

The Metabolism of Starch, Maltose, Glucose and Some Other Sugars by the Rumen Ciliate *Entodinium caudatum*

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SUMMARY

Washed suspensions of *Entodinium caudatum* grown *in vitro* and incubated anaerobically incorporated ^{14}C from [^{14}C]glucose into the cell pool as [^{14}C]glucose, maltose and hexose phosphate and into protozoal polysaccharide and intracellular bacteria as a glucose polymer. The uptake of sugars into the pool was by an active process (predominant at low sugar concentrations) probably into the protozoal endoplasm, and by a passive process (predominant at high concentrations) into another part of the cell. Uptake of glucose by the active process was not sufficiently rapid to increase the level of glucose in the pool by more than 17% per hour; this was probably insufficient to increase appreciably the rate of glucose utilization. These protozoa hydrolysed starch to maltose and glucose and the level of these sugars in the pool of protozoa metabolizing starch grains was controlled in part by the inhibitory effect of these sugars on the enzymes that formed them. Studies on the enzymes involved in the metabolism of starch showed that the glucose in starch was hydrolysed to free glucose before phosphorylation. In experiments on the effect of carbohydrates on the survival of protozoa, starch was the most effective in prolonging protozoal life, followed by maltose and glucose in that order. The greater effectiveness of maltose was correlated with the finding that the rate of uptake of maltose carbon was always greater than that for glucose when the two sugars were present in equimolar concentrations. However, the rate for both sugars was markedly decreased in the presence of penicillin and neomycin. Evidence is presented that the protozoal ectoplasm may be freely permeable to sugars in the medium and that there is a barrier between the ectoplasm and endoplasm. ^{14}C from [^{14}C]starch grains was incorporated by the protozoa but there was no synthesis of protozoal protein from carbohydrate.

INTRODUCTION

It has been known for many years (Sugden, 1953; Abou Akkada & Howard, 1960) that rumen Entodiniomorphid protozoa do not readily metabolize sugars as determined by sugar disappearance, by acid or gas formation or by the synthesis of polysaccharide. In contrast, starch grains as can be engulfed by the protozoa are degraded to volatile fatty acids, hydrogen and CO_2 (Abou Akkada & Howard, 1960) and have been used as principal source of carbohydrate for the maintenance of *Entodinium caudatum*, *in vitro*, for over 8 years (Coleman, 1960*a*). However, there is evidence that this protozoon could metabolize sugars since the life of starved 'bacteria free' suspensions incubated in the presence of penicillin and neomycin could be prolonged by glucose, maltose and starch in increasing order of effectiveness (Coleman,

1962). The present communication describes the metabolism of [^{14}C]labelled starch and sugars by washed suspensions of *Entodinium caudatum* with special reference to the differential effect of glucose, maltose and starch on protozoal survival. There is a permeability barrier between the medium and the protozoal endoplasm and this barrier is more permeable to maltose than glucose and is crossed by starch grains following their engulfment.

METHODS

Source of protozoa. *Entodinium caudatum* was grown and inoculum cultures prepared and treated as described by Coleman (1962) except that inoculum cultures were treated each day with 1.0 ml. 1.5% rice starch suspension and about 10 mg. dried grass.

Preparation of protozoa for inoculation. The protozoa were present as a loose pellet at the bottom of the tube containing the inoculum cultures. After removal of the surface scum and most of the medium, the protozoa and remaining medium were transferred to 8 × 1 in. (20 × 2.5 cm.) tubes and allowed to stand until any grass present had sunk to the bottom, leaving the protozoa in the supernatant fluid. This supernatant fluid was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960*b*) and the washings added to the supernatant fluid. The protozoa were spun down and washed four times in salt solution B through which a gas mixture of 95% (v/v) N_2 + 5% (v/v) CO_2 had been bubbled vigorously for 3 min., on a bucket-head centrifuge for 20 sec. from starting; the maximum speed was equivalent to 200*g*. The protozoa were finally used as an inoculum to give a population density of 5 to 15×10^5 protozoa/ml.

Incubation conditions for incorporation experiments. The medium consisted of 0.15 to 0.4 ml. [^{14}C]sugar (0.0025 to 0.7 M and 0.25 to 6.9 $\mu\text{C}/\text{ml}$.), 1.0 ml. protozoal inoculum (suspended in salt solution B through which 95% (v/v) N_2 + 5% (v/v) CO_2 had been bubbled vigorously for 3 min.), 0.2 ml. of any other additions and 0 to 0.45 ml. salt solution B (chosen so that the final volume was 1.5 ml.). Experiments were made in 4 × 0.5 in. (10 × 1.3 cm.) thick-walled test tubes and after inoculation were gassed with 95% (v/v) N_2 + 5% (v/v) CO_2 , sealed with a rubber bung and incubated at 39°, except where otherwise stated.

Incubation conditions for treatment with antibiotics. The medium consisted of 0.8 ml. penicillin G (25,000 units/ml.), 0.8 ml. 1% (w/v) neomycin sulphate and 20 ml. protozoal inoculum, all in a 100 ml. conical flask which was gassed with 95% (v/v) N_2 + 5% (v/v) CO_2 , sealed with a rubber bung and incubated at 39°. At the end of the incubation (usually 18 hr) the protozoa were isolated as described previously.

Incubation conditions for experiments on protozoal survival. The medium consisted of 3.1 to 5.3 ml. (chosen so that the final volume was 10 ml.) salt solution B and 2.0 ml. water autoclaved (115° for 20 min.) in a 15 ml. centrifuge tube. Immediately after removal from the autoclave the following additions were made aseptically: 0.2 ml. 1% L-cysteine hydrochloride (neutralized and Seitz filtered), 0.2 ml. 5% (w/v) NaHCO_3 (Seitz filtered), 0.4 ml. penicillin G (25,000 units/ml.), 0.4 ml. 1% (w/v) neomycin sulphate, 1.0 ml. autoclaved rumen fluid (PARF; Coleman, 1964*a*) and 0 to 2.2 ml. sugar solution (autoclaved at 115° for 20 min. in water) or soluble starch (autoclaved at 115° for 20 min. in salt solution B) or 0 to 0.3 ml. 7.5% (w/v) rice starch (rice starch heated dry at 120° for 24 hr and suspended in sterile salt solution B). After inoculation

to give a population density of approximately 5×10^5 protozoa/ml. the tubes were gassed with 95% (v/v) N_2 + 5% (v/v) CO_2 , sealed with a rubber bung and incubated at 39°. For each point on a time course a fresh culture was sampled and the tube then discarded.

Incubation conditions for growth of protozoa. The same conditions were used as for experiments on protozoal survival except that neomycin was omitted from the incubation medium. At the end of an experiment in which protozoa had been incubated with a [^{14}C]labelled compound, the culture was centrifuged and the protozoa washed twice at 300g for 30 sec. in salt solution B.

Breakage of protozoa. The protozoa suspended in 2.5 ml. salt solution B (for metabolic experiments) or water (for fractionation of labelled protozoa) were broken by immersion of the tube to the depth of the liquid in the tube in a 80 kc/sec. 40 W. ultrasonic cleaning bath (KG 80/1, manufactured by Kerry's of Chester Hall Lane, Basildon, Essex) for 15 sec. Unless the crude homogenate was required, the whole was then centrifuged at 7000g for 20 min. The supernatant fluid from this centrifugation is hereafter referred to as the 'broken-cell supernatant fluid' and the pellet after washing once in water as the 'broken-cell pellet'. This latter fraction contained all the viable bacteria and the polysaccharide granules in the homogenate.

Estimation of ^{14}C and ^{32}P . ^{14}C or ^{32}P in any material was estimated by pipetting a sample on to an aluminium planchet of area 4.7 cm.² carrying a disc of lens tissue. The sample was spread by one drop of cetyltrimethylammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The disc was dried at 40° and the radioactivity estimated by means of an automatic flow counter (Nuclear-Chicago Corp.) with an efficiency for ^{14}C of about 20%. Over 1000 counts at a rate greater than twice background (usually greater than five times) were recorded for all fractions. Determinations were made with less than 0.5 mg. material/cm.² of disc. The amount of sugar incorporated by protozoa or into the fraction sampled was calculated from the known specific activity of the sugar added initially. To determine the relative ^{14}C or ^{32}P contents of spots on a chromatogram a thin mica end-window GM tube was placed directly on the spot, the position of which had been determined by radioautography.

Fractionation of protozoa. The two fractions obtained by sonic treatment and centrifugation of a protozoal suspension were further fractionated by a method based on that of Roberts *et al.* (1955). The fraction was treated with 5% trichloroacetic acid (TCA) and allowed to stand at 4° for 30 min. The precipitate was centrifuged down and washed once in 5% TCA. The supernatant fluid and the washing formed the 'cold TCA-soluble fraction'. This fraction of the broken-cell supernatant fluid is hereafter referred to as the protozoal 'pool'. The precipitate was then extracted with 75% (v/v) ethanol in water at 40° for 30 min. The residue was centrifuged down and the supernatant fluid formed the ethanol-soluble fraction. The precipitate was then extracted with 75% ethanol+ether (1+1 by vol.) at 40° for 15 min. The residue was centrifuged down and the supernatant fluid formed the ethanol+ether soluble fraction. The precipitate was then extracted twice with 5% TCA at 100° for 30 min. The supernatant fluids formed 'the hot TCA-soluble fraction'. The residue after washing once with acidified ethanol formed the 'residual protein fraction'. The TCA was removed from fractions by washing three times with ether before plating out for the estimation of ^{14}C . The ethanol and ethanol+ether soluble fractions were further

fractionated by the addition of equal quantities of water and ether and the two layers so obtained separated. The aqueous layers after washing once with ether were combined and formed the 'ethanol-soluble protein' fraction. The ether layers plus the washing were combined to form the 'lipid fraction'.

Paper chromatography. The following solvents were used: (A) *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.); (B) *n*-propanol + ethyl acetate + water (24 + 13 + 7, by vol.); (C) *tert*-butanol + ammonia (sp.gr. = 0.880) + water (60 + 30 + 10, by vol.) on paper impregnated with 0.01 M-Na₂B₄O₇ (Agarwal, Sanwal & Krishnan, 1963); (D) phenol + ammonia (sp.gr. = 0.880) + water (80 g. + 0.3 ml. + 20 ml.); (E) methanol + ammonia (sp.gr. = 0.9015) + water (60 + 10 + 30, by vol.; Bandurski & Axelrod, 1951); (F) *n*-butanol + cyclohexane + propylene glycol + water + ammonia (sp.gr. = 0.880) + morpholine (30 + 30 + 10 + 3.5 + 0.7 + 0.07, by vol.; Guillaume & Osteux, 1955); (G) *n*-butanol saturated with 1.5 N-NH₄OH; (H) isopropanol + water (80 + 20, by vol.; Smith, 1960).

Identification of [¹⁴C]glucose in protozoal fractions. The material was first chromatographed in solvent B which separated hexoses from pentoses, disaccharides and phosphate esters, and the position of radioactive spots determined by radioautography. The spot that ran next to marker glucose was eluted and identified in the following way. It was chromatographed against markers in solvents A to H and shown to be glucose. The material was also chromatographed in solvent B before and after treatment with glucose oxidase. As a result of enzyme action the original spot disappeared and was replaced by another which ran with the same *R_f* as that produced from pure [¹⁴C]glucose under the same conditions. When sufficient material was present the spots on the paper after chromatographic separation were shown to give the characteristic hexose reaction when treated with aniline oxalate reagent (Horrocks & Manning, 1949). When insufficient material was present the identity of the glucose was confirmed by the 'fingerprint' technique of Roberts *et al.* (1955). For this method the unknown [¹⁴C]compound was mixed with a relatively large amount of the [¹²C]-form of the compound with which it had been identified and the whole chromatographed. The [¹⁴C] spot was then detected by radioautography and the carrier compound by the aniline oxalate reagent. When the tracer and carrier compound are the same, then the pattern of the radioautogram must agree in every detail with the pattern produced by the chemical spray.

Identification of other [¹⁴C]sugars in the protozoal 'pool'. The method adopted was similar to that for [¹⁴C]glucose with the exception that the glucose oxidase treatment had no effect on the chromatographic behaviour of the [¹⁴C]material.

Identification and estimation of [¹⁴C] in volatile fatty acids. The volatile acids produced during the metabolism of [¹⁴C]carbohydrates were removed from the medium by steam distillation and then the distillate was neutralized, evaporated to dryness *in vacuo* and the residue chromatographed in solvent F, which separated formic, acetic, propionic and butyric acids. Immediately after removal from the tank and while the chromatogram was still wet, it was sprayed with 0.01 M-NaOH to fix the acids on the paper. To determine the position of the various fatty acids, marker mixtures of formic, acetic, propionic, *n*-butyric and *n*-valeric acids containing each acid in turn labelled with [¹⁴C] were chromatographed on the same paper. The relative [¹⁴C]content of the various spots was determined by placing a thin mica end-window GM tube directly on the paper.

Protozoal counts. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Phosphorylase assay. The incubation medium consisted of 1.5 ml. 0.1 M- $\beta\beta'$ -dimethylglutarate buffer (pH 6.4), 0.5 to 1.0 ml. water (chosen so that the final volume was 3.0 ml.), 0.1 ml. ^{32}P -inorganic phosphate, 0.1 to 0.6 ml. 0.001 to 0.1 M-phosphate buffer (pH 6.4), 0.2 ml. carbohydrate solution (usually 2% soluble starch for experiments on glucan phosphorylase) and 0.1 ml. 1% L-cysteine hydrochloride (neutralized). Gas mixture 95% (v/v) N_2 + 5% (v/v) CO_2 was bubbled through the medium for 2 min. immediately after the addition of the cysteine and before the addition of 0.2 ml. broken protozoal preparation. The tubes were then gassed for a further 10 sec. before sealing with a rubber bung and incubation at 39° usually for 30 min. At the end of the incubation 0.2 ml. 0.5 M-NaF and 0.2 ml. 0.2 M-phosphate buffer (pH 6.4) were added and the whole mixed. Then 0.6 ml. magnesia mixture (Leloir & Cardini, 1957) was added, the contents of the tube mixed and the whole allowed to stand at 4° for 1 hr. At the end of this period the precipitate was centrifuged down and 0.2 ml. of the supernatant fluid plated out for the estimation of ^{32}P .

Glucose estimations. Glucose was estimated by the method of Huggett & Nixon (1957) by using a carbohydrase-free preparation of glucose oxidase (Type V of Sigma Chemical Co., 12 Lettice Street, London, S.W. 6).

Maltose estimation. Maltose was estimated as glucose after hydrolysis in $\text{N-H}_2\text{SO}_4$ for 30 min. at 100°, followed by neutralization with NaOH. The results were corrected for the amount of free glucose present before hydrolysis.

Reducing sugar estimations. Reducing sugars were estimated by the ferricyanide method of Park & Johnson (1949).

Hexokinase assay. The incubation medium contained 1.0 ml. salt solution B, 0.6 ml. 0.001 to 0.1 M [^{14}C]glucose or water, 0.1 ml. 0.1 M-adenosine triphosphate (ATP) and 0.2 ml. broken protozoal supernatant fluid. The reaction was usually allowed to continue for 30 min. at 39° and was then stopped by heating at 100° for 5 min. Two ml. of a suspension of Dowex 2 [acetate] was added and the whole shaken for 15 min. to remove the [^{14}C]hexose phosphates. The resin was then washed twice with water and the phosphates removed by treatment with 3 ml. N-HCl a sample of which was plated out for the estimation of ^{14}C . The ^{14}C in the eluate was used as a measure of enzyme activity.

Phosphoglucumutase assay. The reaction mixture contained 1.0 ml. 0.1 M- $\beta\beta'$ -dimethylglutarate buffer (pH 6.4), 0.6 ml. 0.01 to 0.1 M-glucose-1-phosphate or water and 0.2 ml. broken protozoal supernatant fluid. Tubes were incubated at 39° for 30 min. and the reaction stopped by heating at 100° for 5 min. The precipitate was centrifuged down and 0.1 ml. of the supernatant fluid used for the estimation of reducing sugars, with glucose-6-phosphate as a standard. To correct for the reducing materials present in the reagents initially, reducing sugar estimations were done at each glucose-1-phosphate concentration before and after incubation.

Maltase assay. The reaction mixture contained 1.0 ml. salt solution B, 0.6 ml. 0.01 to 0.1 M-maltose or water and 0.2 ml. broken protozoal supernatant fluid. Tubes were incubated at 39° for 30 min. and the reaction stopped by heating at 100° for 5 min. The precipitate was centrifuged down and 0.1 ml. of the supernatant fluid used for the estimation of glucose. To correct for the glucose present in the maltose, glucose estimations were carried out at each maltose concentration before and after incubation.

Preparation of [¹⁴C]labelled starch grains. The method was based on the observation of Oxford (1951) that rumen holotrich protozoa rapidly convert glucose into an intracellular glucosan. A sample (500 ml.) of crude rumen contents from a Clun Forest sheep fed on hay and oats was strained through muslin and allowed to stand in a separating funnel at 39° for 45 min. The sediment, which consisted principally of holotrich protozoa, was run off into an 8 × 1 in. (20 × 2.5 cm.) test tube and washed three times with salt solution B by sedimentation under gravity. The washed protozoa were inoculated into 5 ml. salt solution B (from which the sodium propionate was omitted) containing [¹⁴C]glucose (0.5 mg. and 6 μC/ml.) and 0.2 ml. saturated NaHCO₃ and through which CO₂ had been bubbled for 2 min. After inoculation the tubes were gassed with CO₂ for 10 sec., sealed with a rubber bung and incubated horizontally for 2 hr at 39°. The protozoa were then harvested, washed four times in salt solution B and broken in a Potter homogenizer (Potter & Elvehjem, 1936). The homogenate was centrifuged at 1200g for 15 min. and the pellet of starch grains washed twice in water under the same conditions. Approximately 10% of the ¹⁴C present in the glucose initially was converted into [¹⁴C]starch. On fractionation of these grains (see Methods) over 96% of the ¹⁴C was solubilized on treatment with 5% TCA at 100° for 20 min., and on further hydrolysis of this extract in N-HCl for 1 hr at 100° glucose was found to be the only ¹⁴C-labelled monosaccharide present; only 0.4% of the ¹⁴C was present in the protein fraction. These results indicated that the preparation contained [¹⁴C]starch contaminated with very little protein.

Chemicals. [¹⁴C]compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. The specific activities of the compounds, as supplied, were in μC/mg., [¹⁴C]-D-galactose, 20.8; [U-¹⁴C]-D-glucose, 16.2; [U-¹⁴C]-D-maltose, 13.5; [1-¹⁴C]-D-ribose, 54.9; [U-¹⁴C]soluble starch, 26; [U-¹⁴C]sucrose, 3.5. All sugars were usually used within 6 months of receipt and were always checked for purity by chromatography in solvents A and B.

RESULTS

Metabolism of intact starch grains

Entodinium caudatum suspensions were incubated with the ¹⁴C-labelled starch grains (see Methods) for 48 hr either under standard conditions in the presence of penicillin + neomycin when there was no bacterial growth and the number of protozoa decreased by 26% or under conditions which allowed both protozoal and bacterial growth (Coleman, 1960*b*) when the number of protozoa increased by 45%. Table 1 shows that under both conditions there was no evidence for any synthesis of protein from carbohydrate. Over 80% of the protozoal ¹⁴C was found in the hot TCA soluble fraction of the broken-cell pellet: ¹⁴C from the original starch grains and any other granular polysaccharide would be liberated in this fraction. With non-multiplying protozoa over 50% of the ¹⁴C from the [¹⁴C]starch was liberated into the medium as soluble non-volatile material which was identified as glucose (65%) and maltose (35%). The volatile compounds were acetate (35%) and butyrate (65%); no propionate was detected. In an attempt to demonstrate the presence of [¹⁴C]lactate, the untreated supernatant fluid was chromatographed in solvent A, which separated lactate from glucose and maltose but less than 1% of the total ¹⁴C could have been present as lactate. The remainder of the ¹⁴C in the presence of non-multiplying protozoa and

most of the ^{14}C in the presence of multiplying protozoa was not retained on planchetes in the presence of acid or alkali and was presumed to be CO_2 .

Effect of metabolism of starch on sugar concentrations in the protozoal pool and the medium

On incubation of *Entodinium caudatum* in the absence of added carbohydrate or of antibiotics there was a steady decline in the amount of glucose and maltose in the pool accompanied by a corresponding increase in the amount in the medium such that the total amount of free + bound glucose remained almost constant over the first 90 min.

Table 1. *The metabolism of ^{14}C -starch grains by Entodinium caudatum*

Protozoa were incubated anaerobically for 48 hr in 10 ml. medium containing 10% (v/v) autoclaved rumen fluid (PARF, Coleman, 1964a), 1000 units penicillin G/ml. and $0.14 \mu\text{C}$ ^{14}C -starch grains from holotrich protozoa/ml. in the presence or absence of $400 \mu\text{g}$. neomycin sulphate/ml. With neomycin the number of protozoa decreased by 26% and without it the number increased by 45%. At the end of the experiment the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation.

	Radioactivity (percentage of ^{14}C added initially)				
	No protozoa	Multiplying protozoa (-neomycin)		Non-multiplying protozoa (+neomycin)	
		Initial starch	Supernatant fluid	Pellet	Supernatant fluid
Non-volatile soluble material in medium	1.8	9.9		55.9	
Volatile acid	0	3.9		19.0	
Cold TCA-soluble Lipid	2.8	0.45	0.14	0.33	0.11
Hot TCA-soluble Protein	0.6	0.01	0.06	0.01	0.04
	94.4	0.06	4.4	0.05	7.4
	0.4	0.10	0.30	0.03	0.15

Table 2. *The effect of the metabolism of starch grains on the concentration of maltose and glucose in the medium and pool of Entodinium caudatum*

1.6×10^6 protozoa were incubated anaerobically in 1.2 ml. salt solution B in the presence or absence of 1.5% suspension of rice starch. At the times shown the protozoa were centrifuged down, washed twice in salt solution B, broken by ultrasonic treatment and the supernatant fluid separated from the pellet by centrifugation. The pool was the cold TCA soluble fraction of the protozoal supernatant fluid. The concentration of maltose and glucose in the pool and the incubation medium were determined as described in Methods. All results are expressed in μmoles .

Time (min.)	Protozoal pool				Medium			
	- starch		+ starch		- starch		+ starch	
	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose
0	0.368	0.412	0.378	0.425	0.040	0.100	0.176	0.100
5	0.346	0.388	0.453	0.578	0.044	0.112	0.284	0.140
15	0.382	0.418	0.408	0.480	0.056	0.100	0.306	0.160
30	0.314	0.380	0.404	0.480	0.104	0.112	0.464	0.220
90	0.312	0.328	0.422	0.348	0.080	0.132	0.746	0.408
240	0.248	0.260	0.262	0.248	0.236	0.148	1.50	0.584

In the presence of added rice starch (additional to that already present in the protozoa on harvesting) there was an initial (in first 5 min.) 20 to 25% increase in the amount of glucose and maltose in the pool but thereafter the amount of each declined as in the absence of added starch (Table 2). The amount of glucose in the pool initially was always greater, on a molar basis, than the amount of maltose. In contrast in the presence of added starch there was a large increase in the amount of maltose liberated into the medium and a smaller increase in the amount of glucose. These results indicate that the sugars were probably not liberated into the medium by an 'overflow' from the pool but may have been liberated directly without passage through the pool.

In freshly harvested protozoa the quantity of free glucose and maltose present in 10^6 protozoa was 0.26 and 0.23 μ moles, respectively. Since the volume of each protozoon was $4.7 \times 10^4 \mu^3$ (Coleman, 1967a) the total volume of 10^6 ciliates was $4.7 \times 10^{10} \mu^3$ or 0.047 ml. The minimum concentration of glucose and maltose was therefore 5.5 and 4.9 μ moles/ml., respectively. Since 26% of a packed protozoal pellet was solid material (Coleman, 1967a) and since it is considered likely that these sugars were not present in the 37% of the cell (see below) in and out of which low molecular weight compounds could pass readily, it is probable that these sugars were present in 37% of the cell volume. If this is correct then the concentration of glucose and maltose was 14.8 and 13.2 mM, respectively.

Table 3. *Distribution of ^{14}C in *Entodinium caudatum* after the incorporation of $[\text{U-}^{14}\text{C}]\text{glucose}$*

Protozoa (7.6×10^5) were incubated anaerobically for 5 hr in salt solution B containing 1000 units penicillin + 400 μ g. neomycin sulphate/ml. in the presence of 0.8 mM $[\text{U-}^{14}\text{C}]\text{glucose}$ (670,000 counts/min.) or 110 mM- $[\text{U-}^{14}\text{C}]\text{glucose}$ (670,000 counts/min.). After incubation the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation. These were further fractionated as described in Methods.

	Radioactivity (counts/min.)			
	Broken-cell supernatant fluid		Broken-cell pellet	
	0.8 mM	110 mM	0.8 mM	110 mM
Initial glucose concentration				
Cold TCA-soluble fraction	1380	720	1110	540
Ethanol-soluble protein	15	5	30	0
Lipid	35	20	30	15
Hot TCA-soluble fraction	70	30	2660	1110
Residual protein	50	20	60	15

Metabolism of glucose

Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin + neomycin for 5 hr incorporated ^{14}C from 0.8 mM and 0.11 M $[\text{U-}^{14}\text{C}]\text{glucose}$. Table 3 shows that the distribution of ^{14}C was similar under both conditions and that 26 to 29% was present in the cell pool and 45 to 50% in the hot TCA soluble fraction of the broken cell pellet: after further hydrolysis in N-HCl for 1 hr at 100° the material in the latter fraction was identified as $[\text{U-}^{14}\text{C}]\text{glucose}$. This suggested that in the intact protozoa the ^{14}C was present as a glucose polysaccharide. Less than 3% of the total protozoal ^{14}C was present in the protein fractions. 4% of the glucose-carbon present initially was converted into volatile fatty acids as acetate

(80%) and butyrate (20%). The different proportions of acetate and butyrate produced during the metabolism of glucose and starch could be the result of the differing action of intracellular bacteria on the hydrolysis products of starch digested in the cytoplasm and on glucose taken up from the medium.

Distribution of ^{14}C in the hot TCA soluble fraction of the broken cell pellet. In contrast to the results obtained with [^{14}C]glycine or [^{14}C]bicarbonate (Coleman, 1964*a*) when breakage of the protozoa abolished incorporation of ^{14}C into the pellet, the incorporation of ^{14}C from [^{14}C]glucose into the pellet was increased by up to 60% on protozoal breakage. This suggested that the incorporation under these conditions was into bacteria and that in the intact protozoon [^{14}C]glucose could be incorporated into intracellular bacteria and possibly protozoal polysaccharide granules, both of which would be found in the broken-cell pellet after cell breakage and centrifugation.

Incorporation of ^{14}C from [^{14}C]glucose by bacteria isolated from protozoa. White (1966) found forty viable bacteria inside each ciliate and showed that 90% of these were one or the other of two species of Gram-negative bacilli. These have been shown subsequently to be *Klebsiella aerogenes* (Bacterium 31 used by Coleman 1964*b*, 1967*b*) and *Proteus mirabilis* (R. W. White, personal communication). Cultures of these bacteria were grown anaerobically on a yeast-extract + tryptose + glucose + salts medium (Coleman, 1964*b*) and washed suspensions prepared by washing the bacteria twice on the centrifuge in salt solution B. These suspensions were incubated anaerobically for 20 min. at 39° with 0.5×10^{-3} to 80 mM-[U- ^{14}C]glucose of known specific activity and the incorporation of ^{14}C into washed bacteria measured. Figure 1 is a reciprocal plot of ^{14}C incorporated against substrate concentration and shows that both bacterial species incorporated ^{14}C but that *K. aerogenes* was over four times as active as *P. mirabilis*. Both curves showed a very marked discontinuity at 10 to 20 mM. On incubation of *K. aerogenes* anaerobically in the presence of glucose concentrations in excess of 0.001 M, a fluffy layer of what was presumed to be a glucose polymer was found associated with the bacteria on centrifugation. On fractionation of all the sedimented material over 90% of the glucose-carbon that had been incorporated was released by treatment with hot 5% TCA and after further hydrolysis in N-HCl was found to be glucose. It was essential in these experiments to use short incubation times because after 1 hr, especially at high glucose concentrations, so much 'fluffy' material was formed that it was impossible to remove the supernatant fluid after centrifugation without removing some.

Determination of incorporation of ^{14}C into protozoal polysaccharide and intracellular bacteria. The method used to distinguish between incorporation into protozoal polysaccharide and bacteria was based on the observation that, after centrifugation of the protozoal broken-cell pellet through sucrose, much of the starch but few of the bacteria were present in the pellet as observed microscopically. To obtain a quantitative result it was necessary to assume that on incubation of broken protozoa with [^{14}C]glucose ^{14}C was incorporated only into bacteria. The washed pellet from such an incubation was resuspended in 2 ml. water and samples layered on top of 2 ml. quantities of sucrose of differing molarities and centrifuged at 1900g for 10 min. in a bucket-head centrifuge. The supernatant fluids were removed and the pellets, after washing once in water, plated out for the estimation of ^{14}C . They contained 73, 20, 2.5 and 1.7% of the initial ^{14}C after centrifugation through 1.0, 1.5, 2.0 and 2.5 M-sucrose, respectively. From an examination of this result it was decided to use 2 M-sucrose and by

direct microscopical examination 30% of the original number of protozoal polysaccharide granules were found in the pellet after centrifugation through sucrose. It was therefore possible to obtain an estimate of the amount of ^{14}C incorporated into the protozoal polysaccharide granules by centrifugation of the broken-cell pellet through 2 M-sucrose and multiplying the amount of ^{14}C in this second pellet by 3.3. For the purposes of these calculations the 2.5% of the bacterial ^{14}C that centrifuged through 2 M-sucrose was ignored. The amount of ^{14}C in the intracellular bacteria was the difference between the total ^{14}C in the broken-cell pellet and the ^{14}C in the protozoal polysaccharide measured as described above.

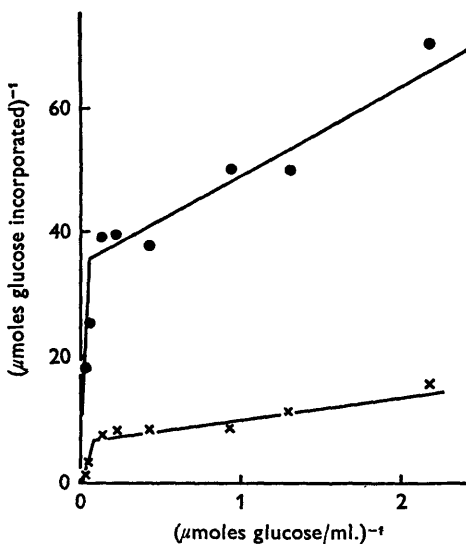


Fig. 1

Fig. 1. Effect of glucose concentration on the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ by washed suspension of *Proteus mirabilis* (●—●) and *Klebsiella aerogenes* (x—x) incubated anaerobically in salt solution B. Duration of experiment 20 min. Results expressed per 10^9 bacteria.

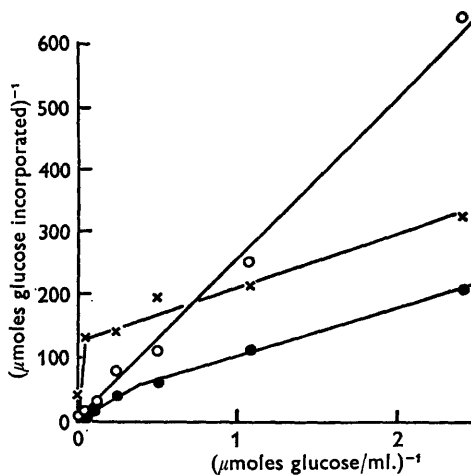


Fig. 2

Fig. 2. Effect of glucose concentration on the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ by *Entodinium caudatum*. After 45 min. the washed protozoa were treated ultrasonically and separated into supernatant fluid and pellet fractions by centrifugation. The cold TCA-soluble material from the broken-cell supernatant fluid formed the pool (●—●). The pellet fraction was further fractionated by centrifugation through 2M-sucrose to give a protozoal polysaccharide (○—○) and a bacterial (x—x) fraction.

Figure 2 is a reciprocal plot of the effect of glucose concentration on the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ and shows that there was a discontinuity in the curves for incorporation into the pool and the bacteria but not for incorporation into protozoal polysaccharide. The break in the curve for uptake into the bacteria occurred at 20 mM, which was at the same concentration as was found with pure cultures of the two predominant bacterial species and may therefore not be associated with any protozoal process. The discontinuity in the curve for incorporation into the pool (at 2.5 mM) and the fact that the left-hand part passed through the origin suggested there may be an 'active' (predominant at low glucose concentrations) and a passive (predominant at

high glucose concentrations) uptake process as was found for amino acids (Coleman, 1967a).

In an attempt to obtain further evidence for the existence of incorporation into both subfractions of the broken-cell pellet, the effect of penicillin (1000 units/ml.) + neomycin (400 μ g./ml.) on incorporation of ^{14}C from [^{14}C]glucose was investigated, since these antibiotics are known to kill the bacteria associated with the protozoa (Coleman, 1962). Unfortunately incubation of the protozoa with these antibiotics for 16 hr before incubation with [^{14}C]glucose decreased the incorporation of ^{14}C into intact protozoa, the broken-cell supernatant fluid and the broken-cell pellet by 84%, and it was not possible to measure incorporation into the bacterial and polysaccharide fractions satisfactorily. However, it was found that, whereas breakage of freshly harvested protozoa before incubation with [^{14}C]glucose stimulated incorporation of ^{14}C into the pellet, breakage of protozoa that had been pre-incubated with antibiotics for 16 hr decreased incorporation of ^{14}C into the pellet by 60%. This result suggests that in freshly harvested protozoa incorporation of ^{14}C was into two fractions, one of which was associated with living bacteria. It was also possible to show a selective effect of penicillin and neomycin when freshly harvested protozoa were incubated in the presence or absence of antibiotics for 4 hr. Under these conditions at low (0.1 mM) glucose concentrations, incorporation into broken-cell supernatant fluid, intracellular bacteria and protozoal polysaccharide was inhibited by 74, 83 and 53% respectively in the presence of antibiotics. These results show that the incorporation into the intracellular bacteria is most sensitive to penicillin and neomycin and are generally consistent with the view that incorporation in the broken-cell pellet is into a bacterial and non-bacterial component.

Effect of other sugars and of sugar analogues. For these experiments the protozoa were incubated anaerobically with 0.4 to 67 mM [U- ^{14}C]glucose in the presence of the inhibitor at 67 mM, and the incorporation into the protozoal pool and polysaccharide and the intracellular bacteria measured. On the basis of results obtained in experiments on the total incorporation into protozoa over 3 to 4 hr the 'sugars' were divided into two groups. Those of group 1 which contained D-galactose and D-2-deoxyglucose inhibited incorporation at concentrations above and below the break (at 2.2 mM) in the reciprocal plot of incorporation of ^{14}C into intact protozoa/substrate concentration. Those of group 2 (i.e. D-sorbitol, D-mannose, D-maltose, D-fructose) inhibited only at concentrations below the break; L-glucose and D-ribose had no effect at any concentration. However, when the experiments were made for only 20 min. then group 2 compounds were not inhibitory and those of group 1 inhibited markedly at concentrations below the break, but only slightly at high concentrations. At low glucose concentrations 2-deoxyglucose inhibited incorporation of ^{14}C from [^{14}C]glucose into all the sub-cellular fractions equally (Table 4) and the same occurred in the presence of galactose although the over-all inhibition was always greater in the presence of 2-deoxyglucose, i.e. 71% compared with 29% (mean of six experiments). However at high glucose concentrations, where the over-all inhibition by both inhibitors was much smaller, the incorporation into the protozoal polysaccharide was always affected more than the incorporation into the other two fractions. The incorporation of ^{14}C from [^{14}C]glucose by washed broken-cell pellet after a 3-hr incubation was decreased to 54% in the presence of galactose and to 37% in the presence of 2-deoxyglucose. However, similar experiments with washed suspensions of the *Klebsiella aerogenes* and *Proteus*

mirabilis isolated from the protozoa showed that incorporation into both bacteria was not affected by 2-deoxyglucose but was decreased to 45% by galactose. As this difference may have been the result of the exposure of the intracellular bacteria, but not the washed suspensions, to ultrasonic treatment, the effect of such treatment of washed suspensions of the bacteria on their ability to incorporate [¹⁴C]glucose was examined. On ultrasonic treatment of suspensions of either bacterium the rate of incorporation of ¹⁴C from [¹⁴C]glucose decreased progressively with time of treatment and 2-deoxyglucose became progressively more inhibitory until after 30 sec. of treatment followed by 30 min. incubation 2-deoxyglucose decreased incorporation by 30%. Under the same conditions galactose inhibited incorporation by 90%. Sorbitol had no effect on the incorporation of ¹⁴C from [¹⁴C]glucose by protozoal washed broken-cell pellet or by either bacterium under any conditions tested.

Table 4. *The effect of galactose and 2-deoxyglucose on the incorporation of ¹⁴C from [¹⁴C]glucose by Entodinium caudatum*

Protozoa (650,000/ml.) were incubated anaerobically in 1.5 ml. medium for 4 hr in the presence of 0.3 mM-[U-¹⁴C]glucose (66,000 counts/min./ml.) or 67 mM-[U-¹⁴C]glucose (66,000 counts/min./ml.) and in the presence or absence of 67 mM-D-galactose (Expt. B) or 67 mM-2-deoxyglucose (Expt. A). At the end of the experiment the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation. The washed pellet fraction was further fractionated by centrifugation through 2M-sucrose, as described in the text, and the incorporation of ¹⁴C into the polysaccharide and bacterial fractions determined. The 'pool' was the cold TCA-soluble fraction of the broken-cell supernatant fluid. Results are expressed relative to incorporation into fraction in absence of inhibitor at given glucose concentrations = 100.

Inhibitor	Glucose concentration	Pool	Bacteria	Poly-saccharide
Expt. A				
2-Deoxyglucose	0.3 mM	7.7	7.8	5.6
2-Deoxyglucose	67 mM	73	54	28
Expt. B				
Galactose	0.3 mM	33	31	23
Galactose	67 mM	81	103	51

Nature of products in protozoal pool. The [¹⁴C]labelled constituents of the protozoal pool after incubation with [¹⁴C]glucose were separated into up to four compounds by chromatography in solvent B. One ¹⁴C-spot remained at the origin and after elution and treatment with acid phosphatase gave rise to [¹⁴C]glucose (for identification of [¹⁴C]glucose see Methods). On chromatography in solvent C the untreated material was resolved into a major component (over 80%) which chromatographed next to marker glucose-6-phosphate, and a minor component which chromatographed opposite glucose-1-phosphate. On elution, this latter component was hydrolysed to [¹⁴C]glucose on treatment with N-H₂SO₄ at 100° for 10 min.; the former was stable under these conditions as determined by chromatography in solvent B. Both components of the origin spot were removed from solution by treatment with Dowex 2 (acetate), showing that they were moderately strong acids. Their identity was confirmed by chromatography in solvents A to G next to marker compounds. The second spot in the original chromatographic separation ran opposite marker maltose. After elution some of this material was hydrolysed in N-HCl at 100° for 1 hr and was converted into glucose only. The remaining material was chromatographed in solvents A to H against marker

compounds and shown to be maltose. The third spot in the original was glucose. The fourth spot which never contained more than 10% of the total ^{14}C in the pool ran slightly faster than glucose in solvent B and was probably fructose, although this was not shown conclusively.

Effect of glucose concentration and inhibitors on incorporation into the pool. After 15-min. incubation in the presence of low (0.4 mM) [^{14}C]glucose 10% of the ^{14}C in the pool was present as hexose phosphate, 36% as maltose and 54% as glucose whereas in the presence of a high (40 mM) glucose concentration the corresponding proportions were 5, 20 and 75%. The proportion of the ^{14}C that was present as hexose phosphate was rather variable and in some experiments was as high as 35%. In the presence of 20 mM-2-deoxyglucose at low glucose concentrations the total incorporation into the pool was decreased by 65% and the distribution of ^{14}C was similar to that at high glucose concentrations. 2-Deoxyglucose had no effect on the pattern of incorporation at high glucose concentrations.

In an attempt to prevent the 'active' uptake of glucose by the protozoa the effect of 0.67 mM-iodoacetate was investigated. At this concentration all ciliary movement of the protozoa was abolished and they began to disintegrate after incubation for 1 hr; all experiments in the presence of iodoacetate were therefore done for 20 min. only. Under these conditions incorporation into the pool was increased up to six times by the presence of iodoacetate and there was a straight-line relationship between incorporation of glucose and glucose concentration on a reciprocal plot, and this straight line passed through the origin. These findings suggested that the 'active' uptake had been abolished by iodoacetate and the passive uptake increased. Incorporation into the hexose phosphate fraction in the pool and into the broken-cell pellet fraction was abolished. The relative incorporation into the glucose and maltose in the pool were unchanged.

Since the increased passive uptake of ^{14}C from [^{14}C]glucose may have been the result of the inhibition of some process for the 'active' removal of glucose from part of the cell, the effect of iodoacetate on the release of ^{14}C from washed protozoa which had been pre-incubated with 67 mM [^{14}C]glucose for 30 min. was investigated. It was not possible to obtain an unambiguous result because, although the addition of 0.67 mM-iodoacetate decreased the rate of loss of ^{14}C from the pool, it stimulated the release of ^{14}C from the pellet fraction of the protozoa into the medium.

Effect of salt concentration and temperature on incorporation into the pool. A decrease of 65% in the salt concentration in the incubation medium killed the protozoa after 1 hr but stimulated the incorporation of ^{14}C from [^{14}C]glucose into the pool by 260% when the incubations were continued for 20 min. only. There was a straight line relationship between incorporation of ^{14}C and glucose concentration on a reciprocal plot, and the line passed through the origin. There was no break in the curve as occurred at standard salt concentration. Almost all the increase in the incorporation of ^{14}C was accounted for by an increase in the amount of [^{14}C]glucose: this increased by 420% compared with only 55% for maltose. This result can probably be explained on the basis of a passive uptake of medium into the protozoa caused by the lowered osmotic pressure. The glucose would be carried into the protozoa with the other medium constituents and be trapped there when the protozoa were washed in salt solution of the standard concentration.

Lowering the temperature from 40° to 30° for a 4-hr incubation had no effect on the

total incorporation of ^{14}C from [^{14}C]glucose into the protozoa but increased the incorporation into the pool by 20% at low (i.e. below the break shown in Fig. 2) glucose concentrations only. Prolonged incubation at 20° was not possible because of death of the protozoa; but in experiments of 30 min. duration the total incorporation at low glucose concentrations was decreased by 35% as compared with incubation at 40° with no change in the amount of ^{14}C in the pool. At high glucose concentrations lowering the temperature to 20° had no effect on the total incorporation of ^{14}C but increased the proportion of the ^{14}C present in the pool from 78% to 88%. These results show that at all glucose concentrations the reactions by which glucose in the pool was incorporated into particulate matter were more sensitive to a decrease in temperature than the reactions by which glucose was incorporated into the pool.

Effect of particulate matter on uptake of glucose. Holter (1965) showed that in *Amoeba proteus* which was taking up protein particles from the medium by pinocytosis only 5% of the liquid in which the protein was suspended was taken up at the same time. Since *Entodinium caudatum* engulfs particulate matter and can take up 200 bacteria/min. (Coleman, 1964*b*) it was of interest to determine the effect of inert particulate matter on the rate of glucose incorporation. The material used was polystyrene latex particles of diam. 1.3 μ (Dow Chemical Co., Midland, Michigan, U.S.A.) which have been shown by light and electron microscopy to be engulfed by the protozoa. In the presence of 0.33% polystyrene latex (5.5×10^8 particles/ml.) there was usually a small and variable increase (0 to 25%) in the incorporation of ^{14}C from 2 to 130 mM-[^{14}C]glucose into the protozoa. However, in one experiment the stimulation increased from 28% in the presence of 130 mM-[^{14}C]glucose to 120% with 4 mM-[^{14}C]glucose. The actual stimulation probably depended on how full of starch grains the protozoa were on harvesting. Latex at 0.03% gave negligible stimulation at any glucose concentration. Since with the higher latex concentration the protozoa were completely filled with particles after incubation for 20 min. and many were still free in the medium, the stimulation obtained must have been the maximum possible associated with the uptake of particulate matter. It is therefore considered that in standard washed suspension experiments particle-stimulated incorporation was unimportant.

Effect of glucose incorporation on the level of glucose in the pool. It has been shown above that glucose in the medium was incorporated into the protozoa and that the freshly harvested protozoon had free glucose in its pool. It was therefore of interest to investigate the effect of glucose incorporation on the concentration of glucose in the pool. Figure 3 is a time course for the effect on the glucose in the pool of the incorporation of ^{14}C from 83 mM-[^{14}C]glucose and shows that after incubation for 6 hr ^{14}C equivalent to 0.18 μ mole glucose was incorporated into a pool that contained 0.20 μ mole glucose; 75% (i.e. 0.135 μ mole) of the ^{14}C incorporated was present as glucose. This shows that over 60% of the glucose in the pool was replaceable by glucose from the medium without increase in the concentration of glucose in the pool. At external glucose concentrations over about 0.08 M the incorporation of [^{14}C]glucose was accompanied by an increase in the total glucose in the cell (Fig. 4). Inhibition of some of the metabolic activities of the protozoa by lowering the temperature to 21° or by adding 0.67 mM-iodoacetate abolished the phase at lower glucose concentrations where the concentration in the pool was not affected by glucose incorporation; for every mole of glucose incorporated there was a corresponding increase in pool glucose over the entire concentration range tested (Fig. 4). The

concentration of glucose in the pool usually decreased on incubation of freshly harvested protozoa (Table 2) but both these treatments produced an increase in pool glucose even in the absence of added glucose (Fig. 4). These results are explicable if the level of glucose in the pool represents a steady state balance between an input of glucose by hydrolysis of maltose derived from intracellular starch or from the medium, and a loss of glucose by release into the medium or conversion into protozoal or bacterial polysaccharide. Inhibition of the biosynthetic reactions by lowering the temperature or the addition of iodoacetate would tend to increase the glucose concentration in the pool because the rate of removal of glucose was decreased without alteration in the

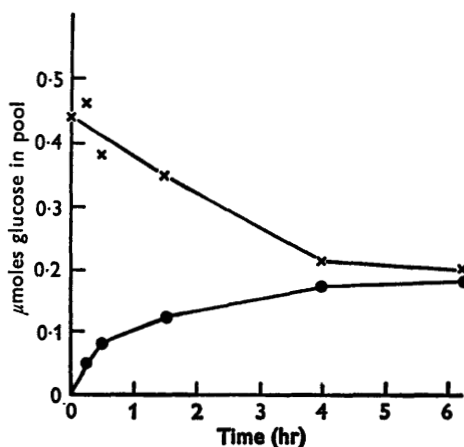


Fig. 3

Fig. 3. Effect of incubation in the presence of 80 mM-¹⁴C glucose on the concentration of glucose in the pool of *Entodinium caudatum*. x—x, Total glucose in pool as determined by glucose oxidase method; ●—●, [¹⁴C]glucose incorporated into pool as calculated from ¹⁴C found in pool and known specific activity of glucose added initially.

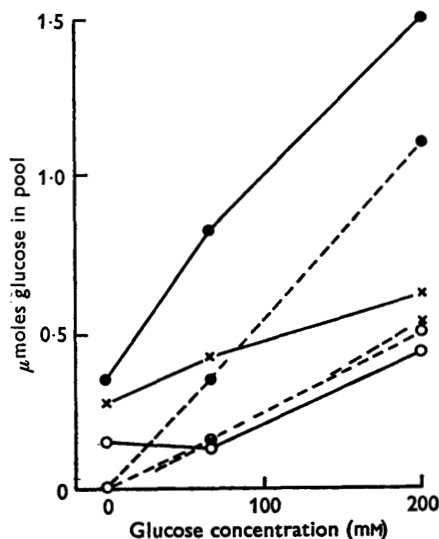


Fig. 4

Fig. 4. Effect of glucose concentration in the medium on the incorporation of [¹⁴C]glucose and the concentration of glucose in the protozoal pool in 1.7×10^6 protozoa contained in 1.5 ml. during a 20 min. incubation. —, Total pool glucose as determined by glucose oxidase method; ---, [¹⁴C]glucose in the pool calculated from the ¹⁴C found in the pool and the known specific activity of the glucose added initially; ●, in presence of 0.67 mM-iodoacetate at 40°; ○, incubated at 40° in absence of iodoacetate; ×, incubated at 21° in absence of iodoacetate. Initial pool glucose was 0.16 μmoles.

rate of input of glucose. With normal protozoa incubated at 40° in the presence of a low concentration of glucose the absence of any increase in pool glucose could be explained by (a) entry of [¹⁴C]glucose by exchange with [¹²C]glucose in the pool or (b) a feed-back mechanism in which the concentration of glucose controls the activity of the maltase which hydrolyses maltose to glucose, but not the rate of glucose uptake from the medium.

In an attempt to show an exchange reaction the protozoa were pre-incubated with 0.02 or 100 mM-¹⁴C glucose for 50 min., then washed three times in salt solution and incubated in the presence or absence of [¹²C]glucose for 5 or 30 min. If an exchange

reaction occurred, the release of ^{14}C from the protozoal pool into the medium would be markedly stimulated by the presence of $[^{12}\text{C}]\text{glucose}$ in the medium. The protozoa labelled at a high glucose concentration released ^{14}C progressively into the medium and there was a decline in the ^{14}C in both the pool and the pellet fractions, but the rate of loss from the pool was not affected by the presence in the medium of 6.7 or 67 mM- $[^{12}\text{C}]\text{glucose}$. With protozoa labelled at low glucose concentration there was a progressive loss of ^{14}C from the pool into the protozoal pellet and the medium but neither of these reactions was affected by the presence of $[^{12}\text{C}]\text{glucose}$. It is therefore considered unlikely that the incorporation of $[^{14}\text{C}]\text{glucose}$ occurred by an exchange reaction. The effect of glucose concentration on the rate of hydrolysis of maltose is considered below.

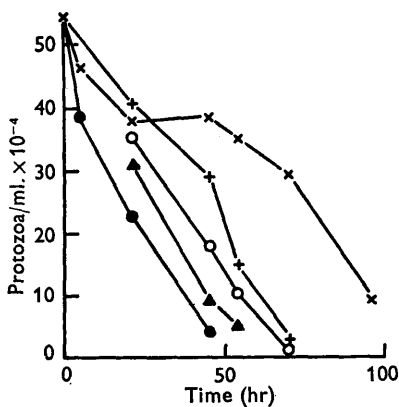


Fig. 5

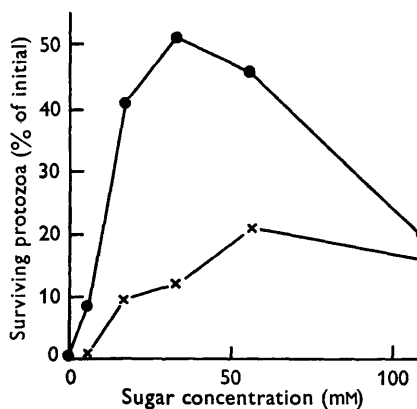


Fig. 6

Fig. 5. Effect of various carbohydrates on the survival of freshly harvested *Entodinium caudatum* incubated anaerobically in the presence of 10% (v/v) autoclaved rumen fluid (PARF, Coleman, 1964a) + 1000 units penicillin G/ml. + 400 μg . neomycin sulphate/ml. ●—●, No added carbohydrate; ▲—▲, 22 mM-galactose; ○—○, 22 mM-glucose; +—+, 11 mM-maltose; ×—×, 0.4% soluble starch.

Fig. 6. Effect of concentration of maltose (●—●) and glucose (×—×) on the survival of freshly harvested *Entodinium caudatum* incubated anaerobically in the presence of 10% (v/v) autoclaved rumen fluid (PARF) + 1000 units penicillin G/ml. + 400 μg . neomycin sulphate/ml. for 50 hr.

Metabolism of maltose

Effect on protozoal survival. Figure 5 shows time courses for the effect of various carbohydrates on the survival of freshly harvested and washed *Entodinium caudatum* incubated anaerobically in the presence of penicillin + neomycin + autoclaved rumen fluid (PARF). Although all the protozoa died in 5 days, soluble starch or maltose were more effective in prolonging the life of the ciliates than were either glucose or galactose. The greater effectiveness of maltose was observed at all concentrations (Fig. 6) whether these were expressed per molecule of maltose or per molecule of glucose. This suggests a difference in mode of uptake or metabolism and not that maltose acts because it contains two molecules of glucose. In experiments with $[^{14}\text{C}]\text{glucose}$ and maltose it was possible to show that their greater effectiveness at high concentrations was not due to the complete utilization of sugars at low concentrations. In no culture in the experiment shown in Fig. 6 was more than 30% of the sugar

utilized. Of the sugar that was metabolized less than 2% appeared in the protozoa and over 80% was converted into material that was volatile on the addition of acid but retained on a planchet in the presence of NaOH, i.e. probably a fatty acid.

Incorporation of maltose. When incubated anaerobically in the presence of equimolar concentrations of glucose and maltose in the absence of antibiotics, *Entodinium caudatum* incorporated into the pool maltose-carbon over four times as rapidly as glucose-carbon. In one experiment 7.4×10^5 protozoa incubated in the presence of 0.67 mM-sugar incorporated in 1 hr 0.87×10^{-2} μ moles glucose and 1.96×10^{-2} μ moles maltose. This higher incorporation of maltose was only apparent at low sugar concentrations (Fig. 7) because on a reciprocal plot of incorporation against sugar concentration both curves showed a break at 2.8 mM and above this the incorporation of both sugars was the same. The maximum 'active' uptake of maltose into the pool as calculated by extrapolation of the right-hand part of the curve to the ordinate was 0.034 μ moles/hr/ 10^6 protozoa, as compared with 0.046 μ moles/hr/ 10^6 protozoa for glucose. However the substrate concentration for half maximum uptake was 3.6 mM for glucose and 0.85 mM for maltose. There was also an increased (40%) incorporation of carbon into the hot TCA-soluble fraction of the broken-cell pellet in the presence of maltose, but this was only into the bacterial fraction, and equimolar amounts of glucose and maltose were incorporated into protozoal polysaccharide at all concentrations. This result can be explained by the observation that although the *Proteus mirabilis* inside the protozoa does not incorporate maltose-carbon *Klebsiella aerogenes* incorporated maltose-carbon at over three times the rate for glucose-carbon when the sugars were present singly at equimolar concentration.

Effect of inhibitors and temperature. As with glucose incorporation, the above results suggested that there may be an active (predominant at low concentration) and a passive (predominant at high concentration) uptake of maltose. An attempt was therefore made to see whether the active process could be inhibited by the use of other sugars or by decreasing the temperature. Like the inhibitors of glucose incorporation, the inhibitors of maltose incorporation could be divided into two groups based on their effect when added at 25 mM in experiments of 3 hr duration. Those of group 1 which contained only 2-deoxyglucose inhibited the incorporation of ^{14}C from [^{14}C]maltose at all concentrations, whereas those of group 2 (galactose, glucose, mannose, cellobiose) inhibited only at concentrations below the break shown in Fig. 7. Similar results were obtained for incorporation into the pool alone and the relative uptakes into the pool and the broken-cell pellet were the same in the presence and absence of inhibitors. In contrast, when the protozoa were incubated at 30° there was an increased incorporation into the pool, especially at substrate concentrations of 0.5 to 1.2 mM and the position of the break in the curve of incorporation/substrate concentration on a reciprocal plot was moved to a lower concentration (Fig. 8). As this result was only obtained clearly after 4 to 5 hr incubation, the experiment was made in the presence of antibiotics in order to prevent the growth of bacteria. Incorporation into the pellet fraction of the protozoa was decreased by 20 to 25% at all substrate concentrations, so that the total incorporation into the protozoa was unchanged or slightly stimulated. The increase in the concentration of ^{14}C in the pool shows that the mechanisms for the incorporation of [^{14}C]maltose into the pool were less sensitive to change in temperature than those for the removal of ^{14}C into the pellet fractions. The change in the position of the break in the curve for incorporation into the pool shown in Fig. 8 means that

the 'passive' uptake reaction was predominant over a longer concentration range at the lower temperature; this suggested that it was less sensitive to change of temperature than the 'active' process. The uniform effect of the temperature change on incorporation of ^{14}C into the pellet fractions at all substrate concentrations may indicate that only the ^{14}C maltose taken up by the active process was incorporated into the pellet fractions. If this were not so, then the increased concentration of ^{14}C in the pool might be expected to nullify the effect of the lower temperature on incorporation of ^{14}C into the pellet fractions from the pool.

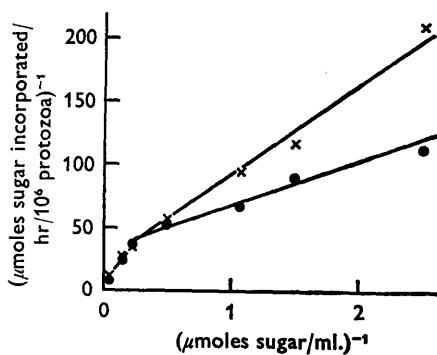


Fig. 7

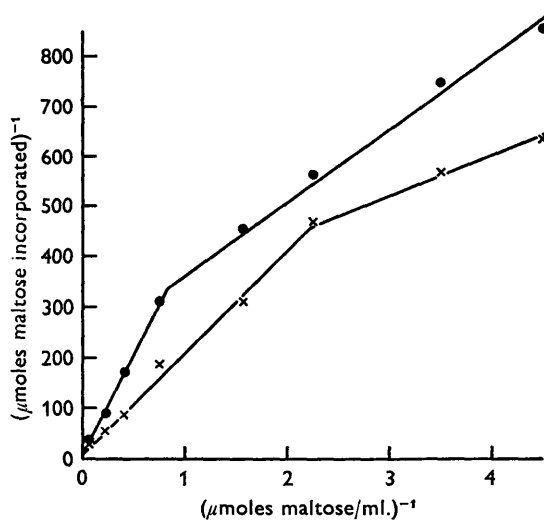


Fig. 8

Fig. 7. Effect of substrate concentration on the incorporation of ^{14}C maltose (●—●) and ^{14}C glucose (×—×) by *Entodinium caudatum* incubated anaerobically in the absence of antibiotics for 20 min.

Fig. 8. Effect of incubation temperature on the incorporation of ^{14}C from ^{14}C maltose into the pool of *Entodinium caudatum* incubated anaerobically in the presence of 1000 units penicillin G/ml. + 400 μg . neomycin sulphate/ml. for 5 hr. ●—●, Incubated at 40° ; ×—×, incubated at 30° .

Effect of antibiotics and of cell breakage. The incorporation of ^{14}C from 0.7 mM- ^{14}C maltose into the pool of protozoa incubated in absence of antibiotics was markedly decreased by pre-incubation for 20 hr of protozoa with 1000 units penicillin/ml. + 400 μg . neomycin sulphate/ml. (Fig. 9). Similar results were obtained with glucose and with both sugars incorporation was progressive for over 4 hr without pre-incubation, whereas with antibiotic-treated protozoa incorporation reached a maximum after 20 min. and then decreased, until after 1 hr the incorporation was less than 15% of that into untreated protozoa. At high sugar concentrations (20 mM) the pre-incubation had no inhibitory effect on the incorporation of ^{14}C from ^{14}C glucose or maltose both of which were incorporated to the same extent on a molar basis. This observation suggested that the antibiotics affected the 'active' uptake only. In contrast, pre-incubation of the protozoa with penicillin and neomycin decreased incorporation of ^{14}C from high concentrations of ^{14}C maltose into the pellet fractions to 30% of that with normal untreated protozoa; at low maltose concentrations incorporation

was decreased to 1 to 2%. This suggested, as before, that only ^{14}C taken up by the 'active' process could be incorporated into the pellet fractions. Breakage of the protozoa before incubation with [^{14}C]maltose decreased the incorporation of ^{14}C into the pellet by between 41% (at 0.3 mM-maltose) and 72% (at 15 mM-maltose).

Nature of products in protozoal pool. Figure 10 shows a time course for the incorporation of ^{14}C from [^{14}C]maltose into various pool constituents. There was a rapid incorporation of maltose into the pool and breakdown of this maltose to glucose during the first minute of incubation. Thereafter the amounts of these [^{14}C]sugars

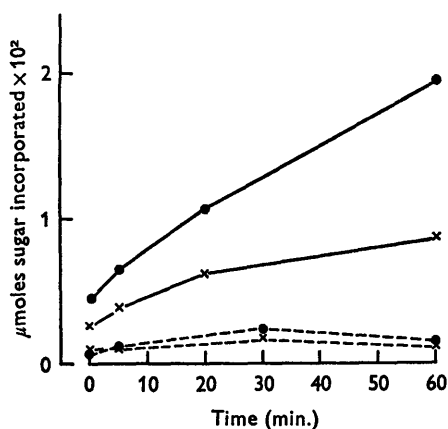


Fig. 9

Fig. 9. Effect of pre-incubation of *Entodinium caudatum* for 20 hr in the presence of 1000 units penicillin G/ml. + 400 μg . neomycin sulphate/ml. on the uptake of ^{14}C from [^{14}C]maltose (●) and [^{14}C]glucose (×) in the absence of antibiotics. —, 4.3×10^8 /ml. freshly harvested protozoa; ---, 4.0×10^8 /ml. protozoa pre-incubated with antibiotics.

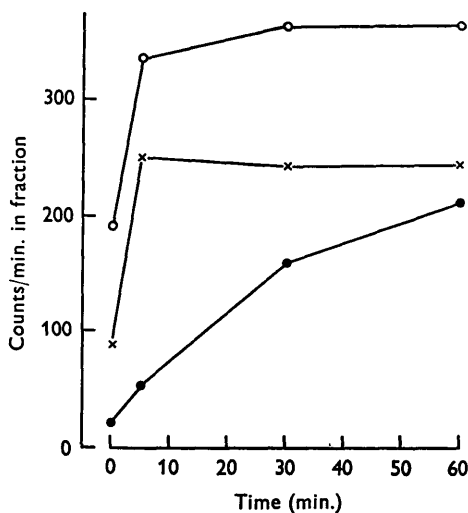


Fig. 10

Fig. 10. Incorporation of ^{14}C from 0.15 mM-[^{14}C]maltose into various fractions in the pool of *Entodinium caudatum*. ○—○, Into glucose; ×—×, into maltose; ●—●, into hexose phosphate.

increased more slowly to reach a maximum after 5 min., after which time the quantities remained constant. In contrast, there was a steady increase throughout the experiment in the amount of glucose phosphate present. During incubation for 5 hr 17 mM-2-deoxyglucose inhibited incorporation into all pool constituents equally, whereas glucose specifically inhibited incorporation into pool glucose and decreased the amount of maltose in the medium that was hydrolysed to glucose from 29 to 3%. The relative incorporation into the various pool constituents was the same at 30° and 40° and at high and low maltose concentrations.

Metabolism of other sugars

Ribose. Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin + neomycin incorporated ^{14}C from [^{14}C]D-ribose progressively for at least 6 hr. After incubation for 5 hr 48% of the ^{14}C incorporated by the protozoa was present in the pool, 5% in the hot TCA-soluble fraction of the broken-cell

supernatant fluid, 25 % in the hot TCA-soluble fraction of the broken-cell pellet and 6% in the combined protein fractions of the protozoa. The ^{14}C in the pool was present as ribose only. After further hydrolysis of the hot TCA-soluble fraction of the pellet in N-HCl for 1 hr at 100° all the ^{14}C was present as glucose. It was not possible to identify [^{14}C]ribose in either of the hot TCA-soluble fractions.

The pellet fraction of protozoa that had incorporated ^{14}C from [^{14}C]ribose was fractionated by centrifugation through 2.0 M-sucrose (as described above) in order to investigate the incorporation of ^{14}C into protozoal polysaccharide and intracellular bacteria. Of the ^{14}C in the pellet, 40 % and 70 % were present in the protozoal polysaccharide on incubation with 0.04 mM and 67 mM [^{14}C]ribose, respectively. Since glucose was the only combined [^{14}C]sugar found in the pellet fraction this suggested that both the protozoa and the bacteria converted ribose into glucose.

As with [^{14}C]glucose and maltose there was a break in the curve for incorporation of ^{14}C from [^{14}C]ribose/substrate concentration on a reciprocal plot; for ribose this was at 3 mM. The maximum rate of uptake of ribose calculated by extrapolation of the right-hand part of the curve was $0.024 \mu\text{moles}/4 \text{ hr}/10^6$ protozoa. During a 4-hr period 25 mM-D-glucose inhibited the incorporation of ^{14}C from 0.3 mM-[^{14}C]ribose by 23 % but had no effect at high ribose concentrations. 2-Deoxyglucose at 25 mM inhibited incorporation by 33 % at 0.3 mM-ribose and by 22 % at 0.05 M-ribose. In contrast to the results on the incorporation of [^{14}C]glucose, breakage of the protozoa before incubation or changing the incubation temperature to 30° decreased the incorporation of ^{14}C from 0.3 mM-[^{14}C]ribose to 28 and 73 %, respectively.

Galactose. ^{14}C from [$1\text{-}^{14}\text{C}$]-D-galactose was incorporated by washed protozoal suspensions and the curve for incorporation of ^{14}C /substrate concentration on a reciprocal plot showed a break at 1.2 mM-galactose. The maximum rate of galactose uptake calculated by extrapolation of the right hand part of the curve to the ordinate was $0.023 \mu\text{mole}/\text{hr}/10^6$ protozoa. After incubation for 4 hr 25 % of the protozoal ^{14}C was present in the pool and 51 % in the hot TCA-soluble fraction of the broken-cell pellet. The only ^{14}C -labelled compound in the pool and in the hot TCA-soluble fraction after hydrolysis in N-HCl for 1 hr at 100° was glucose. Of the ^{14}C in this latter fraction 30 % was associated with the protozoal polysaccharide at low external galactose concentrations and 50 % at high concentrations. As shown in Fig. 5, galactose has little beneficial effect on the survival of protozoa in the presence of antibiotics.

Sucrose. ^{14}C from 0.3 mM-[U- ^{14}C]sucrose was incorporated into the protozoa at two-thirds of the rate for ^{14}C from [^{14}C]maltose at the same concentration. The break in the curve for incorporation of ^{14}C /substrate concentration on a reciprocal plot was 2 mM.

Permeability of protozoa to ^{14}C -labelled sugars

It has been shown (Coleman, 1967a) that 50 to 60% of the packed cell volume of *Entodinium caudatum* is impermeable to amino acids in experiments up to 5 min. duration. It was of interest to determine whether the same is true with sugars and whether or not this is affected by different temperatures of incubation. The experiments were done by a method similar to that described by Coleman (1967a). This involved preparing by the standard method a very thick protozoal suspension such that on centrifugation the packed protozoal pad was about half the total volume. Samples (0.2 ml.) of [^{14}C]sugar (at minimum concentration of 5 mM) were mixed with

1.0 ml. protozoal suspension or 1.0 ml. salts solution in a 10 ml. graduated conical centrifuge tube and incubated for 2 min. The tubes were then centrifuged for 30 sec. from starting, on a bucket-head centrifuge (final speed equivalent to 300g). The volume of the protozoal pellet was measured, then all the supernatant fluid removed and 0.1 ml. of it used for the estimation of ^{14}C . The sides of the tube were wiped with filter paper to remove free medium and then the protozoal pellet suspended in salts solution to a total volume of 5 ml.; 0.25 ml. of this suspension was used for the estimation of the total ^{14}C associated with unwashed protozoa. The suspension was then centrifuged, the protozoa washed once more in salt solution and treated as described under Methods for the estimation of ^{14}C in washed protozoa and in the protozoal pool. In contrast to the results obtained two years ago and reported previously (Coleman, 1967a) it was now possible to incubate *E. caudatum* in a thick suspension at 40° for up to 1 hr without the protozoa showing signs of disintegration. The cause of this change in protozoal behaviour is unknown but it enabled the effect of temperature on protozoal permeability to be measured with greater accuracy. The results were calculated as follows. The total amount of ^{14}C added to the system in the presence or absence of protozoa was calculated from the ^{14}C present in 0.1 ml. of medium in the absence of protozoa. From this value and the amount of ^{14}C present in 0.1 ml. supernatant fluid above the protozoa, the volume of liquid available to the sugar in the presence of protozoa was calculated. The difference between this value and the total volume of liquid gave the volume impermeable to the sugar and this was compared with the volume of protozoa present. The percentage of the protozoal volume impermeable to the sugar was given by

$$100 \times [\text{total volume} - (A/10B)] / \text{protozoal volume},$$

where in counts/min., A = total amount of ^{14}C and B = ^{14}C present in 0.1 ml. supernatant fluid in the presence of protozoa.

Under these conditions $63 \pm 12\%$ (for seven different batches of protozoa; range 46 to 81%) of the packed-cell pad volume was impermeable to glucose at 20°. In an experiment in which 27% of the protozoal volume was permeable to 0.001 M- ^{14}C -glucose at 20°, 4% of this glucose was not removable by washing the protozoa twice in salt solution. If this glucose was isolated in a cellular compartment of the protozoa, then this compartment occupied only 1.7% of the volume of the protozoon. On incubation of the protozoa for 5 min. at 40° the apparent mean impermeable volume decreased to $46 \pm 10\%$; but since the decrease in the ^{14}C in the presence of protozoa was due to a loss of ^{14}C from the complete system, presumably because of the formation of $^{14}\text{CO}_2$, this increased permeability was probably an artifact. However, the proportion of the protozoal ^{14}C that was not removable from the pool by washing increased to 7%. At high glucose concentrations (i.e. 70 mM) the permeable volume remained at the lower value even after 5 min. incubation at 40°, and 5% of the glucose associated with the protozoa was not removed from the pool by washing. Similar results were obtained at low glucose concentrations (1.0 mM) in the presence of 14 mM-2-deoxyglucose. However, in the presence of 0.8 mM-iodoacetate, although the impermeable volume remained unchanged, the proportion of the total ^{14}C in unwashed protozoa that could not be removed from the pool by washing increased to 27% after incubation for 5 min. If this glucose was isolated in a compartment of the protozoa at its original concentration it must have occupied 11% of protozoal volume.

Studies on some protozoal enzymes

A possible explanation for the comparative ineffectiveness of glucose and maltose compared with starch in maintaining protozoal life would be the absence of a glucokinase (ATP:D-glucose-6-phospho-transferase, EC 2.7.1.2) and a maltose phosphorylase (maltose: orthophosphate glucosyltransferase, EC 2.4.1.8) associated with the presence of an α glucan phosphorylase (α 1,4 glucan: orthophosphate phosphorylase, EC 2.4.1.1). Under these conditions glucose could not be phosphorylated to glucose-6-phosphate, the glucose of maltose could not be phosphorylated either directly or after liberation in the free form, but starch could undergo a phosphorolysis to glucose-1-phosphate which could be metabolized by the enzymes of the glycolytic pathway. The presence of these enzymes in the protozoal broken cell supernatant fluid was therefore investigated.

Table 5. *Phosphorylase activity of Entodinium caudatum*

The basal incubation medium contained 1.5 ml. 0.1M- $\beta\beta'$ -dimethylglutarate buffer (pH 6.4), 0.1 ml. 1 mM-phosphate, 0.1 ml. ^{32}P -inorganic phosphate (10 $\mu\text{C}/\text{ml.}$) plus the additions given below. 95% (v/v) N_2 + 5% (v/v) CO_2 was bubbled through the medium for 2 min. (except where cysteine was omitted from the medium) before the addition of 0.2 ml. of broken protozoal preparation. The tubes were then gassed for a further 10 sec. before sealing with a rubber bung and incubation at 39°; total volume was 3 ml. At the end of the experiment inorganic phosphate was precipitated with sodium fluoride and magnesia mixture (see Methods) and a sample of the supernatant fluid plated out for the estimation of ^{32}P .

Additions to the basal medium		Protozoal preparation	Radioactivity (counts/min.)	
2% soluble starch	1% L-cysteine		Initial	30 min.
0.1 ml.	0.2 ml.	BCS	200	1500
—	0.2 ml.	BCS	180	480
0.1 ml.	—	BCS	190	900
0.1 ml.	0.2 ml.	—	200	220
—	0.2 ml.	SP	250	2010
0.1 ml.	0.2 ml.	SP	300	3520
—	0.2 ml.	BCP	270	900
0.1 ml.	0.2 ml.	BCP	130	1940

SP = ultrasonically treated protozoa; BCS = broken-protozoal supernatant fluid; BCP = broken-protozoal pellet.

α -Glucan phosphorylase. Under the conditions described in Methods the values obtained before incubation and in the absence of enzyme represented 0.5% of the total radioactivity and the amount of soluble ^{32}P usually increased linearly up to 10 times during a 2-hr incubation with enzyme (Table 5). When the sodium fluoride that was normally added at the end of the incubation was present throughout, it has been shown on a reciprocal plot of enzyme activity/phosphate concentration to act as a competitive inhibitor (Dixon & Webb, 1964). The effect of cysteine on enzyme activity was very variable and its omission decreased this by 0 to 50%. In the initial experiments the buffer used was 0.03% NaHCO_3 + 5% (v/v) CO_2 + 0.5% NaCl (pH 6.64). The buffering capacity of this mixture was poor: 0.11 M-malate, 0.04 M-citrate, 0.04 M-tris and 0.05 M- $\beta\beta'$ -dimethylglutarate buffers were tested as possible replacements. Phosphorylase activity was detected in the presence of each of these buffers

but with the exception of dimethylglutarate the optimum pH value was at the limit of their buffering ranges. In the presence of $\beta\beta'$ -dimethylglutaric acid + NaOH buffer the optimum was pH 6.4, with 0.13% soluble starch as substrate.

Substrate specificity. The following carbohydrates were tested as substrates in the presence of broken-cell supernatant fluid and 0.04 mM- ^{32}P phosphate; the results are expressed relative to that with 0.13% soluble starch = 100: 0.2% rice starch, 10 to 30; 3.3 mM-maltose, 10 to 36; 3.3 mM-sucrose, 12 to 220; 3.3 mM-glucose, 17; 3.3 mM-lactose, 25; 3.3 mM-cellobiose, 24; 0.06% inulin, 13; no addition, 13 to 23. The activities in the presence of sucrose were very variable. It is possible that the amount of the enzyme responsible varied in different batches of protozoa. The maximum velocities at infinite phosphate concentration and the K_m values (i.e. substrate concentration at which velocity is half the maximum) in the presence of 0.2% carbohydrate are given in Table 6. Although these experiments were all made with broken-cell supernatant fluid as source of enzyme, 60% of the activity present in a complete protozoal homogenate was in the broken-cell pellet (Table 5). The activity found on incubation of the pellet fraction in the absence of added carbohydrate was only stimulated three-fold by the addition of 0.2% soluble starch, presumably because of the presence of rice starch grains and protozoal polysaccharide granules in the pellet fraction. The activity of both the supernatant fluid and pellet fractions in presence of 0.2% soluble starch was decreased by about 20% at all phosphate concentrations on pre-incubation of the protozoa with 1000 units penicillin/ml. + 400 μg . neomycin sulphate/ml. for 18 hr.

Table 6. Kinetic constants of certain enzymes of *Entodinium caudatum*

The enzyme activities of protozoal broken-cell supernatant fluid were measured as described in Methods. To determine the activities present in antibiotic-treated protozoa, the protozoa were harvested from the growth medium and incubated in the presence of 1000 units penicillin/ml. + 400 μg . neomycin sulphate/ml. for 18 hr before preparation of the broken-cell supernatant fluid. Maximum velocities were measured by extrapolation of a reciprocal plot of velocity/substrate concentration, to infinite substrate concentration. The K_m value was the substrate concentration at which the velocity was half the maximum.

Enzyme	Substrate	Protozoa pre-incubated with antibiotics	Maximum velocity ($\mu\text{moles/hr}/10^6$ protozoa)	K_m (mM)
α Glucan phosphorylase	0.2% soluble starch, phosphate	—	4.1–9.8*	5–10
	0.2% rice starch, phosphate	—	0.53*	2.9
	0.2% maltose, phosphate	—	0.15*	1.7
Hexokinase	Glucose, 5 mM ATP	—	1.8†	0.77
	Glucose, 5 mM ATP	+	1.4†	0.54
	Galactose, 5 mM ATP	—	1.2†	7.1
Phosphoglucomutase	Glucose-1-phosphate	—	4.1	0.63
	Glucose-1-phosphate	+	6.2	0.86
Maltase	Maltose	—	22	6.6

* Phosphate esterified at infinite phosphate concentration. † Measured at infinite sugar concentration.

Reaction products. The crude broken-cell supernatant fluid contained an active phosphoglucomutase (see below). After prolonged incubation the products of any reaction in which glucose phosphates were produced were 95% glucose-6-phosphate

and 5% glucose-1-phosphate, which is the equilibrium mixture. To determine which phosphate was formed first during incubation of broken-cell supernatant fluid with soluble starch and [^{32}P]phosphate, samples were taken at intervals, the phosphate precipitated with fluoride+magnesia mixture and then the glucose phosphates separated by chromatography in solvent C. The position of the [^{32}P]phosphates was determined by radioautography and the radioactivity in each measured. After 5 min. incubation 61% of the ^{32}P was associated with the glucose-1-phosphate; this proportion fell progressively until after 3 hr only 10% was present, the remainder being all associated with the glucose-6-phosphate spot. This is taken as evidence that glucose-1-phosphate was the first product of the reaction and that the ^{32}P was not entering the glucose phosphates by exchange with the γ -phosphate of adenosine triphosphate (ATP) followed by phosphorylation of glucose to give glucose-6-phosphate.

Hexokinase. Glucokinase (ATP: D-glucose-6-phosphotransferase) activity was measured by the formation of [^{14}C]glucose phosphate from [^{14}C]glucose in the presence of ATP. In the presence of 1 mM-glucose and the broken-cell supernatant fluid from 2.7×10^6 protozoa, the reaction was linear for 1 hr. Thereafter the rate of hexose phosphate formation decreased markedly and this was associated with the appearance of fructose in the medium. In the absence of ATP the amount of glucose esterified decreased to less than 5%. The optimum value was pH 6.7. The maximum velocities and K_m values for the hexokinase activity of the broken-cell supernatant fluid from normal and antibiotic treated protozoa are given in Table 6.

In view of the possibility that hexokinase might be involved in the incorporation of [^{14}C]glucose by washed protozoa it was necessary to determine whether the pattern of inhibition of the two reactions by other sugars and sugar analogues was the same. For these experiments which were of 30 min. duration [^{14}C]glucose was present at 0.5 mM, ATP at 5 mM and the inhibitors at 25 mM. The results, expressed relative to those in the absence of inhibitor = 100 were: L-glucose, 100; 3-methylglucose, 100; ribose, 100; sorbitol, 96; fructose, 90; mannose, 86; 2-deoxyglucose, 80; maltose, 40; galactose, 38; D-glucose, 6. The decrease in activity in the presence of maltose was probably due to hydrolysis of maltose to glucose as the preparation contained a maltase (see below). These results contrasted with those on the incorporation of glucose by intact protozoa, in that with intact protozoa 2-deoxyglucose was more potent than galactose at low glucose concentration. This suggests that hexokinase did not mediate the incorporation of glucose by intact protozoa. The inhibition of hexokinase activity by galactose was competitive on a reciprocal plot of activity/substrate concentration in the presence and absence of 80 mM-galactose. Galactose was also utilized as a substrate for the hexokinase reaction and the maximum velocity and K_m value are given in Table 6.

Phosphoglucomutase. Phosphoglucomutase (α -D-glucose-1,6-diphosphate: α -glucose-1-phosphate phosphotransferase, EC 2.7.5.1) activity was assayed by estimating the amount of reducing sugar, i.e. glucose-6-phosphate, formed on incubation of broken-cell supernatant fluid with glucose-1-phosphate. The rate of reaction was constant for 30 min. The kinetic constants are given in Table 6.

Maltase. Maltase (α -D-glucoside glycohydrolase, EC 3.2.1.20) activity was assayed by measuring glucose formation from maltose in presence of broken-cell supernatant fluid by means of glucose oxidase. The kinetic constants are given in Table 6. As the concentration of glucose in the protozoal pool could be controlled in part by an

inhibitory effect of glucose on the enzyme hydrolysing maltose to glucose, the effect of glucose and other monosaccharides on this reaction was investigated. This was estimated by incubation of broken-cell supernatant fluid with [^{14}C]maltose in the presence of different concentrations of [^{12}C]glucose or other sugar for 5 min. and then stopping the reaction by immersion in a boiling water bath for 5 min. Samples of the supernatant fluids after removal of the protein by centrifugation were chromatographed in solvent B, the position of the maltose and glucose spots determined by radioautography and the ^{14}C in each estimated by placing a GM tube directly on the paper. From these values the proportion of the maltose hydrolysed to glucose could be calculated. Figure 11 shows that in the presence of 12 mM-maltose the addition of 10 mM-glucose decreased the rate of maltose hydrolysis by 50%. Over the glucose concentration range of 5 to 15 mM (the probable concentration in the freshly harvested protozoan) a change of 1 mM in the glucose concentration altered the rate by 5 to 10%.

Amylase. Since the concentration of glucose in the pool would also depend on the concentration of maltose and the rate of maltose formation from starch, the effect of glucose and maltose on this latter reaction was investigated. The experiment was made in 0.4 ml. volumes containing [U- ^{14}C]soluble starch (0.2 μC ; 7.7 μg .), broken-cell supernatant fluid from approximately 5×10^5 protozoa and 0 to 500 mM [^{12}C]glucose or maltose. After incubation for 5 min. the reaction was stopped by immersion of the tubes in boiling water and the precipitate removed by centrifugation. The glucose and maltose were separated from the starch by chromatography in solvent B and the ^{14}C contents of the compounds measured as described above. The amount of hydrolysis was measured as the ^{14}C present in the two sugars divided by the ^{14}C present in all three compounds. [^{12}C]glucose had little effect on the rate of starch hydrolysis, but small changes in the concentration of [^{12}C]maltose had a marked effect. Over the range 5 to 20 mM a change of 1 mM in the maltose concentration altered the rate by 3 to 5% (Fig. 11).

Probable mechanism of starch metabolism

Although it has been shown above that various enzymes for the metabolism of carbohydrates exist in the cell, it is not clear whether starch was degraded initially by phosphorolysis to glucose-1-phosphate or hydrolysed *via* maltose to glucose and then phosphorylated to glucose-6-phosphate in the presence of a hexokinase. To elucidate this ^{14}C -soluble starch was incubated in salt solution B with broken-cell supernatant fluid in the presence and absence of 6 mM-ATP and the rate of [^{14}C]hexose phosphate formation measured under the two conditions. In the absence of ATP any hexose phosphate must be formed by phosphorolysis of the starch, whereas in the presence of ATP hexose phosphate could also arise by phosphorylation of glucose formed from the starch by hydrolysis.

The experiment was made in 1.7 ml. volumes containing salt solution B, 0.1% [^{14}C] soluble starch (0.06 $\mu\text{C}/\text{ml}$.) and the broken-cell supernatant fluid from 0.86×10^6 protozoa in the presence or absence of 6 mM-ATP. The reaction was stopped by immersing the tubes containing the reaction mixture in boiling water and the protein precipitate then removed by centrifugation. A sample of the supernatant fluid was treated with Dowex 2 (acetate) to remove hexose phosphates which were subsequently eluted by treatment with N-HCl. To obtain a measure of the amount of

starch that had been hydrolysed to maltose and glucose, a reducing sugar determination was made on the reaction mixture after removal of the hexose phosphates with the resin. Under the conditions of the reducing sugar assay the extinction obtained per mole of glucose was only 11% less than that obtained per mole of maltose; the results have been calculated as though all the reducing sugar was glucose. Approximately equal quantities of the two sugars were found. In the experiment shown in Fig. 12 the rate of formation of hexose phosphate in the absence of added ATP was lower than in its presence and had almost ceased after 15 min.; in the presence of ATP the reaction was still proceeding after 60 min. These findings suggested that there

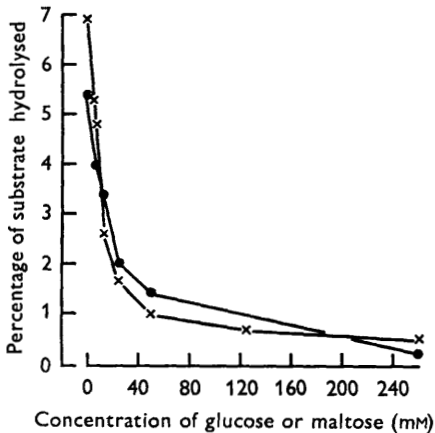


Fig. 11

Fig. 11. Effect of glucose concentration on the hydrolysis of 12 mM-[U-¹⁴C]maltose (x—x) and of maltose concentration on the hydrolysis of 0.2% [U-¹⁴C]soluble starch (●—●) by broken-cell supernatant fluid during a 5 min. incubation.

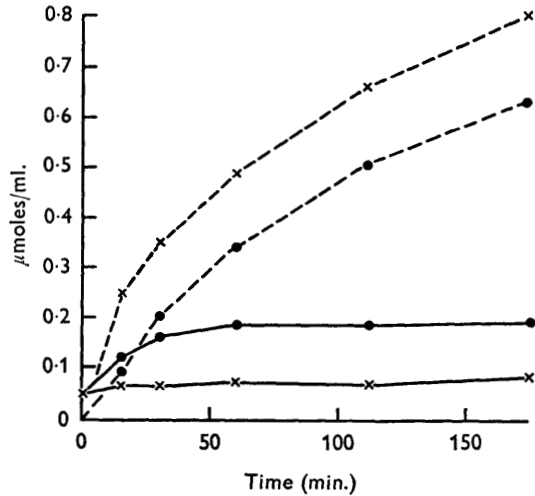


Fig. 12

Fig. 12. Effect of 10 mM-ATP on the formation of hexose phosphate (—) and reducing sugars expressed as glucose (---) from 0.017% [U-¹⁴C]soluble starch by broken-protzoal supernatant fluid. (●), In presence of ATP; (x), in absence of ATP.

might have been a limited amount of ATP present in the broken-cell supernatant fluid and that this was all utilized in the first 15 min. in absence of added ATP. The subsequent slow increase in the amount of hexose phosphate formed is presumed to be due to phosphorylation. It is apparent from these results that, given ATP, the broken-cell supernatant fluid converted the glucose in starch to hexose phosphate after hydrolysis to free glucose, and that the rate of starch hydrolysis was greater than the rate of phosphorylation of glucose.

DISCUSSION

It has been shown that 63% of the volume of a packed protozoal pad was impermeable to sugars on incubation for a few minutes at 20°. The electron microscope studies on thin sections of *Entodinium caudatum* reported previously (Coleman & Hall, 1966) showed that the organism was divided into an outer region (ectoplasm) and an inner region (endoplasm). This latter region contained all the rice starch grains which the

ciliate had engulfed and all the intracellular bacteria. From these findings it is considered likely that the membrane between the endoplasm and the ectoplasm may form the barrier between the regions which are permeable and impermeable to low molecular weight compounds such as sugars. Some information on which of the two regions was freely permeable to sugars was obtained in the investigations of the effect of inhibitors on the incorporation of ^{14}C from [^{14}C]glucose. If the endoplasm were freely permeable and the bacteria therein were exposed to the external medium then the degree of inhibition of incorporation of ^{14}C into the bacteria by any reagent should be the same in intact protozoa as in broken protozoa. If it is the ectoplasm which is freely permeable, then the effect of an inhibitor would be more complicated and would depend in part on the effect of the inhibitor on the passage of glucose through the membrane into the endoplasm, and not necessarily on the effect of the inhibitor on the bacteria. The results obtained showed that 2-deoxyglucose and galactose inhibited the incorporation of ^{14}C from [^{14}C]glucose by bacteria in intact protozoa by 71 and 29 %, respectively, and into washed suspensions of the two most numerous bacteria by 30 and 90 %, respectively, i.e. the pattern of inhibition was different. With intact protozoa in the presence of either inhibitor the effect on the incorporation of ^{14}C into the intracellular bacteria was the same as that on the incorporation into the protozoal pool, suggesting that the glucose utilized by the bacteria came from the pool and not from the medium. Both these results supported the view that it was the ectoplasm that was freely permeable. Unfortunately experiments on the effect of inhibitor on broken protozoa that contained intact bacteria showed that although 2-deoxyglucose and galactose gave about 20% greater inhibition than in intact protozoa, the 2-deoxyglucose was always more effective than galactose. Although this may be interpreted as supporting the view that it was the endoplasm that was permeable, two previously reported findings are not consistent with this. Firstly, the inhibitory effect of a free [^{14}C]amino acid on the incorporation of the ^{14}C -form of that amino acid from bacterial protein into protozoal protein was related to the rate at which the free amino acid was incorporated by the protozoa (Coleman, 1967*a*). Secondly, it proved difficult to kill bacteria inside intact protozoa by incubation in the presence of penicillin + neomycin, although the same bacteria were readily killed under the same conditions after protozoal breakage (Coleman, 1962). Therefore, it is considered more likely that it was the ectoplasm that was freely permeable to glucose and other low molecular weight compounds of the medium.

The incorporation of [^{14}C]glucose into the protozoal pool consisted of two processes as shown by the break in the curve on the reciprocal plot of incorporation of ^{14}C in a certain time/substrate concentration. At high concentrations, above about 2.5 mM, incorporation was proportional to glucose concentration, had an almost infinite maximum velocity and was not affected by inhibitors of group 2 such as sorbitol. It is therefore considered to be a passive uptake. At low concentrations, the reaction had a finite maximum rate, was more rapid than the passive uptake and was inhibited by inhibitors of group 2. It is therefore assumed to be a process that was dependent upon metabolic processes, i.e. 'active'. Both processes are considered to be operative at all glucose concentrations, but the 'active' process is predominant at low concentrations and the passive at high. If the [^{14}C]glucose, in protozoa that had been incubated in the presence of a high concentration of glucose and then washed, was present at its original concentration, it occupied only 5 % of the protozoal volume. This means that

the passive uptake of glucose must represent penetration into a minor cell compartment and not into the complete endoplasm. However, it is considered likely that the 'active' uptake of glucose was into the endoplasm. As there was no discontinuity in the curve for incorporation of ^{14}C into protozoal polysaccharide on a reciprocal plot of incorporation/substrate concentration, it is postulated that only glucose taken up by the 'active' process can be incorporated into protozoal polysaccharide. If glucose taken up by both processes could be utilized the curve might be expected to show a break at about 2.5 mM. At low glucose concentrations more of the ^{14}C that had been incorporated was present as hexose phosphate than at high concentrations and this would be expected if only the 'active' uptake were into a compartment where further metabolism of the glucose could occur. The finding that at low glucose concentrations 2-deoxyglucose decreased the incorporation of ^{14}C into the pool but had no effect on the distribution of ^{14}C between the various pool constituents is interpreted as showing that glucose is taken up actively as such, and then phosphorylated. If the phosphorylation were obligatory for uptake then 2-deoxyglucose might have a specific effect on the concentration of hexose phosphate. This interpretation is supported by the finding that the hexokinase of the broken protozoal supernatant fluid was not very sensitive to inhibition by 2-deoxyglucose.

The action of iodoacetate in markedly increasing the amount of [^{14}C]glucose that could not be removed from the protozoa by washing, without increasing the permeability of the protozoa, was unexpected since it was used in an attempt to inhibit the 'active' uptake of glucose. Since iodoacetate is a metabolic inhibitor then its effect must result from the inhibition of some process such as a metabolic pump for the expulsion of glucose from a cell compartment. If the region of the cell into which glucose penetrated passively were the contractile vacuole then in normal protozoa much of this glucose would be lost by operation of the vacuole during washing of the protozoa. In the presence of iodoacetate the vacuole would be unable to contract and would remain full of glucose. The similar results obtained on incubation of the protozoa at very low salt concentration could be explained by the ectoplasmic/endoplasmic membrane becoming 'leaky', associated with the passage of water into the cell. Glucose would then enter the endoplasm and be trapped there on return of the protozoa to salts of standard concentration.

It has been known for many years (Abou Akkada & Howard, 1960) that the addition of starch but not glucose to washed protozoal suspensions stimulates gas production. This means that any proposed mechanism of glucose incorporation must explain why exogenous glucose and glucose formed by the hydrolysis of starch grains are metabolized differently. For the reasons given above the passive uptake of glucose is assumed to be into some compartment such as the contractile vacuole, where it is not available for further metabolism. The active incorporation of [^{14}C]glucose is probably a net incorporation of glucose into the endoplasm as no evidence was found for an exchange reaction. The maximum rate of glucose uptake at infinite glucose concentration was $0.046 \mu\text{mole/hr}/10^6$ protozoa. As the volume of each protozoon in a packed cell pad was $4.7 \times 10^4 \mu^3$ (Coleman, 1967*a*) the volume of 10^6 protozoa was $4.7 \times 10^{10} \mu^3$, or 0.047 ml. If, as shown above, 37% of this volume was fluid space into which the external medium could not freely penetrate, then the volume of 10^6 protozoa that contained the incorporated glucose was $0.047 \times 0.37 = 0.017$ ml. If all the glucose incorporated in 1 hr remained as free glucose, the concentration at

the end of that hour would be $0.046/0.017 = 2.7$ mM compared with 14.8 mM present initially in the protozoa as the result of the hydrolysis of engulfed starch grains. It is considered that there would be no net increase in the glucose concentration in the endoplasm due to 'active' uptake of glucose on incubation with say 50 mM-glucose in the presence of antibiotics, for the following reasons. Firstly, since the incorporation quoted above was the maximum at infinite glucose concentration, the actual incorporation would be lower. Secondly, as prolonged incubations must be done in the presence of penicillin + neomycin to prevent bacterial growth, the actual rate of glucose incorporation would be decreased under these conditions. Thirdly, any increase in glucose concentration in the pool would tend to decrease the rate of glucose formation from maltose. Even if the glucose concentration were to rise as the result of uptake of free glucose, the rate of glucose phosphorylation would not rise significantly, because at 14.8 μ moles glucose/ml. the activity of the hexokinase was 96% of that at infinite glucose concentration.

The finding that little ^{14}C from [^{14}C]glucose or [^{14}C]starch was incorporated into protozoal protein is of considerable importance in considering the over-all metabolism of the rumen. Bryant & Robinson (1963) showed that many pure strains of rumen bacteria utilized ammonia in preference to amino acids for growth, and when provided with amino acids as sole source of nitrogen incorporated little amino acid-carbon into cellular materials. In contrast, *Entodinium caudatum* incorporated the amino acids of bacterial protein directly into its protein and showed very limited ability to catabolize amino acids (Coleman, 1967*a, b*). This means that if *E. caudatum* is typical of all the Entodiniomorphid protozoa in the rumen, then these protozoa play no role in the synthesis of those 'essential' amino acids commonly present in low concentration in plant proteins.

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