**Incorporation of
¹⁴C-labelled Components of *Escherichia coli* and of Amino Acids
by *Isotricha intestinalis* and *Isotricha prostoma*
from the Sheep Rumen**

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SUMMARY

Studies on the activities of suspensions of *Isotricha intestinalis* and *I. prostoma* freshly isolated from sheep rumen and washed almost free from bacteria were made by using [¹⁴C]-amino acids and ¹⁴C-labelled *Escherichia coli*. Bacteria were taken up by the protozoa at an approximately linear rate of up to 3000 bacteria/protozoon/hr for 24 hr, after which time at least 40 % of the bacterial carbon was no longer in the form of whole bacteria. Amino acids supplied to the protozoa, either free in the medium or as intact bacteria, were incorporated unchanged into protozoal protein. Addition of individual free ¹²C-amino acids to the medium partially inhibited incorporation into protozoal protein of ¹⁴C from *Escherichia coli* labelled with the ¹⁴C form of the same amino acid.

INTRODUCTION

Although some species of entodiniomorphid protozoa from the rumen have been cultured in the laboratory for long periods (e.g. Coleman, 1958, 1960, for *Entodinium caudatum*) the holotrichs have been cultured (Clarke & Hungate, 1966) for only a few months, because of the very laborious techniques necessary. The presence of bacteria appeared to be necessary for the maintenance of the rumen holotrich protozoa *Isotricha intestinalis* and *I. prostoma* and *Dasytricha ruminantium* and it has been suggested that one function of the bacteria may be the maintenance of a low redox potential in the medium (Gutierrez, 1958; Hungate, 1955; Clarke & Hungate, 1966). However, the role of the bacteria has not been investigated in detail. Studies on the carbohydrate metabolism of these holotrich protozoa have been made by using suspensions, freshly isolated from crude rumen fluid, which were more or less free from bacteria (Gutierrez, 1955, 1958; Heald, Oxford & Sugden, 1952). The protozoa maintained their fermentative ability and motility for some days under suitable conditions. Although *E. caudatum* has been shown to engulf bacteria and utilize bacterial amino acids (Coleman, 1964), there is little in the literature about bacterial feeding by holotrich protozoa or the amino acid metabolism of these protozoa. Gutierrez (1958) showed that *I. prostoma* engulfed bacteria but he did not investigate the fate of their cellular constituents. Harmeyer (1965) showed that mixed *I. intestinalis*

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and *I. prostoma* incorporated ^{14}C from $^{14}\text{CO}_2$ into protozoal aspartic acid, alanine, threonine, histidine and glutamic acid, but he did not investigate further the source of protozoal amino acids. The present paper reports studies on the uptake of a single bacterial species, namely *Escherichia coli*, and its metabolism by freshly isolated suspensions of *I. intestinalis* and *I. prostoma*.

METHODS

Isolation of Isotricha intestinalis and I. prostoma. Suspensions of these protozoa were obtained by a method based on those described by Heald *et al.* (1952) and Gutierrez (1955). Rumen fluid was obtained, 1 hr after feeding, from fistulated Clun Forest sheep on a diet of hay and oats, and was strained through muslin to remove large particles. Glucose was then added (to 0.5 %) and the fluid placed in a conical separating funnel at 39° for 60 min. Rapid synthesis of starch led to an increased specific gravity of the protozoa. The white layer which collected at the bottom of the funnel was run off into an 8-inch boiling tube containing 40 ml. of a 'salts solution' containing: (%) NaCl, 0.5; KH_2PO_4 , 0.1; CaCl_2 , 0.01; MgSO_4 , 0.01; NaHCO_3 , 0.5. This solution was steamed in an autoclave for 15 min. to remove dissolved air, then cooled and gassed for 2 min. with CO_2 before use. The tube was closed with a rubber bung and, after most of the protozoa had settled, the supernatant fluid was sucked off and replaced with a further 40 ml. salts solution. This procedure was repeated 4 times giving a final pellet of protozoa, over 95 % of which were *Isotricha intestinalis* and *I. prostoma* and less than 5 % *Polyplastron* spp. The protozoa were resuspended in salts solution (10 ml./500 ml. original rumen fluid) and used to inoculate experimental tubes.

Numbers of protozoa were estimated by fixing samples with 2.5 % (w/v) formaldehyde and diluting, when necessary, with salts solution. The number of protozoa present in 0.1 ml. samples of the suspensions was determined by scanning microscopically the whole area under a cover glass. At least 3 counts were made on each sample, a minimum of 600 protozoa being counted.

Incubation of protozoa. Incubation tubes (8 cm. $\times$ 0.8 cm., closed with polythene stoppers) contained about 2×10^4 protozoa in 2 ml. salts solution + 0.01 % (w/v) cysteine HCl. Additional amino acids, bacteria, penicillin or neomycin were added as required and tubes were gassed for 30 sec. with CO_2 . Incubation was done with the tubes in a horizontal position at 39° unless otherwise indicated. Although actively motile, the protozoa tended to remain near the bottom of the tube. In the absence of antibiotics, less than 10^5 rumen bacteria/ml. were present at the beginning of the incubation.

After incubation, tubes were cooled to 0° and the protozoa were sedimented by centrifugation for 1 min. at low speed in an angle-head centrifuge (accelerating from 0 to 250 g during the 1 min. period). The protozoal pellet was washed 3 times by repeated resuspension in 2 ml. volumes of cold salts solution and centrifugation. The *Escherichia coli* when added could be quantitatively recovered from the supernatant fluid by centrifugation at 7000 g for 30 min. This pellet after resuspension and washing twice on the centrifuge in salts solution was the 'bacterial fraction'.

Preparation of Escherichia coli suspensions. *Escherichia coli* organisms, uniformly labelled with ^{14}C , were prepared by growth in C medium (Roberts *et al.* 1955) con-

taining 2 mg. and $1.6 \mu\text{C}$ [$\text{U-}^{14}\text{C}$]glucose/ml. Incubation was for 16 hr at 39° with a current of air bubbling through the suspension. To obtain *E. coli* labelled with a single ^{14}C amino acid, organisms were grown in C medium (Roberts *et al.* 1955) containing 0.2 % glucose, the appropriate ^{14}C amino acid ($0.8 \mu\text{C} + 10 \mu\text{g./ml.}$) and 0.5 mg./ml. of the ^{12}C form (0.08 mg./ml. for [^{12}C]threonine) of those amino acids (if any) shown by Roberts *et al.* (1955) or by experience to be derived from the ^{14}C amino acid provided. The bacteria were harvested by centrifugation, washed twice in salts solution and resuspended in this solution before addition to incubation tubes. Approximately 10^9 *E. coli* organisms/ml. were present in each tube, unless otherwise indicated.

Bacterial colony counts. Numbers of viable rumen bacteria present in suspensions were determined by the method of Coleman (1962) with medium C. Colony counts of *Escherichia coli* were done on solid (2 % agar) C medium (Roberts *et al.* 1955) + 2 mg. glucose/ml., by the technique of Miles & Misra (1938).

Fractionation of protozoa and *Escherichia coli*. Protozoa suspended in distilled water were broken for 30 sec. in a Potter homogenizer (Potter & Elvehjem, 1936) and were separated into a broken-protozoa pellet (BPP) and a broken-protozoa supernatant fluid (BPS) fraction by centrifugation for 30 min. at $7000 g$ (4°). Chemical fractionation of protozoa and *Escherichia coli* was done as described by Roberts *et al.* (1955). The protein fractions were hydrolysed with 6 N-HCl for 16 hr at 105° in a sealed tube. The tube was then cooled, opened and the acid removed on a boiling-water bath in a current of air. The amino acids were separated by two-dimensional chromatography (sec-butanol + formic acid + water; $70 + 10 + 20$, by vol., 1st dimension; phenol + 0.880 sp.gr. ammonia + water, $80 + 0.3 + 20$, w/v/v, second dimension). Radioactive amino acid spots were located by radioautography, cut out and the amino acids eluted with distilled water for determination of the total radioactivity present. α -Amino-nitrogen in the eluates was determined by the method of Cocking & Yemm (1954).

Radioactivity determinations. Samples were pipetted onto circles of lens tissue on aluminium planchets (2.5 cm. diam.). One drop 0.5 % cetyltrimethylammonium bromide and 1 drop 0.2 % polyvinyl alcohol were added before drying. Samples were counted with a thin mica end-window Geiger-Muller tube or a Nuclear Chicago gas flow counter and over 1000 counts recorded. Counts were corrected to infinite thinness.

Materials. [$\text{U-}^{14}\text{C}$]Glucose, [$\text{U-}^{14}\text{C}$] glycine, [$\text{U-}^{14}\text{C}$]L-leucine, [$\text{U-}^{14}\text{C}$]L-isoleucine, [$\text{U-}^{14}\text{C}$]L-serine, [$\text{U-}^{14}\text{C}$]L-alanine, [$\text{U-}^{14}\text{C}$]L-aspartic acid, [$\text{U-}^{14}\text{C}$]L-glutamic acid, [$\text{Me-}^{14}\text{C}$]L-methionine and [$\text{U-}^{14}\text{C}$]L-lysine were obtained from the Radiochemical Centre, Amersham, England. Benzylpenicillin and neomycin sulphate were obtained from Glaxo Laboratories, Greenford, Middlesex, England.

RESULTS

*Incorporation of radioactivity from uniformly labelled *Escherichia coli**

The method used to study the uptake of bacteria by *Isotricha intestinalis* and *I. prostoma* was to measure the incorporation of ^{14}C from ^{14}C labelled *Escherichia coli* of known specific activity. The bacteria were separated from the protozoa by differential centrifugation as described in Methods. Under these conditions less than 0.5 %

of the ^{14}C in the ^{14}C labelled *E. coli* was incorporated into the 'protozoal fraction' initially, after incubation at 0° for 24 hr or in the absence of protozoa.

At 39° , ^{14}C from uniformly labelled *Escherichia coli* was incorporated into the protozoal fraction at an approximately linear rate for at least 24 hr. This method of measuring bacterial uptake may underestimate the number of bacteria taken up, because as will be shown below, about 25 % of the carbon in these bacteria may be liberated into the medium in a soluble form. Alternatively, the method could overestimate bacterial uptake if some digestion of the bacteria occurred outside the protozoa, followed by

Fig. 1. Effect of *Escherichia coli* population density on uptake by 8.5×10^3 protozoa incubated in 2 ml. salts solution + 0.01 % L-cysteine. The number of bacteria taken up was calculated from the ^{14}C in the protozoal fraction at 5 hr (---) and 24 hr (—) after incubation of protozoa with ^{14}C labelled *Escherichia coli* of known specific activity.

uptake of the liberated low molecular weight compounds. However, since in experiments on the incorporation of free amino acids less than 3 % of total amino acid present was taken up in 24 hr, the error introduced thereby would probably be small. Previous heating of the *E. coli* to 75° for 10 min. (rendering them non-viable) did not decrease the rate of uptake. Incorporation increased with *E. coli* concentration at least up to 1.8×10^{10} *E. coli* organisms/ml., when the rate was equivalent to 3000 *E. coli*/protozoon/hr (Fig. 1), as compared with a rate of 12,000 bacteria/protozoon/hr for *Entodinium caudatum* (Coleman, 1964). In nine simultaneous determinations of incorporation over a 24 hr period, the coefficient of variation of the mean uptake was 4.6 %, indicating that the sampling of the large dense protozoa was satisfactory.

Addition of 5 % (v/v) autoclaved rumen fluid (ARF, Coleman, 1962), previously clarified by centrifugation at 7000 g for 30 min. did not affect incorporation of radioactivity from *Escherichia coli*. 10 or 20 % (v/v) ARF in 24 hr inhibited by 25 or 40 %, respectively. When tubes were gassed with 95 % (v/v) N_2 + 5 % (v/v) CO_2 instead of pure CO_2 (the concentration of NaHCO_3 being adjusted to maintain the same pH value), the protozoa were considerably less active after incubation for 24 hr and incorporation of radioactivity from *E. coli* was decreased by about 50 %.

To determine whether the bacteria taken up by the protozoa were still viable, the following experiment was done. For 24 hr, 2×10^4 protozoa were allowed to take up ^{14}C labelled *Escherichia coli* from a suspension that contained 1×10^9 bacteria/ml. At intervals during the experiment, determinations were made of the ^{14}C in the protozoa and of the number of viable bacteria (colony count) in the medium, in washed intact protozoa, and in washed protozoa broken in a Potter homogenizer. At 4 and 24 hr, respectively, each protozoon contained ^{14}C from 1700 and 11,000 bacteria (as determined from ^{14}C in the protozoa), but there were only 110 and 0 viable bacteria inside each protozoon (as determined from the difference in colony count between intact and broken protozoa). Viability of *E. coli* incubated under the same conditions in the absence of protozoa fell by 50 % in 24 hr. These results show that *E. coli* were rapidly rendered non-viable after uptake by the protozoa.

After incubation for 24 hr of protozoa with uniformly ^{14}C labelled *Escherichia coli*, 19 % of the ^{14}C was found in the protozoal fraction, 20 % in the 7000 g supernatant fluid and 61 % in the bacterial fraction. 13 % of the ^{14}C was found in the supernatant fluid in the absence of protozoa, presumably the result of lysis of bacteria. The appearance of ^{14}C in the medium in the presence of protozoa might result from digestion of the bacteria and release of soluble products.

Table 1. *Distribution of radioactivity among fractions of sheep rumen protozoa (Isotricha species) and Escherichia coli*

Protozoa were incubated in the presence of 10^9 uniformly labelled *Escherichia coli* organisms/ml. for 24 hr before harvesting, washing, homogenising and separation into supernatant fluid (BPS) and protozoal pellet (BPP). The distribution of radioactivity among the chemical fractions was determined as shown in the text.

Fraction	Protozoa		<i>Escherichia coli</i>	
	BPP	BPS	Before incubation	After incubation
	Radioactivity (counts/min.)			
	5,330	3,640	45,100	39,600
	Fractionation (% total counts/min./fraction)			
Pool (cold TCA*)	1.5	10.4	6.4	2.7
Lipid	16.4	3.5	7.2	6.3
Ethanol-soluble protein	22.3	15.8	4.5	5.0
Nucleic acid	4.4	9.4	17.4	16.7
Residual pellet	43.9	50.2	44.5	50.3

* TCA = trichloroacetic acid.

Uniformly ^{14}C labelled *Escherichia coli* (before and after incubation) and the broken-protozoa pellet and broken-protozoa supernatant fluid fractions which had been incubated for 24 hr with uniformly ^{14}C labelled *E. coli* were fractionated (Table 1). 40 % of the total radioactivity incorporated into the protozoa was recovered in the broken-protozoa supernatant fluid. Since any whole bacteria present would have been recovered in the broken-protozoa pellet, this result shows that at least this proportion of the bacteria which were taken up had been disrupted. A higher proportion of the radioactivity was found in the ethanol-soluble protein and a lower proportion in the

nucleic acid fractions in both the broken-protozoa supernatant fluid and protozoal pellet than in *E. coli*. The amount of radioactivity in the amino acids of the residual protein fractions from the broken protozoa supernatant fluid and *E. coli* were compared. The total radioactivity present in each amino acid spot after two-dimensional chromatography of the hydrolysed protein in broken-protozoa supernatant fluid relative to that in glutamic acid (= 100) was as follows (results for *E. coli* are given in parentheses): alanine 6 (53); arginine 44 (39); aspartic acid 65 (93); glutamic acid 100 (100); glycine 17 (29); leucine + isoleucine + phenylalanine 196 (199); lysine 104 (62); methionine + valine 44 (68); proline 29 (41); serine 24 (31); threonine 30 (32); tyrosine 38 (29). It is noticeable that the alanine in the protozoa contained very little radioactivity although amino acid determinations confirmed the results of Harmeyer (1963) that the protozoal protein hydrolysate was not low in alanine. Some differences in the distribution of radioactivity in the other amino acids can also be seen in Table 1. If radioactivity in the protozoa was present only in whole *E. coli* the distribution of radioactivity among the amino acids would be expected to be the same in both protozoa and bacteria. This is therefore further evidence that the bacteria were broken down by the protozoa.

Incorporation of amino acids by protozoa

Incorporation of ^{14}C amino acids provided either free in the medium or in the form of *Escherichia coli* labelled with specific amino acids was investigated. Free ^{14}C amino acids were tested at 100 $\mu\text{g./ml.}$ in the presence or absence of a mixture of the other 17 ^{12}C amino acids. The full 18 amino acid mixture contained glycine, L-aspartic acid, L-glutamic acid, L-arginine, L-methionine, L-lysine, L-threonine, L-proline, L-tryptophan, L-tyrosine, L-cysteine, L-serine, L-phenylalanine, L-histidine, L-valine, L-alanine, L-leucine, L-isoleucine, each at final concentration 100 $\mu\text{g./ml.}$ To determine whether the amino acids were taken up by the protozoa directly or after incorporation into any bacteria present, the incubations were carried out in the presence or absence of 100 $\mu\text{g.}$ benzylpenicillin + 40 $\mu\text{g.}$ neomycin sulphate/ml.; 0 and $1-2 \times 10^6$ viable bacteria/ml., respectively, were found after 24 hr. These antibiotics did not affect the motility of the protozoa or their rate of uptake of radioactivity from ^{14}C -labelled *Escherichia coli*. Incorporation of each ^{14}C amino acid, tested alone was ($\mu\text{g./24 hr./} 2 \times 10^4$ protozoa): glycine 4.6 (5.2); leucine 4.0 (4.4); isoleucine 4.4; serine 3.7; methionine 1.7 (1.8); lysine 0.95 (1.6); aspartic acid 0.11; glutamic acid 0.08 (2.1); alanine 0.07 (0.08). The figures given in parentheses are values found in the absence of benzylpenicillin + neomycin and show that killing of bacteria by these antibiotics did not decrease the rate of uptake of most amino acids by the protozoa. Incorporation was not significantly affected by the presence of other amino acids and took place at a constant rate during the 24 hr incubation period. After chemical fractionation of protozoa which had incorporated glycine, leucine, serine, methionine or lysine, over 75% of the recovered radioactivity was found in the protein fractions in each case, with some in the cold trichloroacetic acid-soluble pool. Radioautography of chromatograms of the hydrolysed protein fractions indicated that no major interconversion of amino acids took place, although the possibility of leucine-isoleucine-phenylalanine, serine-glycine and lysine-arginine interconversions could not be ruled out because of incomplete resolution of these amino acids. The ^{14}C amino acids provided were the only radioactive compounds detected in the medium after removal of the protozoa.

From the results with uniformly-labelled *Escherichia coli*, it was apparent that bacterial carbon was incorporated into protozoal material; but these experiments provided no information on the metabolic fate of individual bacterial amino acids. As it was possible that the metabolism of free and bacterial amino acids might be different, samples of *E. coli* labelled singly with ^{14}C leucine, isoleucine, alanine, glutamic acid or lysine were prepared and the incorporation of radioactivity into the broken protozoa supernatant fluid fraction studied. Very low degrees of incorporation from ^{14}C alanine-labelled *E. coli* were found. Over 70% of the ^{14}C in the broken protozoa supernatant fluid was present in the protein, analysis of which showed that no amino acid was labelled in the protozoal protein which was not labelled in the bacteria fed. Unfortunately, under the conditions used, it was not possible to prepare *E. coli* which contained ^{14}C only in aspartic acid, glycine or serine, and some ^{14}C was found in other amino acids. After incubation of *E. coli* labelled principally with aspartic acid, glycine or serine, the majority of the ^{14}C was present as the same amino acid in the protozoa. It was not possible to eliminate amino acid conversions in the protozoa similar to those which had already occurred in the bacteria.

Incorporation of radioactivity from *Escherichia coli* labelled with specific ^{14}C amino acids was inhibited as follows by the addition to the medium of 500 $\mu\text{g./ml.}$ of the ^{12}C form of that amino acid: leucine 69%, glycine 40%, isoleucine 81%, alanine 37%, lysine 38%, serine 5%, aspartic acid (–16%). To show that this was not a general effect of free amino acids on the rate of uptake of *E. coli* by the protozoa, it was shown that single free amino acids did not decrease the incorporation of ^{14}C from uniformly ^{14}C -labelled *E. coli* into protozoa and that $[^{12}\text{C}]\text{L-leucine}$ did not decrease the incorporation of ^{14}C from *E. coli* labelled with $[^{14}\text{C}]\text{isoleucine}$. Addition of 100 $\mu\text{g. } [^{12}\text{C}]\text{L-leucine/ml.}$ to incubations of protozoa with *E. coli* labelled with $[^{14}\text{C}]\text{leucine}$ decreased incorporation of ^{14}C into the protozoal fraction to the same extent as it increased the radioactivity in the medium. The amount of ^{14}C present in the bacterial fraction was the same at the end of the incubation, whether in the presence or absence of $[^{12}\text{C}]\text{leucine}$. These results are consistent with the hypothesis that the protozoa digested at least part of the bacteria which they took up with the formation of free amino acids which were then incorporated into protein or released into the medium.

DISCUSSION

As far as the authors are aware, these studies represent the first quantitative demonstration of the uptake of bacteria by rumen holotrich protozoa and show that these protozoa are similar in this respect to *Entodinium caudatum* (Coleman, 1964). Gutierrez (1958) suggested that *Isotricha prostoma* selectively ingested certain rods from among many types of rumen bacteria, although several bacterial species were isolated from crushed protozoa. The present results show that *I. intestinalis* and *I. prostoma* took up a bacterium not isolated from the rumen, namely *Escherichia coli*, and then rapidly killed and digested this bacterium. As different bacterial species may have different survival times inside the protozoa, erroneous results may be obtained in attempts to determine, by examination of the bacteria in crushed protozoa, which bacterial species, if any, are important to the protozoa.

In a discussion of these initial results, obtained with the *in vitro* system described, two aspects of the biosynthetic metabolism of *Isotricha intestinalis* and *I. prostoma*

may be considered. One is whether these protozoa are capable of taking up bacteria and using bacterial components for synthesis of their own cellular materials. The second concerns the utilization of amino acids, either free or provided in bacterial form, for protein synthesis and the pathways which may be involved in interconversion of amino acids. Complete answers to these questions would obviously include valuable information about the biosynthetic pathways present in these protozoa.

It is clear that radioactivity from ^{14}C labelled *Escherichia coli* was taken up by the protozoa under the conditions described. Since 40% of the radioactivity from uniformly-labelled bacteria was found in the broken-protozoa supernatant fluid this could not have been in the form of whole *E. coli*. The differences in the distribution of ^{14}C between the chemical fractions of protozoa and bacteria (particularly among the amino acids of the protein fraction) after metabolism of $[\text{U-}^{14}\text{C}]\text{E. coli}$ by the protozoa and also the apparent competition between single free ^{14}C amino acids and *E. coli* labelled with the ^{14}C form of that amino acid, support the hypothesis that bacterial protein was broken down and utilized for the synthesis of protozoal protein. Such an interpretation of the results would be greatly strengthened if a ^{14}C amino acid from *E. coli* could be demonstrated in an isolated specific protozoal protein. *Escherichia coli* was chosen for the present studies because of the wealth of information available about its metabolic pathways (e.g. Roberts *et al.* 1955). It would obviously be of considerable interest to compare incorporation of components of this bacterium with that of others, particularly those normally found in the rumen.

Amino acids provided either free or in *Escherichia coli* were incorporated into protein fractions of the protozoa, suggesting that some protein synthesis or turnover occurred under the conditions used. However, the ability of the protozoa to interconvert amino acids appeared to be limited, since only the ^{14}C amino acids provided were found to be labelled in protozoal protein fractions. It seems unlikely that incorporation of free amino acids by the protozoa occurred through initial incorporation by rumen bacteria followed by their ingestion by protozoa, since benzylpenicillin + neomycin eliminated bacterial growth but did not greatly affect amino acid incorporation.

Without a defined medium which supports growth of the holotrich protozoa of the rumen for prolonged periods, useful information may be still gained from studies of the activities of these organisms under the conditions described above. In particular, the utilization of bacterial components should be investigated further, in view of the observation that bacteria are essential for growth of these protozoa outside the rumen. However, as it is possible that biosynthetic pathways of the protozoa may not be the same *in vivo* as in these washed suspensions, the development of a growth medium for the organisms is of great importance in further understanding of their metabolism.

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