The Metabolism of ^{14}C -Glycine and ^{14}C -Bicarbonate by Washed Suspensions of the Rumen Ciliate *Entodinium caudatum*

By G. S. COLEMAN

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(Received 9 October 1963)

SUMMARY

Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin and neomycin incorporated ^{14}C -glycine in the cell 'pool' as *N*-acetylglycine and into the cell protein as glycine without conversion to any other amino acid. At low external concentrations the glycine was present in the protozoa themselves and was not free in the gastric sac or present solely in the intracellular or extracellular bacteria. The incorporation reached a constant value after incubation for 24 hr; after this time the uptake of glycine was balanced by an equal loss of *N*-acetylglycine from the organisms. These suspensions also incorporated ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into carbon atoms 3 and 4 of protozoal glucose which was probably present as starch in the intact protozoa.

INTRODUCTION

Although *Entodinium caudatum* can be grown *in vitro* in the presence of bacteria on a substrate of rice starch grains, dried grass, autoclaved rumen fluid and chloramphenicol (Coleman, 1960*a*), it has not so far proved possible to grow these protozoa in the absence of bacteria (Coleman, 1962) and very little is known about the biosynthetic capabilities of this or any other Entodiniomorphid protozoon. Abou Akkada & Howard (1962) found that although chloramphenicol-treated *E. caudatum* taken directly from the rumen rapidly hydrolysed casein, there was little simultaneous increase in cell nitrogen. However, DL-alanine, DL-leucine and DL-valine were incorporated by *Ophryoscolex caudatus* (Williams, Davis, Doetsch & Gutierrez, 1961) and *Epidinium ecaudatum* (Gutierrez & Davis, 1962) although with neither organism was the cellular distribution of the incorporated amino acid investigated. The present investigation was undertaken in an attempt to determine if uniformly labelled ^{14}C -glycine, ^{14}C -glucose, ^{14}C -maltose, ^{14}C -starch and ^{14}C -bicarbonate were incorporated by washed *E. caudatum* grown under controlled conditions *in vitro* and whether any such uptake was into the intracellular bacteria or protozoal material.

METHODS

Source of protozoa. The protozoa were grown and 'inoculum cultures' prepared and treated as described by Coleman (1962) except that 'inoculum cultures' were treated each day with 15 mg. rice starch and about 10 mg. dried grass. Where the

metabolism of ^{14}C -starch or ^{14}C -glucose was studied the amount of rice starch was reduced to 5 mg. on the day before the experiment.

Preparation of protozoa for inoculation. The protozoa were taken from the 'inoculum cultures' after removal of the surface scum and most of the supernatant liquid, and allowed to stand in 9 in. $\times$ 1 in. tubes until any grass present had sunk to the bottom. The supernatant fluid containing the protozoa 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 five times in salt solution B, which contained 0.03 % (w/v) L-cysteine, on an angle-head centrifuge for 45 sec. from starting. The maximum speed was equivalent to 300 g. The organisms were finally inoculated at a population density of 100,000–250,000/ml. incubation medium.

Incubation conditions for incorporation experiments. Except where otherwise stated, the basal medium consisted of 1.0–2.0 ml. (chosen so that the final volume was 4.0 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 0.8 ml. water, 1.5 ml. 2 % soluble starch (dissolved in salt solution B) and glycine (when added at the beginning of the experiment) in a 5 in. $\times$ $\frac{1}{2}$ in. cotton-plugged test tube. Immediately after removal from the autoclave and cooling, the following additions were made aseptically: 0.1 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered), 0.1 ml. 5 % NaHCO_3 (Seitz filtered), 0.2 ml. penicillin G (25,000 units/ml.), 0.2 ml. 1 % neomycin sulphate solution, 0.2 ml. autoclaved rumen fluid residue (AR) and any other additions. After inoculation, the tubes were gassed for 10 sec. with 95 % (v/v) N_2 + 5 % (v/v) CO_2 , sealed with a sterile rubber bung and incubated at 39° . Where larger quantities of protozoa were required 5–10 times these quantities were used. For experiments on the metabolism of carbohydrates the soluble starch was omitted and for experiments on the incorporation of ^{14}C from $\text{NaH}^{14}\text{CO}_3$ the NaHCO_3 was omitted and the tubes gassed with 100 % N_2 . ^{14}C -glucose and $\text{NaH}^{14}\text{CO}_3$ were always added immediately before the tube was sealed. At the end of experiments where the uptake of a ^{14}C -compound was investigated the protozoa were centrifuged and washed twice at 300 g for 1 min. in salt solution B containing about 0.005 % of the ^{12}C form of the ^{14}C -compound added initially.

Bacterial viable counts. These were carried out on media A and C as described by Coleman (1962). The number of viable bacteria inside each protozoon was determined after breakage of washed protozoa in a Potter (Potter & Elvehjem, 1936) homogenizer. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Sterile rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay and oats. Protozoa-containing autoclaved rumen fluid (PARF) was prepared from fresh rumen contents by straining through two layers of muslin and autoclaving under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in sealed McCartney bottles at 115° for 20 min. Autoclaved rumen fluid (ARF) was prepared similarly to PARF except that the strained material was centrifuged at 500g for 3 min. before autoclaving. Autoclaved residue (AR) was the pellet from the above centrifugation resuspended in 15 % of the original volume and autoclaved at 115° for 20 min.

Estimation of ^{14}C . ^{14}C in whole protozoa was estimated by washing the organisms with water on to an aluminium disc of area 4.7 cm.² carrying a disc of lens tissue.

The sample was spread by one drop of cetyltrimethyl-ammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The disc was dried *in vacuo* and the ^{14}C estimated by using a thin mica end-window GM tube and conventional scaler equipment. Over 1000 counts at a rate greater than five times background were recorded for all fractions. Most determinations were carried out with less than 0.5 mg. of material per cm^2 of disc. Where more material was present, the results were corrected to infinite thinness by using corrections determined from adding known weights of glycine, mineral salts or rumen fluid fractions to tracer quantities of ^{14}C -glycine. The amount of glycine incorporated by protozoa was calculated from the known specific activity (usually 1.0–4.0 $\mu\text{C./mg.}$) of glycine added initially. To determine the relative ^{14}C 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. $^{14}\text{CO}_2$ was estimated by the method of Coleman (1956).

Fractionation of organisms. The protozoa after harvesting, washing as described above and resuspension in a small volume of salt solution, were first broken by treatment in an all-glass Potter homogenizer at room temperature until 98–100 % of the protozoa were broken (usually about 90 sec.) and then homogenate made up to a known volume and centrifuged at 7000g for 20 min. The supernatant liquid 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 in the homogenate.

These two fractions were further fractionated by a method based on that of Schneider (1945) and 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 with 5 % TCA. The supernatant fluid and the washing formed the 'cold TCA soluble fraction'. The precipitate was then extracted with 5 % TCA at 100° for 30 min. The residue was centrifuged down and washed once with 5 % TCA. The supernatant fluid and washings formed the 'hot TCA soluble or nucleic acid fraction'. The TCA was removed from both fractions by washing three times with ether before plating out for the estimation of ^{14}C . The residue after further washing, once with acidified ethanol and once with ether, formed the protein fraction.

The complete acid hydrolysis of any fraction was done by heating to 105° in 6 N-HCl for 16 hr in a sealed tube. At the end of this period the tube was cooled and opened and the acid removed on a boiling water bath in a current of air.

Paper chromatography. The following solvents were used: A, *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.); B, phenol + ammonia (sp.gr. 0.880) + water (80 g. + 0.3 ml. + 20 ml.; Roberts *et al.* 1955); C, *n*-propanol + ethyl acetate + water (24 + 13 + 7, by vol.); D, *n*-butanol saturated with 1 % (w/v) aqueous ethylamine (Elsden & Lewis, 1953); E, *n*-butanol + ethanol + water (10 + 1 + 2 by vol.); F, *n*-butanol + acetone + water + diethylamine (10 + 10 + 5 + 2, by vol.; Hardy, Holland & Nayler, 1955); G, isopropanol + HCl solution (sp.gr. = 1.18) + water (162 + 42 + 46, by vol.); H, *n*-butanol + cyclohexane + propylene glycol + water + NH_4OH (sp.gr. = 0.880) + morpholine (30 + 30 + 10 + 3.5 + 0.7 + 0.07, by vol.; Guillaume & Osteux, 1955); J, benzene + isopropanol + propylene glycol + water + NH_4OH (sp.gr. = 0.880) (30 + 20 + 10 + 3 + 0.6, by vol.; Guillaume & Osteux, 1955).

Protein hydrolysates were chromatographed in two dimensions in solvents A and B (Roberts *et al.* 1955).

Purification of glycine-X. The method finally adopted was as follows. The supernatant fluids obtained after incubation of protozoa for 3 days in the standard medium containing 0.05M ^{12}C -glycine or tracer [^{14}C]glycine were combined and shaken with Zeo Karb 225 (H^+) and the resin discarded. The supernatant fluid was neutralized with 8% $\text{Ba}(\text{OH})_2$ and the precipitate centrifuged down. Glycine-X was then absorbed on to a column of Dowex 2 (OH^-) which was then washed successively with water, N-acetic acid, water. Glycine-X was then eluted with N-HCl and the eluate evaporated to dryness *in vacuo*. The material was further purified by chromatography in solvent A and its position determined by radioautography. The spot was eluted and this material used for analytical tests.

Estimation of soluble glycine in protozoa. Washed protozoa were broken in a Potter homogenizer and the homogenate centrifuged at 7000g for 20 min. Free glycine was then estimated in the supernatant fluid, after the removal of protein with tungstic acid, by the method of Alexander, Landwehr & Seligman (1945). Combined and free glycine were estimated together after hydrolysis of the protein-free supernatant in 6N-HCl for 16 hr at 105° in a sealed tube.

Chemicals. ^{14}C -compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. All the compounds were uniformly labelled except for [^{14}C] sodium acetate. The specific activity of the compounds as supplied was in μC . per mg.: glucose 450, glycine 41, maltose 16, sodium acetate 260, sodium carbonate 250, starch 23. The specific activity of these compounds was usually lowered to a more convenient level by the addition of carrier ^{12}C -compound before use. N-Acetyl-glycine was supplied by L. Light and Co. Ltd.

RESULTS

Metabolism of glycine

Washed suspensions of *Entodinium caudatum* prepared from growing cultures and incubated anaerobically in the presence of soluble starch, penicillin and neomycin incorporated ^{14}C -glycine. Initially each protozoon contained 10–100 viable bacteria in its gastric sac and there were 10–20 viable bacteria/protozoon free in the medium as determined by growth on media A and C (Coleman, 1962). The number of viable bacteria in these cultures measured immediately after inoculation was only 10% of that found previously (Coleman, 1962). This finding is probably associated with the addition of less rice starch each day to the protozoal cultures and the use of lower centrifuge speeds when washing the protozoa. When the basal medium was supplemented by 5% autoclaved residue (AR) 80–100% of the protozoa survived for over 3 days compared with 40% in its absence, during which time the bacterial viable count on the complete medium decreased to 10% of the initial. Figure 1 shows that ^{14}C -glycine added to the culture at any time during the first 24 hr was incorporated into the protozoal fraction although the initial rate and the final level of incorporation dropped the later the glycine was added.

Effect of salt concentration and rumen fluid fractions. The concentration of salts in the medium had a marked effect on the incorporation of glycine measured after incubation for 7 hr although there was no effect on the number of apparently

healthy protozoa over this time and only a small effect over 48 hr. The uptake of ^{14}C -glycine was stimulated by the addition of 10 % of all the rumen fluid fractions tested of which 10 % AR was the most effective (Fig. 2). However, the magnitude of this stimulation with AR varied from 380 % in salt solution B to 55 % in salt solution B diluted by 20 %. AR was therefore added to all media for incorporation experiments. Control experiments carried out in the absence of protozoa showed that there was no adsorption of ^{14}C on to the particles of AR. The supernatant obtained after centrifugation of AR had only 10–20 % of the incorporation-promoting activity of the original material.

Fig. 1

Fig. 2

Fig. 1. Incorporation of glycine into *Entodinium caudatum* incubated in presence of penicillin and neomycin. ●—●, 28 μg ^{14}C -glycine/ml. added initially; ×—×, 28 μg ^{14}C -glycine/ml. added after 9½ hr; ○—○, 28 μg ^{14}C -glycine/ml. added after 23 hr. Results calculated from uptake of ^{14}C from ^{14}C -glycine of specific activity 180 counts/min./ μg .

Fig. 2. Effect of salt concentration on incorporation of ^{14}C -glycine during the first 7 hr of incubation. The concentration of cysteine, NaHCO_3 , penicillin and neomycin were maintained constant throughout and only the concentration of the constituents of salt solution B varied. ○—○, no addition; ×—×, +10 % autoclaved residue (AR).

Effect of glycine concentration. To determine whether the glycine incorporated by the protozoa was free in the gastric sac, the protozoa were incubated with various concentrations of $[\text{u-}^{14}\text{C}]\text{glycine}$ and the uptake measured. At the lowest concentrations of glycine (up to 20 $\mu\text{g}/\text{ml}$. in the presence of 2×10^5 protozoa/ml.) over 7 % of the total ^{14}C was taken up in 4 hr by organisms, the packed cell pad volume of which was less than 1 % of the total. This shows that glycine was concentrated inside the protozoa. At low external glycine concentrations most of the non-protein glycine was in a combined form, but as the external concentration was increased there was a marked increase in the amount of free glycine present (Fig. 3). It is possible that some of this glycine was not intracellular but was present in the

gastric sac, as the method used does not separate glycine in the cell 'pool' from that in the gastric sac.

Effect of antibiotics and breakage of the protozoa. Although the above results show that glycine was incorporated into the micro-organisms in the suspensions, this uptake might have been into the extracellular or intracellular bacteria rather than into the protozoal protoplasm. In an attempt to eliminate this possibility the uptake of ^{14}C -glycine by intact protozoa was compared with that obtained with protozoa which had been broken under conditions that did not break the bacteria, i.e. in a Potter homogenizer. As a further test for the possible uptake of ^{14}C -glycine into bacteria, all the experiments were done in duplicate; one tube was centrifuged under

Table 1. *Incorporation of ^{14}C -glycine by broken and intact Entodinium caudatum in the presence and absence of antibiotics*

Protozoa were incubated at a concentration of 1.4×10^5 protozoa/ml. in salt medium containing 0.75 % soluble starch, 0.025 % L-cysteine, 0.12 % NaHCO_3 , 25 μg . ^{14}C -glycine (130 counts/min./ μg ./ml. in the presence or absence of 0.05 % neomycin sulphate and 1250 units penicillin G/ml. for $5\frac{1}{2}$ hr under 95 % N_2 + 5 % CO_2 . To determine ^{14}C in intact protozoa the culture was centrifuged for 45 sec. from starting the centrifuge, at the end of which time the speed was equivalent to 300 g. To determine ^{14}C in protozoa + bacteria free in the medium the culture was centrifuged at 7000 g for 20 min. and the pellet washed twice in salt medium in each case. All results are expressed per ml. culture.

Protozoa	Antibiotics	Viable bacteria		^{14}C in protozoal fraction (counts/min.)	^{14}C in protozoa + bacteria (counts/min.)
		Medium A	Medium C		
Initial					
Intact	—	10^5	5×10^5	.	.
Broken	—	10^5	30×10^5	.	.
Final					
Intact	+	10^5	$< 10^5$	196	194
Broken	+	10^5	5×10^5	10	12
Intact	—	10^8	150×10^6	222	226
Broken	—	10^7	300×10^6	144	624

* The viable counts were carried out on media A and C respectively (Coleman, 1962).

conditions such that only protozoa were sedimented (45 sec. at up to 300 g) and the other such that protozoa + free bacteria were sedimented (20 min. at 7000 g). Table 1 shows that in the presence of antibiotics breakage of the protozoa decreased the incorporation into the protozoal fraction to 5 % and that the same result was obtained under both sets of sedimentation conditions, indicating that uptake by intracellular and extracellular bacteria was negligible. In the absence of antibiotics there was an increase (range 10–100 % depending on the experiment) in the incorporation by intact protozoa. However, the experiments made in the absence of antibiotics are difficult to interpret due to the rapid bacterial growth under these conditions.

Intracellular products of glycine metabolism. As a further test as to whether the uptake of ^{14}C -glycine was into the bacteria, protozoal suspensions which had incorporated glycine were broken in a Potter homogenizer. The homogenate was then centrifuged to produce a supernatant fraction free of bacteria and a pellet fraction which contained all the bacteria. Table 2 shows that after 1 hr incubation in the

presence of ^{14}C -glycine over 80 % of the ^{14}C incorporated into intact protozoa was present in the broken-cell supernatant fluid and that 88 % of that was in the cold 5 % TCA soluble or small molecular weight fraction. The low incorporation of ^{14}C into the pellet shows that less than 20 % of the total incorporation could have been into the bacteria. Incubation in the absence of antibiotics resulted in a tenfold increase in the ^{14}C in the pellet fraction and this was probably due to increased incorporation by the intracellular bacteria which were present in this fraction; the incorporation into the supernatant fraction was unchanged.

Table 2. *Distribution of ^{14}C in *Entodinium caudatum* during the incorporation of ^{14}C -glycine*

Protozoa were incubated at a concentration of 3.5×10^5 protozoa/ml. in 8 ml. salt medium containing 0.75 % soluble starch, 0.025 % L-cysteine, 0.12 % NaHCO_3 , 0.05 % neomycin sulphate, 1250 units penicillin G/ml. and 28 μg . ^{14}C -glycine (440 counts/min./ μg ./ml. under 95 % N_2 + 5 % CO_2). One 8 ml. tube was harvested for each time point. All results are expressed in counts/min.

Time (hr)	Broken-cell supernatant fluid			Broken-cell pellet		
	Cold TCA soluble	Hot TCA soluble	Protein (counts/min.)	Cold TCA soluble	Hot TCA soluble	Protein
1	1710	64	140	95	123	180
5½	2170	168	580	230	232	380
23	1120	160	1440	110	76	700

In antibiotic-treated cultures all the protein ^{14}C was present as glycine. This was determined by chromatography in solvents A, B, C, D and E and by the fingerprint method of Roberts *et al.* (1955). Solvent F separated glycine and serine better than the other solvents and no trace of ^{14}C was found at the R_F of the serine marker. The cold TCA-soluble material from the supernatant fraction prepared from protozoa incubated in the presence or absence of antibiotics contained only one ^{14}C -component at low external glycine concentrations and this had different chromatographic properties from glycine (see below).

Extracellular products of glycine metabolism. Figure 1 shows that although glycine uptake apparently proceeded for only 24 hr when glycine was added initially, glycine first added after 24 hr was still incorporated. This suggested that the amount of ^{14}C found in the protozoa at any time may have represented a balance between the uptake of glycine and the liberation of glycine or a glycine metabolite by the organisms. To test this theory the following experiments were carried out.

Protozoa were incubated under otherwise identical standard conditions in tubes containing: (a) 3 μmole ^{12}C -glycine + 0.2 μC . ^{14}C -glycine; (b) 3 μmole ^{12}C -glycine; (c) no glycine. When the rate of ^{14}C incorporation in tube (a) began to decrease, i.e. after 10 hr, 0.2 μC . ^{14}C -glycine was added to tube (b) and 3 μmole ^{12}C -glycine and 0.2 μC . ^{14}C -glycine to tube (c). The rate and total uptake of ^{14}C was the same in tubes (b) and (c). Similar results were obtained when the additions were made after 24 hr. This shows that even when there was an equilibrium amount of ^{12}C -glycine in the protozoa, uptake of glycine, presumably balanced by an equivalent loss of 'glycine', still occurred.

In an attempt to demonstrate this loss of glycine an excess of ^{12}C -glycine was

added to protozoa that contained an amount of ^{14}C -glycine in equilibrium with ^{14}C -glycine in the medium. Under these conditions decrease in the specific activity of the medium glycine to about 10 % of the initial value decreased the ^{14}C in the protozoa by 50 % after 7 hr. In the absence of any added ^{12}C -glycine there was no loss of ^{14}C . This shows that when the rate of uptake of ^{14}C was decreased by lowering the specific activity of the medium glycine, then the protozoa lost ^{14}C presumably due to the liberation of high specific activity 'glycine'. If the plateau in the glycine incorporation were due to the inability of the protozoa to take up further glycine, then this loss would not have occurred. The addition of equivalent amounts of L-alanine, L-aspartic acid or sarcosine produced no fall in the protozoal ^{14}C .

Fig. 3

Fig. 4

Fig. 3. Effect of glycine concentration on the amount of free (○—○) and combined (×—×) glycine present in *Entodinium caudatum* after removal of protein. The results are expressed per 9.26×10^5 protozoa which were incubated in 5 ml. medium for 6 hr. The initial glycine amounts were: free 10.2 μg.; combined 15.5 μg.

Fig. 4. Metabolism of glycine by *Entodinium caudatum*. ○—○, glycine incorporated into protozoa; ●—●, free glycine in the supernatant fluid; ×—×, glycine-metabolite in supernatant fluid. Results calculated from uptake of ^{14}C from ^{14}C -glycine (440 counts/min./μg.) by 1.84×10^6 protozoa suspended in 10 ml. medium.

It was not possible to demonstrate a glycine-induced loss of ^{14}C from washed equilibrium-state protozoa because after washing once in salts 0.02 % L-cysteine medium and resuspension in the standard medium, the protozoa lost 50 % of their ^{14}C in 7 hr in the absence of external glycine. The rate of ^{14}C release under these conditions was the same at 38°, 33°, 27° and 20°. That this was not the result of damage to the protozoa was shown by the finding that washed equilibrium-state protozoa inoculated into medium containing glycine of the same specific gravity as that present in the first incubation medium lost no ^{14}C .

Chromatographic separation of the materials present in the incubation medium from equilibrium-state protozoa revealed two ^{14}C spots only. One was glycine and the other ran with the same R_F as (a) ^{14}C -material found in the cold TCA-soluble fraction from the broken-cell supernatant fluid, and (b) the material liberated from equilibrium-state protozoa by ^{12}C -glycine or washing. The amount of this material increased throughout the incubation and could account for 30 % of the ^{14}C -glycine added initially (Fig. 4). This unknown material was also produced when the sodium

propionate in the medium was replaced by an equimolar amount of sodium acetate or sodium chloride.

The composition of the glycine-metabolite. Preliminary experiments carried out with incubation media taken after 3 days showed that this material was not absorbed by Zeo Karb 225 (H^+) which removed free glycine, but was absorbed by Dowex 2 (OH^-) from which it was eluted by N-HCl but not by N-acetic acid ; this shows that the metabolite was a comparatively strong acid. By using a method based on these properties considerable purification was achieved. The ^{14}C -material in the final eluate was chromatographed in solvent A where it ran as a discrete spot with R_F value 0.9. This spot, which was detected by radioautography or by spraying with bromocresol green, was eluted, found to be a single spot when chromatographed in solvents A, B, C, D, E, J, and analysed.

After complete acid hydrolysis, the material was chromatographed in two dimensions in solvents A and B. The only ^{14}C - and ninhydrin-positive spot was glycine. When 0.5 % collidine was incorporated in the ninhydrin spray the spot had the characteristic glycine colour. The absence of serine was shown by chromatography in solvent F. However, the same quantity of the unhydrolysed material, after chromatography in solvent A, was ninhydrin negative, indicating the probable absence of a free amino group, contained no phosphate as shown by spraying with molybdate + perchloric acid (Bandurski & Axelrod, 1951) and no carbohydrate as shown by spraying with aniline + oxalate (Horrocks & Manning, 1949).

These properties suggested that the glycine metabolite might be a compound such as an *N*-acylglycine, possibly *N*-acetylglycine. The unknown material was chromatographed in solvents A, B, C, D, F, H and J and shown to run with the same R_F as marker *N*-acetyl glycine, which was detected by spraying with bromocresol green, in all solvents. The unknown had different chromatographic properties from *N*-formylglycine in solvents A and J. To obtain satisfactory results it was essential to adjust the unknown material and the *N*-acetylglycine to the same pH value before developing the chromatograms. The glycine metabolite prepared from tracer quantities of ^{14}C -glycine only also corresponded with *N*-acetylglycine by the finger-print technique of Roberts *et al.* (1955) after chromatography in solvents A and B.

The presence of an acetyl group in the glycine metabolite was shown in the following way. The protozoa were incubated for 3 days under standard conditions in the presence of 0.02 M- ^{12}C -glycine and 1.44 μg (0.4 μC .) 1- ^{14}C -sodium acetate/ml. The metabolite in the medium was purified and finally chromatographed in solvent J (opposite *N*-acetylglycine). The ^{14}C spot was eluted, shown to have the same chromatographic properties as *N*-acetylglycine in solvents A, B, H and J, hydrolysed in N-NaOH for 2 hr and finally chromatographed, without removal of the alkali, in solvent H which separates the lower fatty acids. The ^{14}C material which was acid volatile chromatographed with the same R_F as marker sodium acetate.

Incorporation of ^{14}C from ^{14}C -bicarbonate

^{14}C (added to the medium as $\text{NaH}^{14}\text{CO}_3$) was incorporated linearly by *Entodinium caudatum* for 10 hr (Fig. 5) before the uptake reached a plateau. The addition of further $\text{NaH}^{14}\text{CO}_3$ at 10 or 24 hr in order approximately to double the original specific activity (of the CO_2) produced a further incorporation of ^{14}C . Diminution in the specific activity of the CO_2 to approximately 20 % of that of the original $^{14}\text{CO}_2$

by the addition of $\text{NaH}^{12}\text{CO}_3$ at 10 hr produced a small decrease in the ^{14}C in the protozoa, but the effect was smaller than in the case of glycine. As there was a uniform increase in the amount of non-volatile ^{14}C -material in the medium over 24 hr these results suggested that ^{14}C was incorporated by the protozoa and then liberated again into the medium.

The ^{14}C uptake was dependent on the presence of intact protozoa and breakage of the protozoa reduced it by over 90 %. The results obtained in the presence and absence of antibiotics with protozoa harvested at the end of the incubation by high- or low-speed centrifugation were similar to those obtained with glycine.

Fig. 5. Incorporation of ^{14}C into *Entodinium caudatum* incubated ●—● in presence of $\text{NaH}^{14}\text{CO}_3$ (100 $\mu\text{g.}$ and 4000 counts/min./ml.). ●---●, Additional $\text{NaH}^{14}\text{CO}_3$ (0.2 $\mu\text{g.}$ and 4000 counts/min./ml.) added; ●·····●, 500 $\mu\text{g./ml.}$ $\text{NaH}^{12}\text{CO}_3$ added.

Table 3 shows that over 60 % of the ^{14}C incorporated from $\text{NaH}^{14}\text{CO}_3$ was found in the hot TCA-soluble fraction of the broken-cell pellet. After removal of the TCA with ether the material was hydrolysed in N-HCl for 1 hr at 100° and chromatographed in solvent G. The ^{14}C material, which ran as a single spot, as detected by radioautography, did not correspond with any nucleic acid components as detected by ultraviolet photography. The spot was eluted and identified in the following way. When ^{12}C -glucose was added to the ^{14}C material and an osazone prepared the ^{14}C was found in the precipitated osazone, indicating that the ^{14}C material was glucose or fructose. This material also gave the characteristic hexose reaction when treated with aniline oxalate reagent (Horrocks & Manning, 1949). Chromatography against markers in solvents A, B, C, E, G and H indicated that the unknown material was glucose which was probably present in the intact protozoon as starch. As confirmation of its identity the unknown material was chromatographed in solvent E 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 ^{14}C -glucose under the same conditions.

To determine which of the glucose carbon atoms were labelled with ^{14}C the glucose was fermented under nitrogen by baker's yeast (Barnet & Wick, 1950) in a Conway unit and the $^{14}\text{CO}_2$ collected on an aluminium disc containing $\text{Ba}(\text{OH})_2$ (Coleman, 1956). At the end of the reaction the baryta was dried *in vacuo* and the ^{14}C estimated. Under these conditions 33 % of the ^{14}C in uniformly labelled glucose and 95 % of the ^{14}C in glucose isolated from protozoa incubated with $^{14}\text{CO}_2$ for $\frac{1}{2}$, 4 or

Table 3. *Distribution of ^{14}C in Entodinium caudatum after incorporation of ^{14}C -bicarbonate*

Protozoa were incubated at a concentration of 2.0×10^5 protozoa/ml. in salt medium containing 0.6 % soluble starch, 0.025 % L-cysteine, 0.05 % neomycin sulphate, 1250 units penicillin G/ml. and 500 μg . $\text{NaH}^{14}\text{CO}_3$ (270,000 counts/min.) under N_2 for 6 hr.

	Cold TCA soluble (counts/min.)	Hot TCA soluble (counts/min.)	Protein (counts/min.)
Broken-cell supernatant fluid	890	105	270
Broken-cell pellet	430	4005	510
Non-volatile ^{14}C in incubation medium	5300		

11 hr was liberated as CO_2 . As CO_2 is produced only from glucose carbon atoms 3 and 4 by this method, the result shows that *Entodinium caudatum* was similar to higher animals (Abraham & Hassid, 1957) in that ^{14}C from $^{14}\text{CO}_2$ was incorporated only into these carbon atoms of glucose. The protein from the supernatant fluid and pellet contained ^{14}C -aspartic acid, ^{14}C -glutamic acid, ^{14}C -alanine and ^{14}C -leucine, isoleucine or phenylalanine which were not separated by the chromatographic solvents used. Insignificant amounts of ^{14}C were found in lipid extracted by the method of Roberts *et al.* (1955).

Incorporation of ^{14}C from uniformly labelled ^{14}C -starch, ^{14}C -maltose and ^{14}C -glucose. Soluble ^{14}C -starch was rapidly taken up by *Entodinium caudatum* and the maximum incorporation was reached inside 15 min. Thereafter the ^{14}C was slowly lost from the protozoa until only 10 % remained after 24 hr. At this time over 85 % of this residual ^{14}C was in the hot TCA-soluble fraction and none was present in the protein amino acids. Any starch present in the protozoa would be extracted by hot TCA and appear in that fraction. The total ^{14}C in the culture was constant over 24 hr, at which time the principal ^{14}C materials in the medium were found to be maltose and glucose. It seems probable therefore that the soluble starch was absorbed by the protozoa and then slowly hydrolysed to maltose and glucose without appreciable conversion into cellular material. Incorporation of ^{14}C from [$u\text{-}^{14}\text{C}$]glucose and [$u\text{-}^{14}\text{C}$]maltose was small; less than 0.2 % of the carbon was incorporated at sugar concentrations of 1 μg .–10 μg ./ml. Glucose disappearance at these concentrations was also measured by the method of Huggett & Nixon (1957) but none was detected.

DISCUSSION

Although Williams *et al.* (1961) and Gutierrez & Davis (1962) showed that *Ophryoscolex caudatus* and *Epidinium ecaudatum* washed free from most of their extracellular bacteria but probably still containing bacteria in their gastric sacs, incorporated three amino acids, these authors gave no evidence that the uptake was directly into protozoal material. Although materials in the gastric sac can obviously

be digested by a protozoon, they must be regarded as extracellular. The present studies show that at low external glycine concentrations at least, much more glycine (free and combined) was found inside the protozoa than could be accounted for by free diffusion of external medium into the gastric sac. Since after incubation for 1 hr over 70 % of the glycine had been metabolized to *N*-acetylglycine and no free glycine could be detected in the broken-cell supernatant fluid, this suggests that, if the glycine entered the organism via the gastric sac, it was rapidly metabolized from it. Although this evidence and the finding that breakage of the protozoa almost completely abolished incorporation indicate that the reactions probably represent protozoal metabolism, it is impossible to be certain that the incorporation was not mediated by the bacteria in the gastric sac. It has been assumed in these studies that the material in the broken-cell supernatant fluid was of protozoal origin, but it is uncertain to what extent protozoal protein and nucleic acid were carried down with bacteria, starch and other cell debris in the pellet fraction.

The liberation of *N*-acetylglycine into the medium is not considered to be the result of lysis of the protozoa because: (a) as much *N*-acetylglycine was released every 7–10 hr as was found in the protozoa at any one time (Fig. 4); (b) the number of protozoa remained constant over the period of the experiment, although this may have been the result of the death of some protozoa balanced by the division of an equal number of other protozoa; (c) after washing equilibrium-state protozoa there was only a decrease in protozoal ^{14}C when the protozoa were inoculated into fresh medium containing no glycine or glycine of a lower specific activity to that used in the first incubation. The observation that *N*-acetylglycine was liberated from protozoa after washing and that the rate of release was independent of temperature suggests that the process was passive diffusion rather than active secretion. In view of the known action of penicillin on bacterial cell-wall synthesis (Park, 1958) it is of interest that *N*-acetylglycine was also produced in the absence of penicillin. Although glycine is often converted into serine in mammals (Bach, 1952), some bacteria (e.g. Roberts *et al.* 1955) and in *Tetrahymena* (Dewey & Kidder, 1955), there is no evidence for such a reaction in *Entodinium caudatum*.

Although the protozoa were capable of incorporating glycine into their cellular protein no evidence was obtained for the synthesis of protein amino acids from carbohydrate. The protozoa were therefore either unable to synthesize amino acids under any conditions or only when multiplying. As shown previously (Coleman, 1962) high concentrations of some carbohydrates prolonged the life of starved, almost bacteria-free protozoa. The results quoted here with protozoa containing bacteria show that if this effect was concerned with the metabolism of the carbohydrates then the proportion of the carbohydrate that was metabolized by or incorporated into the protozoa was very small. In contrast there was rapid incorporation of ^{14}C from ^{14}C -bicarbonate into carbon atoms 3 and 4 of protozoal glucose, probably in the form of starch in the living organism, suggesting that the enzymes of the Embden-Meyerhof pathway are present in these protozoa.

It is not claimed that the conclusions reached here with non-multiplying protozoa in the presence of antibiotics can be used as good evidence for the source of the amino acids required for the growth of these protozoa. However, the results do show that certain biosynthetic reactions can probably be carried out by these entodinia without assistance from their associated bacteria.

I wish to thank Miss J. G. Pearson, Miss J. King and Miss E. V. Holgate for their valuable technical assistance.

REFERENCES

- ABOU AKKADA, A. R. & HOWARD, B. H. (1962). The biochemistry of rumen protozoa. 5. The nitrogen metabolism of *Entodinium*. *Biochem. J.* **82**, 313.
- ABRAHAM, S. & HASSID, W. Z. (1957). The synthesis and degradation of isotopically labelled carbohydrates and carbohydrate intermediates. *Methods in Enzymology*, Ed. by S. P. Colowick & N. O. Kaplan, vol. 4. New York: Academic Press Inc.
- ALEXANDER, B., LANDWEHR, G. & SELIGMAN, A. M. (1945). A specific micromethod for the colorimetric determination of glycine in blood and urine. *J. biol. Chem.* **160**, 51.
- BACH, J. S. (1952). *The Metabolism of Protein Constituents in the Mammalian Body*. Oxford: Clarendon Press.
- BANDURSKI, R. S. & AXELROD, B. (1951). The chromatographic identification of some biologically important phosphate esters. *J. biol. Chem.* **193**, 405.
- BARNET, H. N. & WICK, A. N. (1950). The formation of glycogen from ¹⁴C-labelled glycine. *J. biol. Chem.* **185**, 657.
- COLEMAN, G. S. (1956). The dissimilation of amino acids by *Rhodospirillum rubrum*. *J. gen. Microbiol.* **15**, 248.
- COLEMAN, G. S. (1958). Maintenance of oligotrich protozoa from the sheep rumen *in vitro*. *Nature, Lond.* **182**, 1104.
- COLEMAN, G. S. (1960*a*). The cultivation of sheep rumen oligotrich protozoa *in vitro*. *J. gen. Microbiol.* **22**, 555.
- COLEMAN, G. S. (1960*b*). Effect of penicillin on the maintenance of rumen oligotrich protozoa. *Nature, Lond.* **187**, 518.
- COLEMAN, G. S. (1962). The preparation and survival of bacteria-free suspensions of *Entodinium caudatum*. *J. gen. Microbiol.* **28**, 271.
- DEWEY, V. C. & KIDDER, G. W. (1955). Threonine aldolase in Tetrahymena. *Résumés communs. 3^e Congr. int. Biochem. Brussels*, p. 92.
- ELSDEN, S. R. & LEWIS, D. (1953). The production of fatty acids by a Gram-negative coccus. *Biochem. J.* **55**, 183.
- GUILLAUME, J. & OSTEUX, R. (1955). Détermination des acides aliphatiques volatils de C₁ à C₆ dans les liquides biologiques par chromatographie sur papier. *C.R. Acad. Sci., Paris*, **241**, 501.
- GUTIERREZ, J. & DAVIS, R. E. (1962). Culture and metabolism of the rumen ciliate *Epidinium caudatum* (Crawley). *Appl. Microbiol.* **10**, 305.
- HARDY, T. L., HOLLAND, D. O. & NAYLER, J. H. C. (1955). One-phase solvent mixtures for separation of amino acids. *Analyt. Chem.* **27**, 971.
- HORROCKS, R. H. & MANNING, G. B. (1949). Partition chromatography on paper. Identification of reducing substances in urine. *Lancet*, i, 1042.
- HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. *Lancet*, ii, 368.
- PARK, J. T. (1958). Selective inhibition of bacterial cell wall synthesis: its possible application in chemotherapy. In *Strategy of Chemotherapy. Symp. Soc. gen. Microbiol.* **8**, 49.
- POTTER, V. R. & ELVEHJEM, C. A. (1936). A modified method for the study of tissue oxidations. *J. biol. Chem.* **114**, 495.
- ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1955). *Studies on Biosynthesis in Escherichia coli*. *Publ. Carneg. Inst.* no. 607.
- SCHNEIDER, W. C. (1945). Phosphorous compounds in animal tissue. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.
- WILLIAMS, P. P., DAVIS, R. E., DOETSCH, R. N. & GUTIERREZ, J. (1961). Physiological studies of the rumen protozoon *Ophryoscolex caudatus* (Eberlein). *Appl. Microbiol.* **9**, 405.