract of Merino sheep in South Africa. VI. The role of infusoria in ruminant digestion with some remarks on ruminant bacteria. *Onderstepoort J. vet. Sci.* **17**, 61.

(Received 29 March 1950)