The Metabolism of *Escherichia coli* and other Bacteria by *Entodinium caudatum*

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(Received 2 June 1964)

SUMMARY

¹⁴C-labelled *Escherichia coli* and other bacteria were used to measure their uptake by washed suspensions of *Entodinium caudatum*. All the bacteria tested were engulfed by the protozoa with a maximum uptake of 1.1×10^4 *E. coli*/protozoon at an initial rate of over 200 bacteria/protozoon/min. After 30 min. only 12% of the engulfed bacteria were still viable. 50% of the bacterial carbon was retained by the protozoa and, after breakage of the protozoa and centrifugation of the homogenate, 40% of this carbon was present in the supernatant fluid, principally as protein. Competition experiments where the protozoa were offered two different species of bacteria showed that the protozoa engulfed bacteria in the proportion in which they were present in the medium. The growth of *E. caudatum* in the presence of rice starch, autoclaved rumen fluid and penicillin was stimulated by several species of bacteria, including *E. coli*, *Clostridium welchii*, *Lactobacillus casei*.

INTRODUCTION

Sheep rumen Entodiniomorphid protozoa freshly isolated from the rumen (Gutierrez & Davis, 1959) or grown *in vitro* (Coleman, 1962) contain bacteria in their gastric sacs. However, Gutierrez & Davis (1959) were able to isolate only 3–10 *Streptococcus bovis* organisms per ciliate, as compared with the 100–150 bacteria which were visible microscopically. The ciliates were also shown to ingest *S. bovis* and it was suggested that the protozoa might have been digesting the bacteria. It was found previously (Coleman, 1962) that *Entodinium caudatum* did not grow in the absence of bacteria. The present paper extends this work and by the use of ¹⁴C-labelled bacteria it has been shown that many species of bacteria are engulfed and one species at least digested by the protozoa. The addition of bacteria under appropriate conditions stimulated the growth of the protozoa.

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.

Preparation of protozoa for inoculation. The protozoa were taken from the 'inoculum cultures', in which they were present as a loose pellet at the bottom of the tube, after removal of the surface scum, and most of the medium and allowed to

stand in 8 in. $\times$ 1 in. tubes 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 three times in salt solution B which contained 0.03 % L-cysteine on a bucket-head centrifuge for 30 sec. from starting. The maximum speed was equivalent to 300*g*. The organisms were finally used as inoculum to give a population density of $5\text{--}10 \times 10^4$ protozoa/ml. for experiments with non-multiplying protozoa and $1\text{--}2 \times 10^4$ /ml. for growth experiments.

Incubation conditions for engulfment of bacteria by non-multiplying protozoa. The medium consisted of 4.0–7.0 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 2.0 ml. water in a 15 ml. centrifuge tube. Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0.2 ml. 1 % (w/v) L-cysteine hydrochloride (neutralized and Seitz filtered), 0.2 ml. 5 % (w/v) NaHCO₃ (Seitz filtered), 0.1–2.0 ml. suspension of ¹⁴C-bacteria in salt solution B (washed once and suspended at a concentration of $10^8\text{--}10^{10}$ bacteria/ml.), and any other additions. After inoculation the tubes were gassed for 10 sec. with 95 % N₂ (v/v) + 5 % CO₂ (v/v), sealed with a rubber bung and incubated at 39°.

Incubation conditions for growth of protozoa. The medium consisted of 3.5–5.5 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 2.0 ml. water and soluble starch (if any) in a 15 ml. centrifuge tube. Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0.2 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered); 0.2 ml. 5 % NaHCO₃ (Seitz filtered); 0.4 ml. penicillin G (25,000 units/ml.); 0.2 ml. 7.5 % sterile rice starch (dry rice starch heated at 120° for 24 hr. and then suspended in salt solution B), 1.0 ml. protozoa-containing autoclaved rumen fluid (PARF), 0.2–2.0 ml. suspension of bacteria in salt solution B (washed once and suspended at a density of $10^8\text{--}10^{10}$ bacteria/ml.); any other additions. After inoculation the tubes were gassed for 10 sec. with a mixture of 95 % N₂ (v/v) + 5 % CO₂ (v/v), sealed with a rubber bung and incubated horizontally at 39°. For experiments on the effect of carbohydrates, the rice starch was omitted and replaced by soluble starch which was dissolved in salt solution B and autoclaved, or by sugars which were autoclaved as 10 % solutions in water and then added to the incubation media.

At the end of experiments in which the uptake of ¹⁴C-bacteria was being investigated the protozoa were centrifuged and washed twice in salt solution B on a bucket-head centrifuge for 30 sec. from starting (maximum speed was equivalent to 300*g*), and plated out to determine radioactivity. The residual bacteria were spun down from the first supernatant fluid obtained after removal of the protozoa and washed once at 2000*g* for 10 min. on an angle-head centrifuge. Samples from the supernatant fluid after removal of the bacteria were placed on planchets for estimation of the ¹⁴C which was free in the medium.

Sources of bacteria used. *Bacillus megaterium* KM, *Clostridium welchii* SR 12, *Escherichia coli* B, *Leuconostoc mesenteroides* P 60 (NCIB 8952), *Nadsonia elongata*, *Salmonella typhimurium* SW 1061, *Staphylococcus aureus* (Duncan), *Streptococcus faecalis* (Dunn) and *Vibrio metchnikovii* were kindly supplied by Professor E. F.

Gale, Dr D. Kerridge and Dr K. McQuillen (of the Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge). *Lactobacillus casei* var *rharnosus* (NCIB 9282) and *Serratia marcescens* (NCIB 1377) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. *Streptococcus bovis* 2B (Bailey & Oxford, 1958) was obtained from the National Collection of Dairy Organisms, Shinfield, Reading (NIRD 1251). Bacterium D (a Gram-positive coccus) and Bacterium 31 (a Gram-negative rod) were isolated from standard protozoal cultures (Coleman, 1960*a*).

Bacterial growth media. *Escherichia coli* and *Bacillus megaterium* were grown and maintained in C medium (Roberts *et al.* 1955) containing 0.2% glucose + 0.05 μ C [$U-^{14}C$]-glucose/ml. (if any) at 37°. The cultures were aerated during incubation by passing sterile air into the medium through a Pasteur pipette.

Clostridium welchii, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus bovis*, *Streptococcus faecalis*, *Vibrio metchnikovii* and Bacteria D and 31 were grown at 37° for 16 hr in a medium that contained, per litre: salt solution (Coleman, 1958) 250 ml.; Difco yeast extract, 2 g.; Difco tryptose, 2 g.; glucose, 2 g. and [$U-^{14}C$]-glucose, 200 μ C. (if any). All the bacteria were grown aerobically in static culture in cotton-plugged flasks except *Clostridium welchii* which was grown under CO_2 .

Nadsonia elongata was grown in cotton-plugged flasks at 30° for 16 hr in a medium that contained per litre: $(NH_4)_2SO_4$, 2 g.; KH_2PO_4 , 2 g.; glucose, 2g.; Difco yeast extract, 5 g.

Bacterial viable counts. Total viable counts were carried out on the medium of Bryant & Robinson (1961; hereafter referred to as Bryant medium) as described by Coleman (1962). Viable *Escherichia coli* counts were carried out by serial 100-fold dilutions in C medium + 0.2% (w/v) glucose (Roberts *et al.* 1955) followed by plating on C medium containing 0.2% glucose and 1% agar and incubation at 37°. Where no *E. coli* were added to protozoal cultures the bacterial counts on this medium were less than 0.01% of those carried out in Bryant medium.

Total bacterial counts. The total number of bacteria in a washed bacterial suspension was estimated by counting an appropriate dilution in a Helber counting chamber of depth 0.02 mm. and square size 0.0025 mm².

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.

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 muslin and autoclaving under 95% (v/v) N_2 + 5% (v/v) CO_2 in sealed McCartney bottles at 115° for 20 min. Rumen fluid without protozoa was prepared by straining the fluid through muslin followed by centrifugation at 500*g* for 3 min. to remove protozoa. This material was then used fresh or autoclaved (ARF). Autoclaved supernatant rumen fluid was prepared similarly to PARF except that the strained material was centrifuged at 12,000*g* for 45 min. before autoclaving. Seitz-filtered rumen fluid was prepared similarly to autoclaved supernatant rumen fluid except that sterilization was by Seitz-filtration.

Estimation of ^{14}C . ^{14}C was estimated by washing the protozoa with water on to an aluminium disc (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 planchet 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. material/cm.² of planchet. Where more material was present, the results were corrected to infinite thinness by using corrections determined from known weights of the appropriate pure organic compounds, mineral salts or rumen fluid fractions added to tracer quantities of ^{14}C compounds. 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 as determined by radioautography.

Fractionation of protozoa. The protozoa were first broken by treatment in an all-glass Potter homogenizer (Potter & Elvehjem, 1936) at room temperature until 98–100 % of the organisms were broken (usually about 90 sec.) and then the homogenate was made up to a known volume and 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 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 % (w/v) 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 washings 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 + washings formed the 'hot TCA soluble or nucleic acid fraction'. The TCA was removed from both fractions by washing three times with ether before placing samples on planchets for the estimation of ^{14}C . The residue after further washing, once with ethanol acidified with 0.01N-HCl and once with ether, formed the protein fraction. This protein was hydrolysed completely by heating at 105° in 6N-HCl for 16 hr 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 paper chromatography in *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.) and phenol + ammonia (sp.gr. 0.880) + water (80 g. + 0.8 ml. + 20 ml.). ^{14}C -Amino acids were detected by radioautography.

Chemicals. ^{14}C -compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. The specific activities of the compounds as supplied were, in $\mu\text{C./mg.}$: [$\text{U-}^{14}\text{C}$]-glucose 47.7; [$8\text{-}^{14}\text{C}$]-guanine 70.7; [$\text{U-}^{14}\text{C}$]-L-leucine 320.

RESULTS

The metabolism of Escherichia coli by non-multiplying Entodinium caudatum

The method used to study the uptake of bacteria by *Entodinium caudatum* was to measure the incorporation by the protozoa of ^{14}C from ^{14}C -labelled bacteria of known specific activity. For most of this work the bacterium used was *Escherichia coli* B because: (a) it could be prepared uniformly labelled with ^{14}C by growth on [$\text{U-}^{14}\text{C}$]-

glucose as sole source of carbon; (b) it grew aerobically on plates on glucose ammonia salts agar medium under which conditions less than 0.1 % of the bacteria which grew from the protozoal inoculum during 24 hr in the protozoal growth medium formed colonies. It was therefore possible to measure the incorporation of bacterial carbon into the protozoa and the disappearance of viable *E. coli* from the medium. The bacteria were separated from the protozoa by differential centrifugation as described under 'Methods'. Table 1 shows that under the conditions used

Table 1. *Effect of various incubation conditions on the engulfment of ^{14}C -Escherichia coli by Entodinium caudatum*

Experiment carried out with 1.3×10^9 *Escherichia coli* organisms/ml. (3710 counts/min.) in the presence or absence of 6.2×10^4 protozoa/ml. at the temperature indicated. The 'protozoal fraction' was the pellet (if any) obtained by centrifugation for 30 sec. from starting at a maximum of 300 g and washing twice in salt solution B under the same conditions.

Time (hr)	Conditions	^{14}C found in 'protozoal fraction' (counts/min.)
0	Bacteria only	12
3	Bacteria only, 39°	24
0	Bacteria + protozoa	5
3	Bacteria + protozoa, 39°	368
3	Bacteria + protozoa, 4°	3
3	Bacteria + broken protozoa*, 39°	10
3	Bacteria + protozoa boiled for 5 min., 39°	15

* Protozoa broken in a Potter homogenizer.

there was little incorporation of ^{14}C from ^{14}C -*E. coli* into the 'protozoal fraction' initially, in the presence of broken or boiled protozoa, after incubation at 4°, or in the absence of protozoa. The incorporation as measured over a 3-hr period had a temperature optimum at 35–40° and the rate dropped to less than 5 % of the optimum at 21° and 45°. The ^{14}C incorporation was also sensitive to decreases in the salt concentration of the medium. At a salt concentration 25 % of normal the incorporation as measured over 2 hr decreased to 10 % as did the number of protozoal survivors at the end of the experiment. These results indicate that incorporation of ^{14}C from ^{14}C -labelled *E. coli* into the protozoal fraction occurred only in the presence of intact metabolizing protozoa. Although the same result might have been obtained if the protozoa caused extensive clumping of the bacteria, no large clumps of bacteria were visible in washed protozoal suspensions at the end of experiments. It is therefore suggested that the bacteria were engulfed by the protozoa.

Attempts to recover viable Escherichia coli inside protozoa. To determine whether the bacteria engulfed by the protozoa were still viable the following experiment was carried out. During 5 hr, 8.4×10^4 protozoa/ml. were allowed to engulf ^{14}C -labelled *Escherichia coli* from a suspension that contained 65×10^7 bacteria/ml. At the beginning and at intervals during the experiment the ^{14}C was determined in the protozoa and the viable bacteria in the medium, in washed intact protozoa and in washed protozoa broken in a Potter homogenizer. At 30 min. and 5 hr, respectively,

each protozoon contained ^{14}C from 770 and 1520 bacteria (as determined from ^{14}C in the protozoa) but there were only 95 and 15 viable bacteria inside each protozoon (as determined from the difference in viable count between intact and broken protozoa). These results show that *E. coli* was rapidly rendered non-viable after engulfment by the protozoa.

Time course of metabolism of Escherichia coli. This is shown in Fig. 1. After 22 hr incubation of protozoa with ^{14}C -labelled *Escherichia coli*, over 90% of the free bacteria had disappeared (as determined by ^{14}C estimations and bacterial viable counts) and approximately half the bacterial carbon then present was found in the protozoa and half in the supernatant fluid.

Fig. 1

Fig. 2

Fig. 1. Metabolism of $[\text{U-}^{14}\text{C}]$ -*Escherichia coli* (9.0×10^8 organisms/ml.; 7550 counts/min./ml.) by 7.3×10^4 *Entodinium caudatum*/ml. $\bullet$ — $\bullet$ ^{14}C in protozoa; $\circ$ — $\circ$ ^{14}C in free bacteria; $\times$ — $\times$ ^{14}C free in medium.

Fig. 2. Effect of *Escherichia coli* population density on engulfment by 7.2×10^4 *Entodinium caudatum*/ml. in the presence and absence of 10% supernatant fluid from centrifugation of PARF at 7000 g for 20 min. $\bullet$ — $\bullet$ incubation for 30 min. without PARF supernatant; $\circ$ — $\circ$ incubation for 30 min. with PARF supernatant; $\times$ — $\times$ incubation for 3 hr with or without PARF supernatant.

Effect of bacterial concentration on the uptake of Escherichia coli. The uptake of bacteria increased with increasing initial concentration of bacteria (Fig. 2). After 30 min. at an initial bacterial concentration of 7.0×10^9 *Escherichia coli*/ml. in the presence of 7.2×10^4 protozoa/ml. the uptake was 6.5×10^3 bacteria/protozoon; this was increased to 7.9×10^3 bacteria/protozoon in the presence of 10% PARF (clarified by centrifugation). To determine the maximum uptake of bacteria the reciprocal of the bacterial uptake at 30 min. and 3 hr was plotted against the reciprocal of the bacterial concentration and the graphs extrapolated to infinite bacterial concentration. The correction was a small one for the 3-hr results and at both times the maximum uptake of *E. coli* was 1.1×10^4 bacteria/protozoon. Although 10% clarified PARF stimulated the rate of uptake of *E. coli*, 10%

PARF (as prepared) and which contained dead bacteria, decreased by 30% the uptake of *E. coli* from a suspension of 1.3×10^9 bacteria/ml. In eight experiments on the engulfment of *E. coli* the highest value of the maximum uptake was 11×10^3 , the lowest 4×10^3 and the average 6.8×10^3 *E. coli*/protozoon. The actual value may depend on the volume of the gastric sac occupied by starch grains on harvesting the protozoa, since a large number of starch grains might be expected to decrease the volume available for the engulfment of bacteria.

The volume occupied by each *Escherichia coli* organism when closely packed was determined as $2 \mu^3$, by centrifugation of a suspension containing a known number of bacteria for 10 min. at 3000g and measuring the volume of the cell pad. The maximum and minimum gastric sac volumes as determined from the number of bacteria and the volume of each bacterium were therefore 2.2×10^4 and $8.0 \times 10^3 \mu^3$, respectively. At a suspension concentration of 6.5×10^8 *E. coli* organisms/ml. there was one bacterium per $1.54 \times 10^3 \mu^3$ medium. At this bacterial concentration after 30 min. in the presence of PARF supernatant fluid, 1.7×10^3 bacteria/protozoon had been engulfed (Fig. 2). Therefore the bacteria from $2.6 \times 10^6 \mu^3$ medium had been removed by each protozoon. If it be assumed that there is a continuous passage of medium through a protozoon from mouth to anus, and that any bacteria in this medium are filtered off and remain in the protozoon, and if A = 'volume of medium which contained the bacteria engulfed by each protozoon', B = 'gastric sac volume' and C = 'number of gastric sac volumes of medium passing through the protozoon', then $A/B = C$. Therefore in 30 min. $2.6 \times 10^6 \div 2.2 \times 10^4 = 120$ gastric sac volumes of medium have passed through each protozoon. Since the most rapid uptake of bacteria occurred during the first 30 min. of incubation, these volumes are maximum values; it is possible that when the protozoa withdraw their cilia and become quiescent no medium passes through them.

Uptake of various particulate fractions prepared from ^{14}C Escherichia coli. Live bacteria, bacteria that had been boiled for 5 min. and then washed, bacteria treated with 5% trichloroacetic acid and then washed and bacterial protein prepared by the method of Roberts *et al.* (1955) and then broken-up in a Potter homogenizer, were all taken up by protozoa and digested to about the same extent when suspended in equivalent amounts. In contrast, the uptake of ^{14}C from the hot trichloroacetic acid extract of the bacteria (after removal of the trichloroacetic acid with ether) was less than 3% of that of any particulate preparation.

Table 2 shows that in an experiment where all the protein (prepared from *Escherichia coli* grown on $[\text{U-}^{14}\text{C}]$ -glucose) was engulfed in the first 30 min. there was subsequently an increase in the ^{14}C in the protein of the broken-cell supernatant fluid and the medium, whereas the ^{14}C in the broken-cell pellet protein and cell 'pool' diminished. This result is consistent with an engulfment of the protein particles (which in the undegraded form would appear in the broken-cell pellet fraction) into the gastric sac, followed by digestion to small molecular-weight compounds such as amino acids, which were then released into the medium or incorporated into protozoal soluble protein.

Metabolism of ^{14}C -leucine and ^{14}C -guanine labelled Escherichia coli. In an attempt to study the metabolism of protein and nucleic acid of living bacteria by protozoa, *Escherichia coli* was grown on C medium containing ^{12}C -glucose and either $[\text{U-}^{14}\text{C}]$ -L-leucine or $[8\text{-}^{14}\text{C}]$ -guanine. Roberts *et al.* (1955) found, and it was confirmed in

the present work, that growth in the presence of [U-¹⁴C]-L-leucine produced bacteria labelled only in the leucine of the bacterial protein, and that growth in the presence of ¹⁴C-guanine produced bacteria labelled in nucleic acid, guanine and to a lesser extent adenine.

Table 2. *The metabolism of Escherichia coli protein by Entodinium caudatum*

Protozoa (*Entodinium caudatum*) incubated anaerobically in the presence of *Escherichia coli* protein (8800 counts/min.) prepared from *E. coli* grown on [U-¹⁴C]-glucose by the method of Roberts *et al.* (1955). The results are expressed in counts/min. After incubation the washed protozoa were broken in a Potter homogenizer and the supernatant and pellet fractions separated by centrifugation.

Time (hr)	Protozoa present	¹⁴ C in 'protozoal fraction'	¹⁴ C in medium	Cold tri- chloroacetic acid soluble fraction	Nucleic acid	Protein
Radioactivity (counts/min.)						
0	±	732	200	—	—	—
0.5	+	6480	1580	—	—	—
		Broken cell supernatant	—	2180	52	350
		Broken cell pellet	—	105	150	3650
5.5	+	3590	3630	—	—	—
		Broken cell supernatant	—	400	55	925
		Broken cell pellet	—	110	190	1910
5.5	—	240	1000	—	—	—

The course of the digestion of ¹⁴C-leucine labelled *Escherichia coli* was similar to that obtained with bacteria labelled by growth in the presence of [U-¹⁴C]-glucose (Fig. 1). Of the ¹⁴C retained by the protozoa approximately 40 % was in the broken-cell supernatant fraction, and over 90 % of the ¹⁴C in the supernatant fluid and pellet fractions was present as protein-leucine. In the absence of protozoa less than 5 % of the bacterial ¹⁴C was liberated into the medium in 60 hr. The presence of 0.01 M-¹²C-L-leucine in the medium during the experiment decreased the incorporation of ¹⁴C into the protozoa by only 20 % in the first hour, but thereafter the ¹⁴C in the protozoa remained constant, while in the absence of ¹²C-leucine it steadily increased. It was not possible to abolish completely the incorporation of ¹⁴C by the addition of increased amounts of ¹²C-leucine. The lowest concentration that gave maximal effect was 0.005 M and there was no increased effect when this was increased to 0.02 M.

The metabolism of ¹⁴C-guanine-labelled *Escherichia coli* differed from that of ¹⁴C-leucine labelled *E. coli* in that after incubation for 27 hr, when 98 % of the bacteria had been engulfed (i) over 70 % of the ¹⁴C was present in the medium, and (ii) 75 % of the protozoal ¹⁴C was present in the broken-cell supernatant fluid, and only 30 % of this was in the nucleic acid, the remainder being in the cold trichloroacetic acid soluble material. However, these results were difficult to interpret because in the absence of protozoa 25 % of the bacterial ¹⁴C was liberated into the

medium. The nucleic acid ^{14}C was present only in the adenine and guanine which were labelled in the same proportions as in the engulfed bacteria.

The engulfment of other micro-organisms by non-multiplying protozoa

The method used to study the engulfment of other bacteria and a yeast was similar to that used for *Escherichia coli* except that the micro-organisms were grown on complex organic materials + $[\text{U-}^{14}\text{C}]$ -glucose and were therefore probably not uniformly labelled with ^{14}C . When the organisms were incubated for 3 hr at an organism/protozoon ratio of 50–500, 21–45% of a suspension of the following micro-organisms was taken up by the protozoa: *Bacillus megaterium* KM, *Clostridium welchii* SR 12, *Leuconostoc mesenteroides*, *Nadsonia elongata* (a yeast), *Salmonella typhimurium* sw 1061, *Serratia marcescens*, *Streptococcus bovis* 2B, *Streptococcus faecalis* (Dunn), *Vibrio metchnikovii*, Bacterium D, Bacterium 31. All the organisms tested were engulfed by the protozoa.

Competition between bacteria for engulfment by Entodinium caudatum. In an attempt to determine whether the protozoa engulfed some bacteria preferentially, a washed suspension of protozoa was incubated for 3 hr in the presence of a concentration of ^{14}C -*Escherichia coli* such that any further increase in the concentration of bacteria caused negligible increase in the number of bacteria taken up per protozoon, i.e. almost a saturating concentration. The effect on uptake of ^{14}C -*E. coli* of adding varying volumes of suspensions of ^{12}C -bacteria, containing a known number of bacteria, to a fixed saturating concentration of *E. coli* suspension, was then studied. The volume required to decrease the uptake of ^{14}C into the protozoa to 50% of that in the absence of ^{12}C -bacteria was determined. From the relative numbers of *E. coli* and other bacteria present the number of *E. coli* equivalent to each of the other bacteria under these conditions was determined. For example, Fig. 3 shows an experiment in which ^{12}C -*E. coli* and ^{12}C -*Lactobacillus casei* were in competition with 25×10^8 ^{14}C -*E. coli*/ml. The number of these bacteria required to decrease the engulfment of ^{14}C -*E. coli* to 50% was 24×10^8 and 8.5×10^8 /ml. respectively. Therefore each ^{12}C -*E. coli* and ^{12}C -*L. casei* was equivalent to 1.04 and 2.9 ^{14}C -*E. coli*, respectively. It is of interest that each *L. casei* occupied 2.4 times as much space as *E. coli* as determined from the packed volumes of the bacteria. The following list gives, first the number of *E. coli* equivalent to each bacterium as determined under these conditions, and second the volume occupied by each bacterium relative to that of *E. coli*: *Bacillus megaterium* KM, 27, 24; *Clostridium welchii* SR 12, 4, 5; *Lactobacillus casei*, 2.9, 2.4; *Nadsonia elongata*, 22, 21; *Staphylococcus aureus* (Duncan), 2, 1.3; *Streptococcus bovis* 2B, 2, 1.4; *S. faecalis* (Dunn), 0.6, 0.72; Bacterium D, 1.1, 0.9; Bacterium 31, 2.2, 1.8. These results suggest that the efficiency of any bacterium in diminishing the engulfment of ^{14}C -*Escherichia coli* was approximately proportional to its size.

Growth of Entodinium caudatum in the presence of Escherichia coli

It was reported previously (Coleman, 1960b) that in the presence of rice starch, dried grass, autoclaved rumen fluid (PARF) and penicillin, *Entodinium caudatum* which had been maintained *in vitro* culture for 18 months grew for 1–2 generations in 4–7 days. However, after 3 years of maintenance *in vitro* maximal growth of the protozoa only occurred when living bacteria were added to the culture; there was no

stimulation in the presence of boiled bacteria. This effect was not specific to one bacterial preparation and was found with crude rumen contents from which protozoa had been removed by centrifugation (Fig. 4), and with *Bacillus megaterium* KM, *Escherichia coli* B (Fig. 4), *Clostridium welchii* SR12 (Fig. 4), *Nadsonia elongata*, *Lactobacillus casei*, *Streptococcus faecalis* (Dunn) and *S. bovis* 2B at population densities of 10 – 100×10^7 organisms/ml. In contrast, Bacterium 31 (an unidentified Gram-negative rod) which was isolated from a protozoal culture and which produced slime on addition to incubation media, inhibited protozoal growth. Growth in the

Fig. 3

Fig. 3. Effect of population density of ^{14}C -*Escherichia coli* and ^{14}C -*Lactobacillus casei* on the engulfment of $[\text{U-}^{14}\text{C}]$ -*Escherichia coli* by 8.8×10^4 *Entodinium caudatum*/ml. after 8 hr. The total volume was 10 ml. ●—●, effect of ^{14}C -*Escherichia coli* (25×10^8 /ml. and 1510 counts/min./ml.) population density on ^{14}C incorporated by protozoa; ○—○ effect of ^{14}C -*E. coli* (14×10^8 /ml.) population density on incorporation of ^{14}C from 1.0 ml. ^{14}C -*E. coli*; ×—×, effect of ^{14}C -*Lactobacillus casei* (9.4×10^8 /ml.) on incorporation of ^{14}C from 1.0 ml. ^{14}C -*Escherichia coli*.

Fig. 4

Fig. 4. Effect of various bacterial preparations on the growth of *Entodinium caudatum* under standard conditions. No added bacteria ×—×; 5% crude rumen contents (after removal of protozoa) +—+; 3.5×10^8 *Clostridium welchii*/ml. ●—●; 1.4×10^9 *Escherichia coli*/ml. ○—○ (the figures indicate the number of viable *E. coli* at each point).

presence of stimulatory bacteria was decreased by 25% when tubes were incubated vertically instead of horizontally. As the protozoa in tubes that were incubated vertically contained less starch (as determined microscopically after staining with iodine) this result may be associated with the inability of protozoa to obtain sufficient starch for maximal growth when the starch is as a pellet at the bottom of a tube. During growth in the presence of *E. coli* the number of viable *E. coli* organisms in the medium decreased rapidly until after 72 hr less than 0.01% of the original number remained (Fig. 4). In the absence of protozoa the number of viable *E. coli* organisms decreased by 25 and 75% in 48 hr and 96 hr, respectively.

Growth of other bacteria during growth of protozoa in presence or absence of Escherichia coli. The number of other bacteria as determined by viable counts in Bryant

medium increased from $1-3 \times 10^5$ to about 50×10^7 /ml. over the first 36 hr in the presence or absence of *Escherichia coli* and usually remained constant thereafter for more than 60 hr. However, in some experiments the numbers decreased to $1-10 \times 10^7$ /ml. after 48 hr and then stayed constant or increased slowly to 50×10^7 /ml.

Effect of *Escherichia coli* concentration on protozoal growth. Fig. 5 shows that the lowest initial population density of *Escherichia coli* that produced the maximum growth stimulation was 4×10^4 bacteria/protozoon inoculated, and that higher bacterial concentrations did not increase the growth rate further, although at the highest concentration tested it was occasionally depressed.

Fig. 5. Effect of initial population density of *Escherichia coli* on growth of *Entodinium caudatum* from a 1.8×10^4 /ml. inoculum after 50 hr.

Effect of autoclaved rumen fluid fractions on protozoal growth in presence of *Escherichia coli*. There was no increase in the number of protozoa after 48 hr in the absence of protozoa-containing autoclaved rumen fluid (PARF) and 2 and 5 % PARF gave 28 and 75 %, respectively, of the growth obtained with 10 % PARF. Higher concentrations of PARF were inhibitory. Autoclaved rumen fluid supernatant and Seitz filtered rumen fluid supported no growth, and ARF had only 60 % of the activity of PARF. To determine whether the soluble or insoluble materials were important PARF was fractionated by centrifugation into a clear supernatant fluid and a pellet fraction which was washed free from supernatant fluid. When tested at 10 % in the presence of bacteria the supernatant fluid fraction contained 0-80 % of the growth-promoting activity in PARF and the number of protozoa never doubled, however long the incubation period. In contrast, the pellet fraction contained 60-70 % of the total activity and the number of protozoa usually doubled in 60 hr. In the absence of bacteria the number of protozoa declined in the presence of both fractions. These results suggest that particulate matter, which was presumably engulfed by the protozoa, was more important for growth under these conditions than were soluble materials.

Replacement of starch grains by other carbohydrates. Of the carbohydrates tested, growth comparable to that in the presence of starch grains was found only with soluble starch and maltose (Fig. 6). Replacement of the starch grains by 1 % cellobiose, glucose or sucrose produced approximately half maximum growth and

there was no growth during 72 hr in the presence of a 1% solution of arabinose, fructose, galactose, inulin, mannose, melibiose or xylose, although in their absence the protozoa died in 48 hr. The growth of bacteria other than *Escherichia coli*, as determined by viable counts in Bryant medium, in the presence of soluble starch or maltose was usually the same as, and never more than double, that in the presence of starch grains.

Metabolism of uniformly labelled ^{14}C -Escherichia coli. During growth of protozoa in the presence of uniformly labelled ^{14}C -*Escherichia coli* about 30% of the bacterial-carbon was assimilated by the protozoa and the remainder appeared in the medium.

Fig. 6

Fig. 6. Effect of carbohydrates on the growth of *Entodinium caudatum* in the presence of 2.2×10^9 *Escherichia coli*/ml. under standard conditions except that rice starch was omitted or replaced by other carbohydrates. Δ — Δ , no addition; $\times$ — $\times$, +0.15% rice starch grains; $\bullet$ — $\bullet$, +0.6% soluble starch; $\circ$ — $\circ$, +1% maltose; +—+, +1% glucose; $\blacktriangle$, +1% fructose.

Fig. 7

Fig. 7. Effect of 0.01 M- ^{12}C -L-leucine on the incorporation of ^{14}C from ^{14}C -leucine labelled *Escherichia coli* during growth of *Entodinium caudatum*. $\bullet$ — $\bullet$ no added ^{12}C -L-leucine; $\circ$ — $\circ$ +0.01 M- ^{12}C -L-leucine. 1.1×10^4 protozoa/ml. and 1.7×10^9 *E. coli*/ml. (8800 counts/min./ml.) present initially.

After breakage of these protozoa in a Potter homogenizer 45% of the ^{14}C was in the broken cell supernatant fluid and 70% of this was present in the protein fraction. In the absence of protozoa only 10–13% of the bacterial-carbon was liberated into the medium over 48 hr.

Metabolism of ^{14}C -leucine labelled Escherichia coli. In an attempt to follow the metabolism of *Escherichia coli* protein during protozoal growth, the bacteria were labelled with [U- ^{14}C]-leucine and the incorporation of ^{14}C from these bacteria was compared with the uptake of ^{14}C from [U- ^{14}C]-L-leucine free in the medium in the presence and absence of ^{12}C -*E. coli*. In the experiment shown in Fig. 7, the number of protozoa increased by 140% in the presence of, and 85% in the absence of *E. coli*

during 48 hr.; 24 % of the ^{14}C from the ^{14}C -*E. coli* was found in the protozoa and the remainder in the medium. Over 95 % of the protozoal ^{14}C was in the protein fraction as leucine. In the absence of protozoa the amount of ^{14}C that appeared in the medium was decreased by 85 %. The addition of 0.01 M- ^{12}C -L-leucine to the incubation medium diminished the incorporation into the protozoa to 3 %, suggesting that, as with non-multiplying protozoa, the leucine in the bacterial protein was hydrolysed in the gastric sac to the free amino acid before incorporation into protozoal protein. From the results of Roberts *et al.* (1955) on the composition of *E. coli* it was calculated that the 1.7×10^8 bacteria/ml. present initially in this experiment contained 23 μg . leucine of which 5.5 μg . (24 %) was incorporated into the protozoa. In contrast only 4 % of the ^{14}C from 0.013 μg . [U- ^{14}C]-L-leucine/ml. and less than 1 % from 13 μg . leucine/ml. free in the medium was taken up by the protozoa. In the presence of ^{12}C -*E. coli* the uptake of ^{14}C -leucine was increased, probably due to incorporation after assimilation by the bacteria. These results show that leucine was utilized much more economically from intact bacterial protein which was broken down in the gastric sac than from the free amino acid which could probably only be taken up during passage of the medium through the gastric sac.

Attempts to replace living bacteria for protozoal growth

Since protozoa can engulf living bacteria, dead bacteria and particles of bacterial protein, the necessity for living bacteria for growth must be associated with some other property, possibly the accelerated reduction in the redox potential of the medium. To test this possibility tubes of medium with soluble starch as source of carbohydrate were pre-incubated anaerobically for 16 hr in the presence and absence of 4×10^8 *Escherichia coli*/ml. Two of the tubes that contained *E. coli* were then heated at 80° for 5 min. to kill the bacteria. 4×10^8 *E. coli*/ml. was then added to one of the heated tubes and also to one of the tubes pre-incubated in the absence of *E. coli*. At the same time fresh tubes of medium with soluble starch as source of carbohydrate were set up in the presence and absence of 4×10^8 *E. coli*/ml. and all the tubes inoculated with 2.3×10^4 protozoa/ml. These tubes were gassed with a mixture of 95 % (v/v) N_2 + 5 % (v/v) CO_2 and sealed. After 24 hr the number of protozoa had increased by 45 % in all tubes that on protozoal inoculation contained live *E. coli*, by 10–15 % in tubes pre-incubated with bacteria, then heated and inoculated in the absence of viable *E. coli* and by 2–6 % in tubes incubated (whether pre-incubated in the absence of bacteria or not) without added *E. coli*. The addition of the redox indicator resazurin (0.0001 %) showed that although the indicator was reduced in all tubes, in the tubes with living bacteria it reduced more rapidly and re-oxidized more slowly on exposure to air than in those which contained heated or no *E. coli*. These results suggest that a low redox potential was important for protozoal growth in the given conditions.

Effect of addition of Escherichia coli after 48 hr. Protozoal cultures incubated without added bacteria contained about 50×10^7 viable bacteria/ml. after 48 hr. It was therefore of interest to measure the rate of disappearance of a bacterium which did not grow under these conditions, namely *Escherichia coli*, in order to obtain an estimate of the rate at which other bacteria were being engulfed. In one experiment 49×10^7 *E. coli* organisms/ml. were added at 48 hr to a culture that contained 1.87×10^4 protozoa and 60×10^7 bacteria/ml. After 6 hr and 25 hr the numbers of viable

E. coli organisms were 19×10^7 and $< 10^6$ /ml., respectively and the number of other bacteria were 50×10^7 /ml. after 25 hr. The number of viable *E. coli* organisms in control tubes incubated without protozoa for 25 hr decreased by less than 15%.

DISCUSSION

The results reported show that the engulfment and digestion of bacteria by *Entodinium caudatum* is a more important process than was previously thought and that the protozoa engulf most bacteria with which they come in contact. Theoretically, when the protozoa are offered *Escherichia coli* and, for example, a Bacterium X which was ten times the size of *E. coli*, Bacterium X would take up the space of ten *E. coli* organisms in the gastric sac and the presence of one Bacterium X to ten *E. coli* organisms in the medium would decrease the uptake of *E. coli* to 50% if neither bacterium were taken up preferentially. Since the efficiency of a bacterium in decreasing the total uptake of *E. coli* was found experimentally to be proportional to its size, this suggests that *Entodinium caudatum* engulfed different bacteria in the proportion in which they were present in the medium. It is of interest that *Streptococcus bovis*, Bacterium D and Bacterium 31, which were isolated from the sheep rumen or from protozoal cultures, were not engulfed preferentially to organisms isolated from other habitats.

Although several species of bacteria stimulated the growth of protozoa more than the bacteria which grew from those associated with the protozoal inoculum, it is not claimed that under these conditions, *Escherichia coli*, for example, was the only bacterium taken up. It seems certain that the other bacteria growing in the medium were also engulfed and it is hoped that the results obtained with *E. coli* are representative of what occurred with other bacteria. The rapid disappearance of *E. coli* added to growing cultures after incubation for 48 hr suggests that other bacteria were also being rapidly engulfed and that the number present represents a balance between multiplication of the bacteria and their engulfment by the protozoa. Although this stimulatory effect occurred only with living organisms it was obtained with micro-organisms of widely differing properties, namely a short Gram-negative aerobic nutritionally non-exacting rod (*E. coli*), a large Gram-positive anaerobic nutritionally-exacting rod (*Clostridium welchii*) and a yeast. This suggested that one or more of the following three factors may be important. Since the protozoa require a low redox potential in the medium for survival and growth, all the micro-organisms when alive would probably reduce the potential below that obtainable by the use of cysteine. Secondly, if the protozoa require solid nucleic acid and protein particles in order to obtain a sufficiently high concentration of purines, pyrimidines and amino acids in the gastric sac for nucleic acid and protein synthesis, then living or dead bacteria could supply this requirement. This may be of particular importance in the rumen where the concentration of free amino acids is low but the concentration of bacteria high. Thirdly, either some low molecular weight compounds normally found in the metabolic 'pool' of bacteria or a bacterial enzyme may be required and both these would be lost on boiling. However, a requirement for any proteinaceous material is thought to be unlikely in view of the rapid digestion of protein which occurs in the gastric sac.

I thank Miss E. V. Holgate for her valuable technical assistance.

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