Fermentation of Soluble Sugars by Anaerobic Holotrich Ciliate Protozoa of the Genera *Isotricha* and *Dasytricha*

By P. J. HEALD and A. E. OXFORD
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 21 *June* 1952)

The glycolytic mechanisms of the obligatory anaerobic ciliates, which occur for example in great numbers in the rumens of sheep, oxen and goats, have never been adequately investigated. Hitherto the prevailing view has been that such Protozoa, even if seeming to ferment fodder carbohydrates, really do so by virtue of bacterial action in their body cavities (see, for example, van der Wath & Myburgh, 1941). It has recently been shown, however (Oxford, 1951; Masson & Oxford, 1951), that one important group of large rumen Protozoa, the holotrich ciliates, can very rapidly ferment glucose and certain other soluble hexoses, and oligo- and poly-saccharides, when the cells are separated from rumen contents and washed relatively free of extraneous bacteria. Such a fermentation will still take place even if the living Protozoa have been in contact with bactericidal concentrations of streptomycin for several days (Heald, Oxford & Sugden, 1952).

Since no evidence has been obtained by cultural studies that these holotrichs normally contain or ingest appreciable numbers of bacteria (Sugden & Oxford, 1952*a*, *b*), the inference seems clear that they must possess a powerful glycolytic mechanism of their own. This paper is mainly concerned with the action of washed, streptomycin-treated and starved sheep's rumen holotrichs on various soluble carbohydrates, together with a detailed study of the products of the fermentation of glucose by these Protozoa.

Some points of difference between these fermentation studies and a conventional bacterial fermentation must be mentioned at the outset in order that unprofitable comparisons between protozoan and bacterial action may be avoided:

(1) The protozoan suspensions used, even although virtually bacteria-free, were not a pure culture of one species only. There are three species of holotrichs present in the sheep's rumen, namely, *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*. No easy method of separation or cultivation of any one species has yet been devised (Sugden & Oxford, 1952*b*), nor can it be guaranteed that the same sheep on a standard hay diet will always yield a mixture of holotrichs containing the three species in constant relative proportions. This is probably of little importance in our fermentation study since microscopic examination has shown that all three species behave in the same way towards soluble fermentable sugars (Sugden & Oxford, 1952*b*).

(2) A very much greater proportion of the sugar disappearing from solution during the protozoan fermentation is stored in the cells as reserve starch-like polysaccharide than would be the case in an anaerobic bacterial glucose fermentation. This is mainly due to the size and internal structure of the Protozoa, e.g. the smallest of the three holotrichs, *D. ruminantium*, has dimensions of approximately $60 \times 40 \times 40\,\mu$. This is 100000 times as large as an average rumen coccus of diameter $1\,\mu$. The holotrich polysaccharide storage capacity is so great that the protein content of the fully loaded cell may be as little as 10% of the dry matter (Oxford, 1951).

(3) The ratio, carbon in cells to carbon in substrate at the beginning, is much greater than is usual for a bacterial fermentation. Changes in the carbon in the cells during fermentation have therefore to be taken into account in compiling balance sheets of the fermentation.

METHODS

Material

Sheep as a source of holotrich ciliate Protozoa. Rumen liquor was obtained from 3-year-old Cheviot wethers which were maintained exclusively on a virtually starch-free diet of hay fed at intervals of 12 hr. The sheep had been fitted with rumen cannulae by Dr A. T. Phillipson of this Institute (Phillipson & Innes, 1939).

Preparation of virtually bacteria-free holotrich ciliates. Samples of the rumen contents were taken with wide-bore glass tubes through the rumen cannulae 2 hr. after the sheep had been fed. At this point the storage of carbohydrate in the rumen micro-organisms of hay-fed sheep is at a maximum (Heald, 1951*a*). If samples were taken at a later period after feeding, poorer separation of the Protozoa was achieved. The samples were strained through six thicknesses of surgical gauze and were allowed to stand at 40° for 1–1·5 hr. During this period, the holotrich ciliates settled to the bottom together with much debris. The supernatant was decanted, the bottom layer was diluted slightly with acetate buffer (see below), and to this was then added glucose equivalent to 4–5 g./l. of original sample. The mixture was stirred, poured into separating funnels and allowed to stand

at 40° for 1–1·5 hr. The holotrich ciliates settled out as a thick white layer (Oxford, 1951) and were run off into sterile 50 ml. tubes containing acetate buffer. These were allowed to stand at 40°, when the gas produced by the ciliates caused any debris to rise to the top of the tubes. This was poured off, and the Protozoa were washed several times with the buffer by decantation. They were finally allowed to stand for 48 hr. in acetate buffer containing streptomycin (0·8 m-mole/l.), the buffer and streptomycin being changed at least twice and preferably three times during this period. In order to prevent excessive bursting of Protozoa, the washed cells from 1 l. of rumen contents were distributed between at least four 50 ml. tubes. Also for this reason, it was desirable that the buffer be changed frequently during the starvation period. After 48 hr. the Protozoa were sufficiently empty of storage polysaccharide to be used for further experiments. Microscopical examination then showed that all the living Protozoa were holotrich ciliates, and that all three species of rumen holotrichs were invariably present.

Composition of buffers

Acetate buffer for separation of the Protozoa from rumen contents. This was based, as regards Na, on the composition of sheep saliva (McDougall, 1948), and contained NaCl, 5·0 g.; CH_3COONa, 1·5 g.; K_2HPO_4, 1·0 g.; KH_2PO_4, 0·3 g.; $MgSO_4.7H_2O$, 0·10 g.; distilled water, 1000 ml.; pH, 7·3–7·4.

Phosphate-saline. Since the Protozoa did not survive in 0·1 M or 0·2 M-sodium phosphate buffer alone, the buffer finally used had the following composition: NaCl, 5·0 g.; K_2HPO_4, 3·0 g.; KH_2PO_4, 1·0 g.; distilled water, 1000 ml.; pH, 7·3–7·4. This was used for fermentation studies and for some manometric experiments.

Bicarbonate-saline. This was the buffer, pH 7·4, gassed with 5 % CO_2 : 95 % N_2 (v/v) described by Krebs & Henseleit (1932). Although suitable for experiments of up to 2 hr. duration it was unsuitable after 4 hr., since many Protozoa burst.

Manometric methods

Measurement of acid and gas production was carried out in standard Warburg flasks fitted with one or two side arms as required. In some experiments requiring two substrates, flasks with a single side arm were used, the second substrate being added from a dangling tube (Keilin, 1929).

Anaerobiosis was obtained by placing a stick of scraped yellow phosphorus in the centre well. H_2 production was measured in phosphate-saline by absorbing CO_2 in KOH contained in the centre well. At the end of the experiment 'bound' CO_2 was liberated by tipping in 0·2 ml. 10 N-H_2SO_4 from a second side arm.

Large-scale anaerobic fermentations

Method. The fermentation liquid (50–100 ml.) was contained in a Büchner flask maintained at 40° in a water bath, and continuously swept by an O_2 and CO_2-free stream of N_2. The gas was delivered below the surface of the liquid. The outgoing gases were passed first through a tube of anhydrous magnesium perchlorate and then through two tared tubes of soda asbestos (British Drug Houses microanalytical reagent). The CO_2-free gases were mixed with dry CO_2-free O_2 and were combusted in a furnace over hot copper oxide, the products of combustion being absorbed in anhydrous magnesium perchlorate and soda asbestos contained in tared tubes. From the increase in the weight of these tubes the amount of H_2 and hydrocarbon gases (if any) could be determined.

Inoculum. This consisted of a suspension of the Protozoa prepared as described above. Usually the washed Protozoa from 4 to 6 l. of rumen liquor served as inoculum for 100 ml. medium, containing not more than 0·4 % (w/v) glucose. They were washed four or five times in the centrifuge with phosphate-saline at 40° and finally resuspended in buffer. A known volume was added to the flask, which was then swept with a slow continuous stream of N_2 for about 3 hr. and the fermentation was ended by the addition of 2 ml. of 10 N-H_2SO_4 to release bound CO_2. The flask was then swept out for a further hour. The contents were centrifuged and the Protozoa were made up to their original volume in fresh buffer. The samples were analysed as described below.

Fermentation with [^{14}C]glucose. The technique adopted was that outlined by Umbreit, Burris & Stauffer (1949) and used by Cochrane & Gibbs (1951) and Wiame & Doudoroff (1951). Protozoa were allowed to metabolize glucose to which randomly labelled [^{14}C]glucose (10 or 30 μc.) had been added, and were then washed and placed in Warburg manometers and allowed to ferment in the presence and absence of exogenous unlabelled glucose. The CO_2 evolved was collected in 10 N-NaOH in the centre well. After a suitable time the fermentation was stopped, by the acid-tip method, and the carbonate solution removed from the centre wells of the manometers. $Ba^{14}CO_3$ was precipitated in the presence of carrier carbonate from these solutions and counted at infinite thickness using an end-window counter.

Analytical methods

Streptomycin. Streptomycin was estimated by the colorimetric method of Boxer, Jelinek & Leghorn (1947) as used for quantities of 200–1000 μg.

Carbohydrates. Glucose was estimated by the method of Hagedorn & Jensen (1923) by measuring the reducing value before and after fermentation of the solutions with Distiller's Company yeast (Mann, 1946).

Protozoan polysaccharide, after conversion to glucose by acid hydrolysis under the conditions described by Heald (1951 *a*), was estimated similarly.

Total carbon. The method of Heald (1951 *b*) was used with the following modification. Since the fermentation solutions contained large amounts of NaCl, it was found necessary to remove all Cl_2 formed on oxidation by absorption in H_2O_2 (100 vol.), contained in a spiral bubbler.

Volatile acids. These were distilled in the Markham (1942) still. The acids were separated and identified by means of gas-liquid partition chromatography (James & Martin, 1952).

Lactic acid. This was determined by the method of Friedemann & Graeser (1933) after removal of glucose by copper-lime (Van Slyke, 1917).

Succinic acid. The method was that of Krebs (1937) using sheep-heart succinoxidase.

Non-volatile acids other than lactic acid. The method used was that of Birkinshaw & Raistrick (1931), after first concentrating the metabolism solution, under reduced pressure, to a small volume.

Total nitrogen. This was estimated by the method of Chibnall, Rees & Williams (1943).

Ammonia and urea. The method of Conway (1947) was used.

Volatile neutral and non-volatile neutral compounds. These were determined as described by Neish (1946).

Substrates, etc. Streptomycin was used as the $CaCl_2$ complex supplied by Glaxo Laboratories Ltd. The carbohydrates were good commercial samples (L. Light or Kerfoot, Biochemical Reagent quality) with the exception of dahlia inulin and rye-grass fructosan, which were supplied by the late Dr E. G. V. Percival and Dr P. C. Arni (of this Institute) respectively.

[^{14}C]*Glucose.* This was supplied by the Radiochemical Centre, Amersham, Bucks.

Units. Since the gas evolved during fermentation may contain H_2 as well as CO_2, results in Fig. 1 *a* and *b* are given as 'μl. gas' produced in bicarbonate saline/0·1 mg. protozoan N. These values were obtained by multiplying mm. pressure change by the appropriate manometer constant for CO_2 (k_{CO_2}). It is recognized that these figures have no absolute meaning but may be used to show the relative rate of gas production by the Protozoa under the experimental conditions.

RESULTS

Effect of streptomycin on the holotrich ciliates

Results with added pure cultures of bacteria have been summarized elsewhere (Heald *et al.* 1952). They showed that a concentration of 0·8 m-mole/l. of streptomycin base (500–600 μg./ml.) was effective in completely suppressing all bacteria, added or otherwise. Furthermore, no bacterial colonies developed if the streaked test plates were incubated anaerobically in a 95 % N_2:5 % CO_2 atmosphere. It was therefore concluded that streptomycin at this concentration had rendered the living Protozoa virtually bacteria-free, and that no substantial interference by bacterial action in any fermentation study restricted to 3 hr. or less need be feared.

The concentration of streptomycin used was many times the usual lethal dose for bacteria (cf. Pulvertaft, 1952). It had no visible effect on the holotrichs in 2–3 days, i.e. neither on their motility nor on the gradual diminution of their polysaccharide reserves. The suppression of bacteria was not due to a dilution effect caused by replacing the buffer, since there was good bacterial growth on plates inoculated from non-streptomycin-treated control cultures containing similarly washed ciliates. The Protozoa did not decompose the antibiotic, since a quantitative recovery of it was obtained after a 24 hr. treatment of the cells with streptomycin (0·8 m-mole/l.).

Manometric experiments with various substrates in bicarbonate buffer

Since the holotrich ciliates stored large quantities of polysaccharide, part of which was still present after 48 hr. of starvation, it was to be expected that the endogenous fermentation would then still be appreciable. For this reason, and because the protozoan suspensions contained three separate species, the relative proportions of which might vary from time to time, it is clear that no simple basis exists for the quantitative comparison of manometric results obtained with different suspensions, even if all were derived from the same sheep. Nevertheless, the fermentation of glucose was strikingly evident. Fructose, sucrose, raffinose, inulin and rye-grass levan were fermented at rates comparable to glucose; cellobiose at a much slower rate; and xylose, galactose, mannose, turanose, maltose, lactose, melibiose, melezitose, soluble starch (as paste) and sodium glucuronate, not at all.

Effect of streptomycin on the rate of acid production from glucose

When the activity of washed and starved cells which had been in contact with streptomycin was compared with a suspension not treated with streptomycin, there was no essential difference in the rate of acid production (Fig. 1*b*). Similarly, the addition of streptomycin during the metabolism of glucose by washed streptomycin-treated cells, did not produce a measurable effect (Fig. 1*a*).

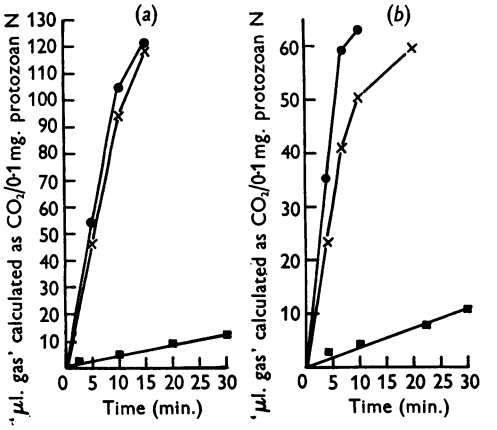

Fig. 1. (*a*) The effect of streptomycin on the rate of fermentation of glucose by Protozoa previously treated with streptomycin. Each flask contained 292 μg. cell N in 2 ml. of bicarbonate-saline. Side arms contained 0·5 ml. of 0·01 M-glucose. ●—●, glucose; ×—×, glucose + streptomycin (1·5 mg.) in buffer (0·2 ml.); ■—■, endogenous substrate.

(*b*) Comparison of the rate of fermentation of glucose by two protozoan suspensions, one treated with streptomycin and one untreated. Each flask contained the cells in 2 ml. of buffer. Side arms contained 0·5 ml. of 0·01 M-substrate. ●—●, cells treated with streptomycin; protozoan N = 336 μg./manometer. ×—×, cells not treated with streptomycin; protozoan N = 414 μg./manometer.

In both experiments the gas phase was 95 % N_2:5 % CO_2; temp., 40°. Anaerobiosis was obtained by placing a stick of yellow phosphorus in the centre well. For method of calculation of 'μl. gas' see text.

Table 1. *The production of hydrogen and carbon dioxide during the fermentation of glucose by holotrich ciliates of the sheep's rumen*

(Each manometer contained 2 ml. of Protozoa in phosphate saline, pH 7·4; gas, N_2; temp., 40°. Glucose added in 0·5 ml. buffer.)

Protozoan suspension no.	Glucose added (μmoles)	Time (min.)	Total gas produced (μl.)	Hydrogen produced (μl.)	Carbon dioxide produced (μl.)	Ratio $\frac{\text{carbon dioxide}}{\text{hydrogen}}$
1	5	10	175	90	85	0·95
2	5	12	96	51	45	0·89
3	5	18	272	130	142	1·09
4	100	30	190	107	83	0·77
		60	290	162	128	0·79
		90	324	149	175	1·13
5	100	60	103	55	48	0·87
		120	196	104	92	0·88
		180	158	75	83	1·12

Manometric studies with glucose in a bicarbonate-free buffer

In order to obtain a measure of true fermentation carbon dioxide and of any other gases evolved, manometric experiments were carried out in the phosphate buffer. Table 1 shows that a gas other than carbon dioxide was in fact obtained under these conditions, and this was later shown, by combustion over copper oxide, always to be entirely hydrogen with no admixture of methane. It appeared probable that hydrogen was reabsorbed during the later stages of the fermentation of a large excess of glucose.

Effect of oxygen on glucose fermentation

Suspensions of Protozoa, when shaken in air, soon lost all power of ciliary motion, and burst. Under these conditions, although still feebly alive for a period, it was found that hardly any glucose was used and that the uptake of oxygen was very small indeed.

Large-scale fermentation of glucose by holotrich ciliates

Fermentation in phosphate buffer. No fermentation was allowed to proceed beyond 3 hr. The only detectable fermentation products were lactic, acetic, butyric and sometimes traces of propionic acids together with carbon dioxide and hydrogen. Volatile fatty acids higher than butyric were not produced. Volatile and non-volatile neutral and non-volatile acidic products (including succinic acid) seemed to be present, if at all, in the merest traces. No formic acid was detected. The results of six fermentations are summarized in Table 2. It will be noted that more than half of the glucose fermented is accounted for by an increase of glucose-containing cellular material, presumably storage glucosan for the most part (cf. Masson & Oxford (1951) who attempted with only moderate success to isolate this starch-like material quantitatively by perchloric acid extraction). The carbon recoveries and the oxidation-reduction index indicated that the bulk of the glucose fermented seemed to be accounted for satisfactorily. This calculation assumes, however, that no loss of cellular material, other than storage glucosan, took place during fermentation. Since lactic and acetic acids, with oxidation-reduction values identical with that of glucose, might conceivably also arise from lower amino-acids by deamination, it was considered advisable to prepare a carbon and nitrogen balance sheet for the whole fermentation system. The results for four glucose fermentations are summarized in Table 3. It will be noted that there was always a considerable loss of cellular nitrogen. Since the fermentation liquid gave no reactions for ammonium salts even when the fermentation was conducted in a closed system (Table 3, Exp. 6), nor for amino-acids (by ninhydrin test) or urea, it was assumed that the lost nitrogen was probably present in solution as soluble protein or polypeptide, derived by breakdown of cellular protein. Hence carbon in organic nitrogenous compounds in solution might reasonably be arrived at by multiplying the weight loss of nitrogen from cells by three. Owing, presumably, to difficulties of sampling on so small a scale, these two independent values for soluble carbon as 'polypeptide' did not always exactly agree. Even so, there was usually still some carbon unaccounted for in solution, the origin of which is obscure. It is conceivable that this is the sum of numerous products arising from cellular autolysis, but individually present in traces only, beyond the limit of detection by the methods used.

The extent of the loss of cellular substance, other than storage polysaccharide during fermentation, was also determined by another method based on a comparison of the extra carbon stored in the cells as reserve glucosan (and liberated as glucose on hydrolysis) with the total increase in cellular carbon

Table 2. *The products of the fermentation of glucose by rumen holotrich ciliates, in phosphate-saline*

(Glucose (100 ml. 0·4 %) fermented in N_2 stream at 40°; initial pH, 7·4; CO absorbed in soda asbestos; H_2 determined by combustion.)

Exp.	Glucose: Fermented (m-mole)	Glucose: Stored as glucosan (m-mole)	Lactic acid (m-mole)	Acetic acid (m-mole)	Propionic acid (m-mole)	Butyric acid (m-mole)	Carbon dioxide (m-mole)	Hydrogen (m-mole)	Apparent carbon recovery (%)	Hydrogen/oxygen index for fermentation products
1	0·96	0·528	0·21	0·128	0	0·128	0·44	0·58*	88·4	2·01
2	1·01	0·568	0·31	0	0	0·22	0·37	0·49*	94·6	2·13
3	1·86	1·18	0·53	0·23	0·056	0·142	0·48	0·61	94·0	1·99
4	2·16	1·26	0·54	0·29	0·08	0·30	0·76	1·05	94·3	2·08
5	1·83	1·42	0·33	0·25	0	0·25	0·56	0·75*	104·5	2·01
6	0·338	—	0·086	0·065	0	0·065	0·122	0·16*	—	2·13

* Calculated on the basis that carbon dioxide/hydrogen = 0·75.

Table 3. *Overall fermentation balance sheet for holotrich ciliate fermentation of glucose, attention being paid to changes in cellular carbon and nitrogen and also storage polysaccharide*

(For details see Table 2.)

Ref. no. of Exp. (see Table 2)	Carbon in glucose used up from solution (mg.) G	Carbon in carbon dioxide evolved (mg.) M (see Table 2)	Carbon in cells: Before fermentation (mg.) A	Carbon in cells: After fermentation (mg.) X	Increase in total protozoan carbon during fermentation (mg.) $(X-A)$	Carbon in solution at end, other than glucose carbon (mg.) S	Total of carbon accounted for (%) $\frac{(X-A)+S+M}{G}\times 100$
3	134·2	5·4	63·6	123·6	60·0	74·4	104
4	143·3	9·1	130·8	180·0	49·2	80·0	97
5	131·4	6·7	103·2	185·4	82·2	36·8	96·5
6 (closed system)	26·2	1·4	27·8	34·9	—	13·4	83·5

Ref. no. of Exp. (see Table 2)	Carbon in form of protozoan glucosan (mg.): Before fermentation	Carbon in form of protozoan glucosan (mg.): After fermentation	Increase in glucosan carbon during fermentation (mg.) P	Net decrease in carbon in protozoan cellular substance other than polysaccharide (mg.) $P-(X-A)$	Nitrogen in cells: Before fermentation (mg.)	Nitrogen in cells: After fermentation (mg.)	Nitrogen in solution after fermentation (mg.)
3	43·4	129·1	85·7	25·7	11·0	6·0	3·2
4	96·2	187·2	91·0	41·8	16·9	13·7	4·0
5	52·8	155·5	102·7	20·5	14·4	10·9	—
6 (closed system)	—	—	—	—	3·9	2·6	0·8

Table 4. *Endogenous fermentation balance sheet for holotrich ciliate Protozoa in phosphate buffer*

(Each experiment with approx. 100 ml. buffer pH 7·3, ran for approx. 3 hr. in N_2 stream at 40°. CO_2 absorbed in soda asbestos.)

Initial state of Protozoa	Carbon in carbon dioxide evolved (mg.)	Total carbon in cells: Before fermentation (mg.)	Total carbon in cells: After fermentation (mg.)	Carbon in protozoan glucosan: Before fermentation (mg.)	Carbon in protozoan glucosan: After fermentation (mg.)	Carbon in solution at end (mg.)	Carbon as soluble acidic products of fermentation (mg.): Lactic	Acetic	Butyric	Nitrogen in solution after fermentation (mg.)
After 2 days starvation	0·24	27·8	24·9	—	—	3·6	0·6	0·65	0·65	1·0
Immediately after glucose fermentation	0·96*	61·2	46·8	62†	34·8	15·6	3·4	1·1	0·95	2·7

* Gaseous carbon dioxide/hydrogen ratio was 0·6.

† Probably a little too high. The cells nevertheless were extremely well-filled with storage polysaccharide, when examined microscopically after staining with Lugol's iodine.

as determined by wet combustion (see also Table 3). The net decrease in the carbon of non-glucosan cellular substance was rather greater than the apparent production of soluble 'polypeptide'. This is to be expected since some carbon dioxide and a little of the acidic fermentation products have probably arisen from original cellular substance during the fermentation. The possible magnitude of this contribution was indicated by a no-substrate control fermentation (Table 4). As might be anticipated, the soluble products of endogenous fermentation (particularly lactic acid) were relatively much greater in amount when well-filled Protozoa, taken immediately after a glucose fermentation, were used. Even the starved Protozoa, however, still produced acetic and butyric acids as well as 'polypeptide'. It is safe to conclude, therefore, that the endogenous fermentation of glucosan is always qualitatively similar to the fermentation of added glucose.

Fermentation in bicarbonate buffer. An experiment was carried out to determine whether a reasonably high concentration of bicarbonate, such as exists in the rumen, affected the end products from the fermentation of glucose. When a fermentation was carried out in a modified bicarbonate saline containing $3{\cdot}55 \times 10^{-2}$ M-HCO_3^- (pH approx. 6·5) under a 100% carbon dioxide atmosphere, the percentages of the carbon fermented which appeared as lactic, acetic, propionic and butyric acids were 12·9, 4·2, 0·45 and 6·5, respectively. These values did not differ sufficiently markedly from those found in Table 2 (fermentation in phosphate-saline in 100% nitrogen atmosphere) to suggest that a higher bicarbonate concentration had any effect on the fermentation. The pH chosen was within the normal range for actively fermenting rumen contents *in vivo*.

Table 5. *Effect of protozoan fermentation of added glucose on endogenous fermentation of* [^{14}C]-*storage polysaccharide*

(Two experiments (suspensions (*a*) and (*b*)) carried out in phosphate-saline buffer at pH 7·2. The experiments were carried out in Warburg manometers. Each flask contained 2 ml. of the protozoan suspension. The side arms contained either 200 μmoles glucose in 0·5 ml. of buffer, or 0·5 ml. of buffer, as required. The CO_2 evolved was absorbed in KOH (10%, w/v) in the centre well; gas phase, 100% N_2; temp., 40°. Radioactivity measured at infinite thickness to an accuracy of ±5%.)

Substrate	Carbon dioxide produced (μl.)	Activity in carbon dioxide counted as $BaCO_3$ (counts/min.)
Endogenous	(*a*) 133	141
	(*b*) 168	101
Glucose	(*a*) 586	52
	(*b*) 736	35

The effect of glucose fermentation on the endogenous fermentation of storage polysaccharide

In view of the large endogenous fermentation taking place in the absence of added carbohydrate, it was of interest to determine whether this fermentation was suppressed during the fermentation of glucose. A fermentation, first of labelled, and then of unlabelled glucose by the same cells was therefore carried out (see Methods section). The results are shown in Table 5. Taking into account the inevitable dilution of radioactive storage polysaccharide resulting from 'starch' formation in the subsequent fermentation of unlabelled glucose, it is considered that there was no extensive suppression of the endogenous fermentation in this experiment.

DISCUSSION

If it be allowed that streptomycin, as our results seem to show, has no appreciable effect on the protozoan enzymes, it is a remarkable fact that the only soluble sugars from which the bacteria-free holotrich ciliates produce acid rapidly are those liable to occur in the sheep's fodder, namely, glucose, fructose, sucrose, raffinose, inulin and grass fructosan. These Protozoa have also a certain limited power of fermenting cellobiose, and presumably other β-glucosides of true plant origin. In the main these results with washed suspensions are in harmony with those of Sugden & Oxford (1952*b*) on the effect of soluble sugars in prolonging life of the Protozoa beyond 2 days. It is clear that a number of sugars, like maltose, and the pentoses, which are known to be readily fermented by mixed rumen bacteria, are of little use to the rumen holotrich ciliates. Nevertheless, the protozoan fermentation products from glucose, in the absence of bacteria, are qualitatively just those which would arise from glucose *in vitro* by the action of a mixed population of rumen bacteria, namely, lactic, acetic, propionic and butyric acids, carbon dioxide and hydrogen (cf. Elsden, 1945). The relative proportion of propionic acid is, however, much smaller in the protozoan fermentation, and the presence or absence of bicarbonate does not appear to affect the yields of the respective acids. It is doubtful, therefore, whether carbon dioxide absorption plays an important part in the metabolism of these Protozoa, even though in their natural habitat, the rumen, they constantly live in presence of a high bicarbonate concentration.

As regards the host animal, the significance of the rapid rate of glucosan storage by these Protozoa is twofold: (*a*) the rumen bacteria are prevented from fermenting all soluble carbohydrates at a rapid rate: (*b*) when no soluble fodder carbohydrate remains, the endogenous fermentation of the protozoan

storage glucosan releases into the rumen a steady flow of lactic, acetic and butyric acids. The first-named acid is probably converted into volatile fatty acids, chiefly propionic, by known bacterial mechanisms (cf. Elsden, 1945; Johns, 1951).

It may be pointed out that this is the first time that rumen ciliate Protozoa have been shown to possess a vigorous carbohydrate metabolism in absence of bacteria. Furthermore, it is clear that, in this respect at least, the ciliates are beneficial rather than harmful to the host animal.

SUMMARY

1. Suspensions of virtually bacteria-free and starved holotrich ciliates separated from sheep's rumen contents, containing *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*, produced acids and gas at a rapid rate under anaerobic conditions from the following soluble carbohydrates: glucose, fructose, sucrose, raffinose, inulin, rye-grass levan and, to a much lesser degree, cellobiose. No other soluble carbohydrate was attacked. Glucose was not attacked in presence of oxygen.

2. The relatively high concentration of streptomycin used to kill bacteria associated with the Protozoa had little effect upon the viability or fermentative activity of the latter.

3. The fermentation products from glucose were lactic, acetic, propionic (traces) and butyric acids, carbon dioxide and hydrogen. Soluble nitrogenous compounds, which were not ammonia, urea or amino-acids, were also produced.

4. In the first 3 hr. the starved ciliates stored more glucose as polysaccharide than was fermented to acids.

5. The endogenous fermentative activity of the ciliates containing storage glucosan yielded lactic, acetic and butyric acids.

6. Glucose fermentation did not extensively suppress the endogenous fermentation of storage polysaccharide.

7. The use of carbon dioxide instead of nitrogen in the gas phase had little apparent effect upon the protozoan glucose fermentation.

8. The importance of these ciliates as fermentative agents in the rumen is discussed.

We are particularly indebted to Dr W. H. Pfander for carrying out the determinations of volatile acids on the gas-liquid partition chromatogram. Our thanks are due also to Dr T. F. Macrae (Glaxo Laboratories, Ltd.) for a generous supply of streptomycin; Miss M. J. MacPherson for bacterial cultures; Dr Brenda Sugden for confirming the protozoan identifications; Dr P. C. Arni for a gift of rye-grass levan; Dr G. A. Garton for advice and assistance concerning the use of radioactive-carbon compounds; and Mr M. B. Great for much technical assistance.

REFERENCES

Birkinshaw, J. H. & Raistrick, H. (1931). *Phil. Trans.* B, **220**, 11.
Boxer, G. E., Jelinek, V. C. & Leghorn, P. M. (1947). *J. biol. Chem.* **169**, 153.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Cochrane, V. W. & Gibbs, M. (1951). *J. Bact.* **61**, 305.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby, Lockwood and Son.
Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
Friedemann, T. E. & Graeser, J. B. (1933). *J. biol. Chem.* **100**, 291.
Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
Heald, P. J. (1951*a*). *Brit. J. Nutr.* **5**, 84.
Heald, P. J. (1951*b*). *Biochem. J.* **49**, 684.
Heald, P. J., Oxford, A. E. & Sugden, B. (1952). *Nature, Lond.*, **169**, 1055.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Johns, A. T. (1951). *J. gen. Microbiol.* **5**, 317, 326, 337.
Keilin, D. (1929). *Proc. Roy. Soc.* (B), **104**, 206.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
McDougall, E. I. (1948). *Biochem. J.* **43**, 99.
Mann, T. (1946). *Biochem. J.* **40**, 481.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Masson, F. M. & Oxford, A. E. (1951). *J. gen. Microbiol.* **5**, 664.
Neish, A. C. (1946). *Analytical Methods for the Bacillus Subtilis Fermentation. Nat. Res. Coun. Ottawa.* Rep. 46–8–3.
Oxford, A. E. (1951). *J. gen. Microbiol.* **5**, 83.
Phillipson, A. T. & Innes, J. R. M. (1939). *Quart. J. exp. Physiol.* **29**, 333.
Pulvertaft, R. J. V. (1952). *J. Path. Bact.* **64**, 75.
Sugden, B. & Oxford, A. E. (1952*a*). *J. gen. Microbiol.* **6**, ii.
Sugden, B. & Oxford, A.E. (1952*b*). *J. gen. Microbiol.* **7**, 145.
Umbreit, W. W., Burris, R. H. & Stauffer, J. T. (1949). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* 2nd ed. Minneapolis: Burgess Publishing Co.
Van Slyke, D. D. (1917). *J. biol. Chem.* **32**, 455.
van der Wath, J. G. & Myburgh, S. J. (1941). *Onderstepoort J. vet. Sci.* **17**, 61.
Wiame, J. M. & Doudoroff, M. (1951). *J. Bact.* **62**, 178.