Experiments on the Culture and Physiology of Holotrichs from the Bovine Rumen

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(*Received* 16 *November* 1954)

The rumen protozoa can be assigned to three different groups; one consisting of the large protozoa of the genus *Diplodinium* and related types which ingest quantities of fibrous plant material; a second group of smaller protozoa in the genus *Entodinium* which actively digest starch but seldom are found to contain the cell walls of plants; and a third group, the holotrichs, which seldom contain plant materials. This latter group includes the species *Isotricha prostoma*, *I. intestinalis* and the smaller *Dasytricha ruminantium*.

Previous investigators (Heald, Oxford & Sugden, 1952; Heald & Oxford, 1953) gave small amounts of glucose to strained sheep-rumen contents placed in separatory funnels. Metabolizing the carbohydrate very rapidly, the three species of holotrichs settled to the bottom of the funnel and could be obtained free of the other rumen protozoa. Heald & Oxford (1953) analysed the fermentation products of the mixed holotrich protozoa and found carbon dioxide, hydrogen, lactic, acetic and butyric acids, and a trace of propionic acid as the products of glucose fermentation. Cellobiose was utilized by the mixture but at a rate much slower than glucose.

This paper deals with attempts to separate the three species of holotrichs, to determine their individual characteristics, and to estimate their significance to the host.

MATERIALS AND METHODS

Preparation of buffer solutions and rumen fluid. The inorganic salt solution used in the culture media and manometric experiments contained 0·5% (w/v) NaCl, 0·01% (w/v) $CaCl_2$, 0·01% (w/v) $MgSO_4$ and 0·1% (w/v) KH_2PO_4. The composition of this solution is similar to that of the inorganic salt solution described by Hungate (1942) for cultures of *Diplodinium neglectum*, except that he used 0·6% (w/v) NaCl. Anaerobic conditions were maintained with CO_2 gas, and 0·5% (w/v) $NaHCO_3$ was added to give a suitable pH.

Samples of rumen contents were removed by tube and suction bulb (Hungate, 1950) from two fistulated steers. When required for use in culture medium, the rumen fluid was placed in a refrigerator overnight to allow the protozoa and plant debris to settle. The supernatant fluid was found by microscopic examination to be free of protozoa but to contain many bacteria. The upper portion was drawn off and stored in the refrigerator until required. Media containing either heated or Seitz-filtered rumen fluid did not support growth as well as those made up with untreated fluid. When the holotrich protozoa were to be used in Warburg experiments or fermentation product analyses, the rumen contents were filtered through bolting silk to remove the large debris and immediately incubated with glucose.

Separation of protozoa. A portion of the rumen sample (usually about 300 ml.) was placed in a separatory funnel with 0·5% (w/v) glucose and kept for 30–60 min. at 39° according to the method described by Heald *et al.* (1952). Many *Isotricha* and some *Dasytricha* collected in the bottom of the funnel and were drawn off for further purification.

After the remaining rumen contents were drained out of the funnel, streaks of a fine greyish white film were observed on the inner wall which microscopic examination showed to consist of pure *D. ruminantium* in large numbers. The *Dasytricha* were washed off the walls with mild shaking, using anaerobic salt solution which had been warmed to 39°. The loss of motility by the dasytrichs sticking to the wall was only temporary, since they were seen swimming actively immediately after removal from the sides. Sufficient numbers of this species uncontaminated by *Isotricha* were obtained to permit manometric experiments and quantitative analyses of the products of fermentation.

As a means of separating the isotrichs, the protozoa which had been drawn off from the base of the separatory funnel were added at the top of a glass tube filled with 30% (v/v) rumen fluid, plus 70% (v/v) inorganic salt solution. Anaerobic conditions were maintained using CO_2 gas, and 0·5% (w/v) $NaHCO_3$ was added to give a suitable pH. The tube was 1·5 cm. in diameter and 60 cm. long and was fitted with an exit tube and stopcock at the lower end. The larger, heavier *I. prostoma* and *I. intestinalis* settled faster than *Dasytricha*. The first portion to settle could be drawn off through the stopcock uncontaminated by *Dasytricha*.

The degree of success obtained with this method depended in part on the length of time the mixture of holotrichs remained in the solution containing glucose. Thirty minutes gave a good separation; 1 hr. in the sugar solution often caused the protozoa to burst, as was observed by Sugden & Oxford (1952). In certain instances it was noted that soon after transferring the protozoa to a glucose solution, a granular material adhered to the mouth of *I. prostoma* as it swam with the mouth directed posteriorly. The significance of this phenomenon was not ascertained. The appearance suggested that the material was extruded from the cell, though the possibility that it was an accumulation of material from the medium was not excluded.

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The initial procedure for separation of *Isotricha* from *Dasytricha* was later improved by omitting the rumen fluid from the medium and layering 15 ml. of tap water on the top of the salt solution in the column. Most of the *Dasytricha* became swollen, non-motile and did not settle. *Isotricha* settled with fewer convection currents than were encountered in the original method and a larger number of pure isotrichs could be collected.

Counting the protozoa. Counts of the protozoa in the suspensions used in manometric experiments were made by serial dilution of a sample (1 ml.) of the holotrich suspension (originally obtained by glucose sedimentation) into a series of anaerobic buffered saline tubes. The appropriate dilution tube was shaken thoroughly to insure a uniform suspension and 0·1 ml. quickly removed. This was placed in a watch glass under a dissection microscope and the protozoa were counted as they were gently pipetted one by one out of the dish with a finely drawn micro-capillary. Five protozoan samples were counted and an average figure calculated. In order to estimate populations of *Isotricha* and *Dasytricha* in the rumen of different cattle, the same method was used except that glucose was not introduced into the rumen sample.

Preparation of fermentation flask cultures. The non-gaseous fermentation products of mixed *I. prostoma* and *I. intestinalis* were obtained for quantitative analysis by incubating a washed suspension anaerobically with 0·4% (w/v) glucose in 100 ml. of inorganic salt solution at 39°. The protozoa cell count in the fermentation flash was 12000/ml. The suspension contained approximately equal numbers of both species of large *Isotricha*. The flasks used were 200 ml. round-bottomed Pyrex type. Pure CO_2 was passed through the mixture by means of a bent Pasteur pipette connected by rubber tubing to the gas source. As the pipette was withdrawn, a rubber stopper was inserted without allowing entrance of air. Sodium bicarbonate, 0·5% (w/v), was used to give an initial pH of about 7·0.

The protozoan cells in a control flask were killed at the beginning of the experiment with 3 ml. of 3·6 N-H_2SO_4. After incubating for 6 hr. the protozoa in the experimental flask were killed with the same amount of acid as the control and the liquid in both control and experimental cultures was analysed for fermentation acids. The amounts of acid found in the control were negligible. The short incubation period was used in order to allow accumulation of sufficient products for analysis, yet limit the formation of bacterial fermentation products. No indications of significant bacterial growth were observed.

The fermentation flasks for *D. ruminantium* were prepared in a similar manner except that the substrate was decreased to 0·1% (w/v) glucose in 75 ml. of inorganic salt solution. The cell count in the flask was 60000/ml. The suspension contained only *D. ruminantium*.

Since a fermentation balance sheet was not calculated from the large fermentation flask analyses, a determination for residual glucose was not performed. In the manometric experiments, reducing substances were not present at the end of the run, when the vessel contents were tested with Benedict's solution.

Identification of fermentation acids in flask cultures. The method of Friedmann, Cotonio & Shaffer (1927) was used for the quantitative determination of lactic acid. Volatile acids were collected by steam distillation and separated on a chromatographic column prepared by a modification (Carroll & Hungate, 1954) of the method of Elsden (1946). The identity of the acid in each band was confirmed by Duclaux distillation and calculation of the Duclaux constant.

Determination of total nitrogen. Enough protozoan cells were collected to permit a micro-Kjeldahl determination (Hungate, 1940) for an estimation of the total nitrogen per cell.

Manometric methods. The rates of production of acid, CO_2 and H_2 per protozoan cell were determined by the use of Warburg manometers and vessels with two side arms. Two mg. of glucose or cellobiose in 0·2 ml. of distilled water were placed in the first side arm and 0·2 ml. of 3·6 N-H_2SO_4 in the second side arm. The protozoa were washed three times at 39° in the anaerobic salt solution described previously except that 0·1% (w/v) $NaHCO_3$ was used with 5·0% CO_2 + 95% N_2. After the final centrifuging, the suspension was made up to a known volume, usually 8 ml., with salt solution. One ml. of the suspension was diluted and the number of protozoa counted in order to calculate the number used in each run. One ml. was placed in the main compartment of each vessel. Acid production and CO_2 evolved in metabolism were calculated by comparison with a control vessel in which the acid was tipped at the beginning of the run. At the end of the experiment, the H_2 evolved was measured by exposing the gas to palladium contained in a small bulb on the ground-glass joint of each vessel (Hungate, 1943). Temperature for all experiments was 39° and the gas phase was 5·0% CO_2 in N_2.

RESULTS

Carbohydrate utilization. Manometric experiments on *Dasytricha* and on mixed isotrichs were run, testing their ability to use glucose and cellobiose. The results for *Dasytricha* are shown in Fig. 1 and for *Isotricha* in Fig. 2. It will be seen that whereas a utilization of the suspension of *Dasytricha* utilized both glucose and cellobiose the mixture of *I. prostoma* and *I. intestinalis* used only glucose.

Gas evolution by *Dasytricha* with cellobiose as a substrate was not quite as rapid as with glucose, but the rate of fermentation of both substrates was of the same order of magnitude and much greater than in the control. The diminished rate of cellobiose utilization reported by Heald & Oxford (1953) for the mixed protozoa is explained by the fact that only *Dasytricha* can use this sugar.

The utilization of various other carbohydrates by individual species of holotrichs was studied by feeding test carbohydrates to starved suspensions, adding iodine, and examining microscopically to see which species showed storage polysaccharide. In instances where the carbohydrate was utilized, the holotrichs became dark with food reserves within 3–5 min. When these cells were tested with iodine they showed a purple brown colour not exhibited by controls. *I. prostoma*, *I. intestinalis* and *D. ruminantium* all deposited reserve food when fed sucrose, fructose, glucose, inulin and raffinose, the rate of deposition appearing about the same with all these substrates. In addition, cellobiose and salicin were used by *Dasytricha*, though the deposition of reserves

was not as marked as with the other sugars. These latter substrates were not used by *Isotricha*. Starved specimens of *I. prostoma* and *I. intestinalis* ingested small starch grains from a suspension of 'soluble' starch, but *Dasytricha* did not. Examination after 8 hr. showed that most of the starch grains had disappeared and the protozoa then stained purple brown with iodine. The carbohydrates utilized are similar to those reported by Sugden & Oxford (1952).

Determination of fermentation products. Lactic, acetic and butyric acids were identified as products of glucose fermentation by mixed suspension of *I. prostoma* and *I. intestinalis*. The amounts found, in milliequivalents were: lactic acid, 0·953; butyric acid, 0·358; and acetic acid, 0·230. A trace of an unidentified acid appearing between acetic and butyric on the chromatogram was probably the same as the acid reported by Heald & Oxford (1953) to be propionic acid. The amount present was too small to permit a determination of Duclaux values.

With *D. ruminantium* fermentation acids were found in the ratios of 0·147 m-equiv. lactic acid, 0·061 m-equiv. acetic acid, and 0·042 m-equiv. butyric acid. Traces of the unidentified acid showing a band between acetic and butyric acids were also observed with this species.

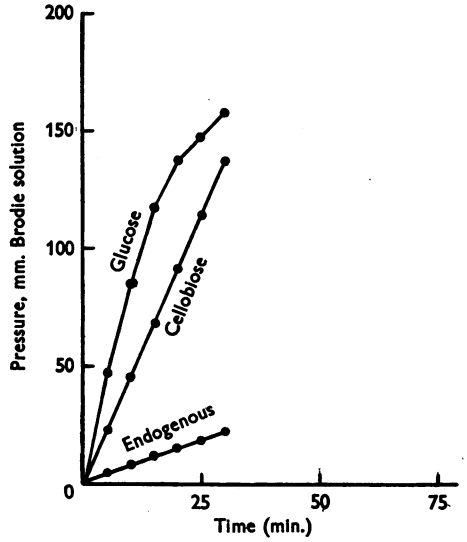

Fig. 1. The fermentation of glucose and cellobiose by a washed suspension of *Dasytricha*. Each flask contained 240000 cells in 1 ml. of 0·1% bicarbonate–saline. 11·1 μ-moles glucose or cellobiose in 0·2 ml. distilled water in the first side arm, and 0·2 ml. of 3·6 N-H_2SO_4 in the second side arm. Total volume of the manometer cups was 19–21 ml. Gas phase 95% N_2 + 5% CO_2; temp. 39°.

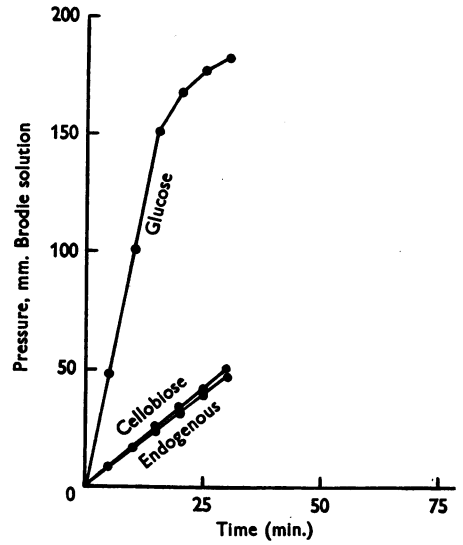

Fig. 2. The comparison of glucose fermentation with cellobiose by a mixed suspension of *I. prostoma* and *I. intestinalis*. Each flask contained 7000 cells in 1 ml. of 0·1% bicarbonate–saline. 11·1 μmoles of glucose and 8·85 μmoles cellobiose provided in the side arms. Other conditions of the experiment similar to those given for Fig. 1.

Table 1. *The fermentation products from glucose by holotrich protozoa*

The experimental flask side arm contained 11·1 μmoles glucose in 0·2 ml. distilled H_2O. Control flasks received no substrate. Each *Isotricha* vessel contained 1·0 ml. protozoan suspension (9600 cells) in 0·1% bicarbonate–saline. *Dasytricha* flasks contained 150000 cells. Gas phase was 95% N_2 + 5% CO_2; temp. 39°. The difference between the glucose and control flask was due to glucose. Applying the percentage of each kind of acid produced in the large fermentation flasks to the total acid found in the above experiment, an approximation of the amounts of each acid was calculated (see text).

		Products (μmoles)			Calculated amounts of different acids (μmoles)
	Flask	H_2	CO_2	Acid	
Isotricha	Plus glucose	5·45	5·94	5·13	Lactic 1·92
	Endogenous	0·81	0·89	2·01	Acetic 0·46
	Difference	4·64	5·05	3·12	Butyric 0·72
Dasytricha	Plus glucose	4·02	3·44	3·7	Lactic 1·88
	Endogenous	0·58	0·89	0·49	Acetic 0·78
	Difference	3·44	2·55	3·21	Butyric 0·53

In manometric experiments, CO_2, H_2 and acid were shown to be formed by both *Dasytricha* and *Isotricha* during the fermentation of glucose. The amounts of CO_2, H_2, and acid formed in the Warburg experimental and control flasks were calculated. The difference between these values was the amount ascribed to the 2 mg. of glucose provided in the experimental cups. The results are shown in Table 1.

The ratios of fermentation acids found in the large fermentation flasks made possible an indirect calculation of the carbon recovery in the Warburg experiments. This was necessary, since the amounts of acid produced in the manometric experiments were too small to allow separation and quantitative estimates of the individual acids.

The carbon in the fermentation products derived from glucose by *Isotricha* (Table 1) amounts to 14·8 μg. atom C and 13·7 μg. atom C by the *Dasytricha* as compared with 66·6 μg. atom C in the glucose fermented. This low recovery indicates that a very large proportion of the substrate is synthesized into reserve polysaccharide, in agreement with the findings of Heald & Oxford (1953).

Table 2. *Endogenous fermentation rates*

Results expressed as μm-moles product/cell/hr. Protozoa were collected from rumen contents by glucose sedimentation, washed with anaerobic bicarbonate–saline and used within 3 hr. to determine fermentation rates. Flasks in the *Isotricha* experiments contained 10 000 cells/ml. The *Dasytricha* manometers held 180000 cells/ml. One ml. of protozoa suspension in 0·1% bicarbonate–saline placed in each vessel. No substrate was provided. One side arm held 0·2 ml. of 3·6N-H_2SO_4. Gas phase 95% N_2+5% CO_2; Temp. 39°. Duration of run, usually 2 hr. Expts. 1, 2*a* and 2*b* were performed with the same protozoa sample.

Expt. no.	Rate of production (μm-moles/cell/hr.) H_2	CO_2	Acid CO_2
1. *Isotricha*, not starved	0·39	0·32	0·43
2*a*. *Isotricha*, not starved	0·30	0·25	0·32
2*b*. *Isotricha*, starved 24 hr.	0·17	0·14	0·14
3. *Dasytricha ruminantium*, not starved	0·02	0·02	0·01

Rate of fermentation per cell

Endogenous. The suspension contained both species of *Isotricha*. In Expts. 1 and 2*a* of Table 2 the organisms were washed free of glucose and the endogenous rate determined immediately. In Expt. 2*b* they were starved 24 hr. before the endogenous rate was measured.

Starved cells have a slower rate of endogenous fermentation than protozoa which have recently been fed but even after starvation for 24 hr. (Expt. 2*b*, Table 2) the rate of acid production was still approximately 30% of that found immediately after feeding.

With substrate. The amounts of acid, CO_2 and H_2 produced from glucose per cell by the *Isotricha* species and *Dasytricha* are given in Table 3. An average acid production from glucose of 0·062 millimicromoles per dasytrich per hr. and 2·35 per isotrich per hr. was found. The results in Table 3

Table 3. *The rates of fermentation of glucose*

Results expressed as μm-moles product/cell/hr. The number of cells in the *Isotricha* experiments ranged from 5000 to 13 000/ml. With *Dasytricha* the numbers used in each cup varied from 150 000 to 240 000/ml. One ml. of protozoa suspension in 0·1% bicarbonate–saline in each vessel. One side arm contained 11·1 μmoles glucose in 0·2 ml. distilled water. Other side arm held 0·2 ml. of 3·6N-H_2SO_4. Acid production was calculated from the CO_2 liberated by bicarbonate. H_2 was determined at the end of the run (usually 30 min.) by absorption with palladium placed in a neck bulb. Gas phase was 95% N_2+5% CO_2; Temp. 39°. The values presented are for protozoa samples collected from different animals on separate occasions.

Species	Rates of formation (μm-moles/cell/hr.) H_2	CO_2	Acid CO_2
I. prostoma	4·05	2·73	2·57
	4·75	1·98	4·44
I. intestinalis and *I. prostoma*	1·90	1·45	2·01
	1·07	1·00	0·88
	1·58	1·27	1·88
D. ruminantium	0·053	0·076	0·077
	0·066	0·080	0·048
	0·068	0·060	0·062

Table 4. *Nitrogen content of rumen holotrichs*

Each determination done in duplicate with protozoa collected at different times. *Dasytricha*; Expts. 1 and 2 were performed with $2·8 \times 10^6$ and $1·3 \times 10^6$ cells respectively. *Isotricha*: Expts. 1 and 2 used $1·6 \times 10^4$ and $1·4 \times 10^4$ cells respectively. Ten ml. of the sample were placed in a 100 ml. Kjeldahl flask with 1·0 g. of K_2SO_4, 0·1 g. Hg, 3·0 ml. of conc. H_2SO_4 and digested for 30 min. The digest was distilled in a Pregl micro-still and the NH_3 titrated as it came over, instead of being trapped in an excess of acid and back-titrated (Hungate, 1940).

Species	Expt. no.	Total N (μmg./cell)
D. ruminantium	1	1·17
		1·15
	2	1·19
		1·12
Mixed *I. prostoma* and *I. intestinalis*	1	26·3
		31·5
	2	23·0
		25·2

suggest that *I. prostoma* has a higher metabolic rate than *I. intestinalis*. Cultures of *I. intestinalis* unmixed with other species could not be collected, making it difficult to prove that the rate of glucose fermentation was in fact less than that found in *I. prostoma*.

Cellular nitrogen content. By counting the protozoa, average micro-Kjeldahl values of nitrogen per cell were determined. The results of different experiments are given in Table 4.

Culture experiments. The best approach to culture of the holotrichs appeared to be one which would duplicate as nearly as possible the conditions existing in the rumen. Undiluted rumen fluid (50 ml.) gave less than 24 hr. survival of holotrichs (*I. intestinalis* and *I. prostoma*) when inoculated with 1 ml. of fresh rumen contents containing protozoa of many species. Since proper osmotic pressure was essential in culturing *Diplodinium* (Hungate, 1942), optimum osmotic pressure conditions were determined for the holotrichs. For this purpose 50 mg. each of dried ground alfalfa and wheat were suspended in 100 ml. of inorganic salt solution in which the NaCl content was varied from 0·1 to 1·0 % (w/v). Anaerobiosis was maintained with 5 % CO_2 + 95 % N_2, and 0·1 % (w/v) $NaHCO_3$ was used to buffer at a pH of 6·8. The 0·6 % NaCl flask had the largest number of isotrichs surviving at 48 hr.

The CO_2 content of rumen gas is 70 % and it seemed possible that better survival of the isotrichs might be obtained with more CO_2 than was present in the 5 % CO_2 + N_2 mixture. Pure CO_2 was used and the bicarbonate buffer was increased to 0·5 % in order to maintain the same pH. In an experiment to determine optimum osmotic pressure in this medium 0·4, 0·5 and 0·6 % (w/v) NaCl showed the best conditions for survival of isotrichs.

Up to this point, survival of the isotrichs beyond 48 hr. had not been achieved, though *Diplodinium* and *Entodinium* were active and healthy. Failure of the isotrichs to survive suggested that some additional nutritional factor, present in rumen fluid, might be missing. The culture medium was modified by incorporating various levels of rumen fluid. The addition of rumen fluid 30 % (v/v) to the hay–grain medium increased the survival time to 96 hr.

An experiment was designed to determine whether toxic elements accumulating in the medium might be responsible for the failure of continued growth of the protozoa. Isotrichs were pipetted out of fresh rumen contents and transferred to alfalfa and wheat medium plus 30 % (v/v) rumen fluid in a large centrifuge tube. At the end of each day the cultures were centrifuged and the supernatant fluid was pipetted off and discarded, leaving the protozoa in the sediment. Fresh substrate and inorganic culture medium plus rumen fluid were then added. Survival time for the isotrichs was increased by this procedure to 18 days, but there was no apparent increase in numbers. In a control flask in which only half the culture medium was removed each 24 hr., no protozoa survived beyond 48 hr.

To avoid the accumulation of sediment, the protozoa were removed individually with a pipette, avoiding taking up any of the debris. This was accomplished by placing 1 ml. portions of the culture in a watch glass and picking the protozoa with a micro-capillary with the aid of a dissecting microscope. Each isotrich was counted and then placed in a tube containing fresh inorganic salts plus 30 % (v/v) rumen fluid, 0·05 % (w/v) alfalfa and 0·05 % (w/v) ground wheat. Transfer was completed as rapidly as possible to prevent damage from oxygen. This individual handling of the isotrichs necessitated the use of small numbers of protozoa.

Beginning with 15 isotrichs freshly removed from the rumen, and placed in 25 ml. of the culture medium, the count went to 20 at 24 hr., to 27 protozoa at 48 hr., and to 36 at 72 hr. Several protozoa were seen dividing transversely. In another experiment, using as an inoculum 15 protozoa which had been carried for 2 weeks in laboratory cultures, 24 isotrichs were counted at 24 hr., 28 isotrichs at 48 hr., and 37 at 72 hr. These numbers are all minimal counts, since some of the protozoa may have been missed in the debris of the ground alfalfa and wheat. The increases appear to indicate that division occurs every 48 hr. in these laboratory cultures. This is probably slower than the rate occurring in the rumen, since the protozoa would have to divide at least once a day to maintain themselves in the host.

DISCUSSION

Significance of the protozoa to the host

Numbers present. Fluctuations in the numbers of holotrichs in an individual steer were evident even when the animal was on a constant diet throughout the period of sampling. On different occasions *I. prostoma* was the predominant large isotrich, while at other times *I. intestinalis* was more numerous in the same host. *D. ruminantium* was consistently found in greater numbers than the isotrichs. The combined isotrich population ranged from 300 to 4000/ml. of rumen contents and the dasytrichs from 2700 to 10000/ml.

Energy supply. The experimental results on rates of acid production from glucose per cell per hour provide a means for the estimation of total acid production by the holotrichs in the rumen. Calculations based on an average of 2·35 μm-moles acid

for each isotrich show that 5·64 m-moles acid would be produced each day in 100 kg. of rumen contents containing one isotrich/ml. Similarly, using a figure of 0·062 μm-moles for each dasytrich, 0·15 m-moles of acid would be the daily contribution if glucose was continuously fermented. In the endogenous metabolism experiments the rate of acid production was approximately one-sixth of the glucose rate, indicating that a total of 0·94 m-moles of acid would be formed per day in 100 kg. of rumen contents with one isotrich/ml. and 0·025 m-moles of acid for a concentration of one dasytrich/ml. The production of 0·94 m-moles of acid with an average molecular wt. of 82 would equal 77 mg. of acid produced in 100 kg. of rumen contents by a concentration of one isotrich/ml. A production of 0·025 m-moles would equal 2·0 mg. of acid for each dasytrich/ml. Using rumen counts of 3000 isotrichs and 5000 dasytrichs/ml. calculations show that approximately 240 g. of fermentation acids are produced by the holotrichs each day. This value is based on the minimum fermentation rate found for endogenous metabolism and on an arbitrary estimate of the protozoa in the rumen.

Carroll & Hungate (1954) have estimated that an average of 2·2 kg. of volatile acids are produced each day in a bovine with a rumen content of 100 kg. The holotrichs thus contribute about 10 % of the total acids in the rumen. It should be emphasized that these estimates are based on rates of acid produced in the endogenous experiments and not from glucose. No data on rates of acid production per cell are available for the oligotrichs but if their fermentation products are estimated as approximately equal to those of the holotrichs, the rumen protozoa contribute about 20 % of the total fermentation acids available to the host.

The acids produced by the rumen protozoa are not identical with those absorbed by the host. Presumably the lactic acid is first converted into propionic acid by certain rumen bacteria (Johns, 1951; Gutierrez, 1953), and is then absorbed. Since relatively little of the energy is lost during the conversion, the final acids have approximately the same energy values as those formed by the protozoa.

Nitrogen supply. With one isotrich/ml. there would be 2·5 mg. of isotrich N or 15 mg. of isotrich protein in a rumen containing 100 kg. of contents. With one dasytrich/ml. there would be 0·11 mg. N or 0·66 mg. dasytrich protein/100 kg. Assuming that 69 % of this is digested daily (Hungate, 1942) values of approximately 10 mg. protein are available to the host for each isotrich per ml. and 0·45 mg. protein for each dasytrich/ml. Using rumen counts of 3000 *Isotricha* and 5000 *Dasytricha*/ml., approximately 33 g. of protein would be supplied to the host by the holotrich protozoa each day.

Cultural requirements of the protozoa. The preliminary culture experiments which have been presented do not permit a satisfactory analysis of the various factors that allow growth of the protozoa under some conditions, but fail to give growth under other conditions which appear quite similar. However, a listing of the possible influencing factors may serve to clarify the problem and suggest the lines along which further experiments may be performed. (1) The protozoa use dissolved carbohydrates rather than the insoluble cellulose or hemicellulose to meet their energy requirements, and in this respect may be competing with certain bacteria. (2) Rumen fluid contains and/or has produced in it toxic materials which cause death of the isotrichs in laboratory cultures, but in the rumen are removed before they reach a toxic level. (3) In addition to the dissolved substances which provide carbohydrates, the protozoa may require bacteria as food to meet their protein needs.

Present results indicate that the isotrich protozoa differ markedly from *Diplodinium* and *Entodinium* in their culture requirements. They are more difficult to handle but can presumably be grown indefinitely in laboratory cultures with the procedures which have been developed. Using these procedures it is hoped that further experiments will permit a more complete evaluation of the rumen isotrichs, and their significance in the nutrition of the host.

SUMMARY

1. Methods are described for the separation of *Dasytricha ruminantium* from *Isotricha prostoma* and *Isotricha intestinalis*, holotrichs found in the bovine rumen.

2. The fermentation products of the protozoa were determined and carbon dioxide, hydrogen, lactic, acetic and butyric acids were found to be produced by both *Isotricha* and *Dasytricha*.

3. All three species of holotrichs deposited reserve food when fed sucrose, fructose, glucose, inulin and raffinose. Cellobiose and salicin were used only by *Dasytricha* and to a lesser extent than glucose.

4. Rates of acid, CO_2 and H_2 production per cell per hour for the holotrichs were obtained from Warburg experiments, and from the average number of holotrichs it was calculated that 240 g. of fermentation acids are produced by them per day in 100 kg. of rumen contents.

5. The holotrich protein yield was calculated from the total nitrogen content per cell and numbers of protozoa present. About 33 g. of protein are supplied each day.

6. *Isotricha* was successfully cultured in small numbers for periods up to 3 weeks. The increase in numbers suggested that under culture conditions it divided once every 48 hr.

The author wishes to express his sincere appreciation to Dr R. E. Hungate for an introduction to the fascinating cattle protozoa and for many stimulating discussions during the course of the experiments. The investigation was supported in part by funds for biological and medical research by the State of Washington, initiative measure number 171.

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A Colorimetric Method for the Estimation of Serum Magnesium

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(*Received* 29 *December* 1954)

In clinical work, where general electrolyte depletion occurs, there may be disturbances of serum magnesium which require correction. These deficiencies have been shown by Flink, Stutzman, Anderson, Konig & Frazer (1954) to occur sometimes in chronic alcoholics and in patients on continuous intravenous therapy. These authors reported neurological symptoms similar to those described in animals by Kruse, Orent & McCollum (1932) and by Greenberg & Tufts (1938). Evidence of change in the magnesium levels of the cerebrospinal fluid in human subjects was found by McCance & Watchorn (1932) in cases of chronic meningeal inflammation, but no symptoms were ascribed to this. Smith (1949) showed that a raised serum magnesium concentration in the dog caused a flaccid paralysis. The importance of magnesium is also evident in intermediary metabolism, since magnesium is needed as an enzyme activator in oxidative decarboxylation and is involved in adenosine triphosphate energy-release mechanisms (Baldwin, 1947). Interest in magnesium levels in clinical work is increasing and there is a need for a method of serum magnesium estimation suitable for a routine clinical laboratory.

The earliest method for the estimation of magnesium was gravimetrically as magnesium ammonium phosphate (McCrudden, 1909), and in the following period many micro-methods applicable to serum were based on the estimation of phosphate in the precipitates by various procedures. Decolorization of ferric thiocyanate was used by Marriott & Howland (1917), and by Kramer & Tisdall (1921), who combined calcium and magnesium estimations by consecutive precipitation of calcium as oxalate, and magnesium as magnesium ammonium phosphate. Denis (1920) used a nephelometric method with strychnine molybdate. Upon the introduction of the colorimetric estimation of phosphate by Bell & Doisy (1920), this was applied to the magnesium ammonium phosphate precipitate by Denis (1922), Hammett & Adams (1922), and with modifications by Briggs (1922*a*, *b*, 1924). The colorimetric method of Fiske & Subbarow (1925) was subsequently used until Simonsen, Westover & Wertman (1947) introduced molybdivanadate for the estimation of phosphate.

A different principle was used by Greenberg & Mackey (1932), in which calcium was removed and magnesium precipitated by 8-hydroxyquinoline, which was estimated volumetrically; but this method has not been generally used owing to difficulty in handling the light precipitate.

Hirschfelder & Serles (1934) introduced the method based on an estimation of magnesium as a specific coloured lake using Titan Yellow in alkaline solution. The lake had, however, to be suspended by a colloidal dispersing agent; soluble starch was used but tends to give opalescent solutions. Garner (1946) used gum ghatti as the dispersing agent to give clear solutions. A further modification was introduced by Kunkel, Pearson & Schweigert (1947) using hydroxylamine hydrochloride with Titan Yellow. The most recent examination of Titan Yellow methods was made by Orange & Rhein (1951), who selected polyvinyl choride as a colloidal dispersing agent.

Buckley, Gibson & Bartolotti (1951) introduced titration with ethylenediaminetetraacetate to estimate magnesium using Eriochrome Black T as an indicator after the determination of calcium