The Preparation and Survival of Almost Bacteria-free Suspensions of *Entodinium caudatum*

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(Received 23 August 1961)

SUMMARY

Suspensions of *Entodinium caudatum* containing less than one viable bacterium per ten protozoa were prepared by aerobic incubation of the protozoa with penicillin, streptomycin, dihydrostreptomycin and neomycin in the presence of autoclaved rumen fluid and rice starch grains. Unfortunately these almost axenic Entodinia could be maintained alive for only 3-4 days. Replacement of the rice starch grains in the medium used for bacterial removal by soluble starch produced almost bacteria-free protozoa which were devoid of food storage materials and died more rapidly than usual. The life of these protozoa was prolonged by adding any of the following: rice starch grains, soluble starch, maltose, glucose and some other sugars.

INTRODUCTION

Sheep rumen Entodiniomorphid (*Oligotrich sensu lato* of Corliss, 1959) protozoa, principally *Entodinium caudatum*, have been maintained dividing every 2 days in the presence of bacteria for over 18 months (Coleman, 1960*a*). There are two reports in the literature of successful attempts to prepare axenic Entodiniomorphid protozoa (Sugden, 1953; Abou Akkada & Howard, 1960), but in each case the demonstration that bacteria were absent depended on the use of a single growth medium. Oxford (1958) treated *Epidinium ecaudatum* with penicillin and neomycin but was unable to obtain the protozoa free from bacteria. The present paper records a successful attempt to obtain almost bacteria-free protozoa, many media being used to test for the absence of bacteria. The protozoa were first incubated with antibiotics to decrease the number of bacteria and then the effects of various incubation conditions on the survival of these protozoa were studied.

METHODS

Source of protozoa. The original source of the protozoa was described previously (Coleman, 1958, 1960*a*) and when used they had been grown on rice starch, grass, autoclaved rumen fluid and chloramphenicol under condition C3 (Coleman, 1960*a*) for over a year. However, whilst most of the experiments described below were being carried out this routine procedure was simplified as follows. The protozoa were grown in 250 ml. centrifuge tubes containing 150 ml. medium and 30 mg. rice starch and about 50 mg. dried grass were added each day. Twice a week two-thirds of the supernatant fluid was sucked off as quickly as possible and replaced by fresh medium (salt solution; Coleman, 1958) to which was added per 100 ml.: 10 ml. auto-

claved rumen fluid (ARF), 5 mg. chloramphenicol, 25 mg. rice starch and about 100 mg. dried grass). After mixing, the culture was divided into two, and one half was made up to the original volume with fresh medium and continued as the stock culture. The other half was combined with a similar half from another culture tube and placed in two 100 ml. centrifuge tubes (to facilitate subsequent manipulations) to form an 'inoculum culture'. These culture tubes were treated each day with 22 mg. rice starch and about 20 mg. dried grass and used on the third day as the source of protozoa. Under these conditions the protozoa formed a white layer at the bottom of the tube and the grass remained as a scum on the surface.

Treatment to remove bacteria from protozoal suspensions. The standard procedure finally adopted is as follows. The medium consisted of 3 ml. salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) in a cotton plugged 5 in. $\times$ $\frac{1}{2}$ in. test 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 with NaOH and Seitz-filtered just before use), 0.2 ml. 5 % (w/v) NaHCO₃, 0.1 ml. 6 % (w/v) rice starch (British Drug Houses Ltd., Poole, heated at 120° for 24 hr.), 0.4 ml. penicillin G (25,000 units/ml.), 0.4 ml. 1 % (w/v) streptomycin sulphate solution, 0.4 ml. 1 % (w/v) dihydrostreptomycin sulphate solution, 0.4 ml. 1 % (w/v) neomycin sulphate solution, and 0.8 ml. protozoa-containing autoclaved rumen fluid (PARF). The antibiotics were dissolved in salt solution B. Where larger quantities of 'bacteria-free' protozoa were required, as in the experiments on the subsequent survival of these protozoa, five times the above quantities were taken in 15 cm. $\times$ 2.2 cm. tubes.

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 a suitable test tube for 2 min. until any grass present had sunk to the bottom. The supernatant fluid containing the protozoa was transferred to centrifuge tubes, the residual grass washed once with salt solution B and then the protozoa washed five times in salt solution B which contained 0.3 % (w/v) yeast extract and 0.03 % (w/v) L-cysteine on an angle-head centrifuge at 400 *g* for 1 min. They were finally inoculated at a population density of 30,000–80,000 protozoa/ml. incubation medium, the culture well mixed and incubated aerobically at 39° for 3 days.

Incubation conditions for 'bacteria-free' protozoa. Under standard conditions the medium consisted of 1–4 ml. (adjusted so that the final volume was 7.6 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 0.9 ml. water and 0.4 ml. 0.05 M-DL-*p*-fluorophenylalanine in a 5 in. $\times$ $\frac{1}{2}$ in. cotton-plugged test tube or 7.5 ml. tube (fitted with a ground glass stopper). 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), 0.1 ml. 6 % (w/v) rice starch, 0.4 ml. penicillin G (25,000 units/ml.), 0.4 ml. 1 % (w/v) streptomycin sulphate solution, 0.4 ml. 1 % (w/v) neomycin sulphate solution, 1.0 ml. Seitz-filtered rumen fluid and any other additions. The 5 in. $\times$ $\frac{1}{2}$ in. test tubes were gassed with 95 % (v/v) N₂ + 5 % (v/v) CO₂ for 1½ min. and then sealed with a sterile rubber bung. Except where stated, all experiments were carried out in these tubes which were inoculated by opening momentarily and then regassing with 95 % (v/v) N₂ + 5 % (v/v) CO₂. The 7.5 ml. glass-stoppered tubes were inoculated before completely filling with sterile salt solution B and sealing with a flamed glass stopper.

The protozoa for inoculation were prepared from the bacterial removal (or first) incubation medium by sucking off 80–90 % of the supernatant fluid (this removed no protozoa) and washing the sedimented protozoa twice on the centrifuge with salt solution B plus 0.03 % (w/v) L-cysteine solution.

Sterile rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay or 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 subsequently centrifuged at 500 g for 3 min. before autoclaving. 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. Autoclaved rumen bacteria fraction was the pellet from the above 12,000 g centrifugation, washed once on the centrifuge in salt solution B, and made up to the original volume before autoclaving. Seitz-filtered rumen fluid was prepared as autoclaved supernatant rumen fluid except that sterilization was effected by Seitz-filtration. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of distintegration were counted.

All gases were freed from oxygen by the method of Stone & Beeson (1936).

Bacterial culture media. Medium A contained (g./100 ml.): K_2HPO_4 , 0.50; KH_2PO_4 , 0.39; NaCl, 0.050; $MgSO_4 \cdot 7H_2O$, 0.007; $CaCl_2 \cdot 6H_2O$, 0.007; Difco yeast extract, 0.5; Difco Bacto-tryptose, 0.5; glucose, 0.5; sodium lactate, 0.35; 4 ml. PARF (centrifuged to remove solid matter) autoclaved at 115° for 20 min. Medium A was always incubated aerobically in cotton-plugged tubes.

Medium B was Oxoid (Oxo Ltd., London, E.C. 4) thioglycollate (fluid) medium (fluid thioglycollate medium U.S.P.) to which was added 10 % (v/v) PARF (centrifuged to remove solid matter) dispensed in 5 ml. quantities in 5 in. $\times$ $\frac{1}{2}$ in. tubes. After inoculation a half-inch layer of sterile liquid paraffin was run on the surface of the medium and the tubes incubated in an anaerobe jar under 95 % (v/v) H_2 + 5 % (v/v) CO_2 .

Medium C was based on that of Bryant & Robinson (1961) and contained per 100 ml.: 40 ml. PARF (centrifuged to remove solid matter); 0.5 g. agar (Davis Gelatine Co. Ltd., London E.C. 3); 0.1 ml. 0.1 % resazurin; 3.75 ml. each mineral salt solutions no. 1 and no. 2 (Bryant & Burkey, 1953); 0.025 g. glucose; 0.025 g. cellobiose; 0.05 g. soluble starch; water to 100 ml. This was autoclaved (115° for 20 min.) in 5 ml. quantities in 5 in. $\times$ $\frac{1}{2}$ in. tubes and after cooling to 50° 0.25 ml. sterile 8 % (w/v) Na_2CO_3 and 0.25 ml. of a mixture of 0.5 % (w/v) L-cysteine and 0.5 % (w/v) $Na_2S \cdot 9H_2O$ (autoclaved together under N_2 after adjustment to pH 11) were added and the medium gassed with CO_2 for 1 $\frac{1}{2}$ min.

Bacterial viable counts. The standard technique for determining the number of viable bacteria in 'bacteria-free' protozoal suspensions treated under standard conditions was as follows: 0.1 ml. of a well mixed suspension was added to 5 ml. medium B and then 0.05 ml. of this diluted suspension inoculated into media A, B and C followed by two serial tenfold dilutions in media A and B. Each colony in medium C represented 10^3 bacteria/ml. of the original suspension. Where more bacteria were present serial dilutions were made in medium B before inoculation

into media A and C. None of the media maintained the inoculated living protozoa which disintegrated and liberated their ingested bacteria.

All cultures were incubated at 36° for at least 10 days.

RESULTS

Suspensions of *Entodinium caudatum* (10^4 – 10^5 protozoa/ml.) prepared from growing cultures as described above by washing five times still contained over 10^6 bacteria/ml., excluding those in the gastric sac. Breakage of the protozoa in a Potter homogenizer to liberate these internal bacteria increased the bacterial count 10–100 times on all media. It was therefore necessary to remove most of these internal and external bacteria before it was possible to study the survival and metabolism of the protozoa. A maximum limit of one viable bacterium per ten intact protozoa was arbitrarily chosen; any experiments in which the 'bacteria-free' protozoa contained more bacteria than this were discarded. To demonstrate that the bacteria had been removed it was first essential to grow all the bacteria present; some experiments on the production of media able to grow the bacteria in antibiotic-treated protozoal suspensions are described below.

Media for bacterial viable counts

Preliminary experiments on the development of media A, B and C to give the highest bacterial viable counts were carried out on crude rumen contents and on penicillin-treated protozoa (Coleman, 1960*b*). Subsequent experiments on protozoal cultures treated under standard conditions confirmed that media A, B and C gave higher results than other media tested.

For an aerobic viable count medium A grew 10^8 bacteria/ml. crude rumen fluid and 10^7 bacteria/ml. of growing protozoal cultures. Medium A grew more bacteria from antibiotic-treated protozoal suspensions than did medium B, Oxoid thioglycollate medium (Brewer), Oxoid thioglycolate (fluid) medium, Oxoid reinforced clostridial medium or glucose peptone water incubated aerobically. The addition of 0.05 % (w/v) L-cysteine to medium A and incubation in an anaerobe jar under 95 % (v/v) H_2 + 5 % (v/v) CO_2 decreased bacterial count. Solidification of medium A with 1 % (w/v) Davis agar and incubation aerobically in plates produced no colonies although liquid medium inoculated with the same inoculum grew well. Growth in medium A always began on the surface and sometimes spread throughout the medium.

Media B and C were used routinely for the growth of anaerobes. They grew respectively 10^8 – 10^9 and 10^9 – 10^{10} bacteria/ml. crude rumen contents and 10^7 – 10^8 and 10^8 – 10^9 bacteria/ml. growing protozoal cultures. Omission of PARF or agar from medium B decreased the bacterial count in penicillin and streptomycin-treated suspensions from, for example, 10^5 to 10^2 . The number of colonies produced on medium B solidified with agar was at best 25–50 % of those in medium C and they were almost too small to count under a lens. Medium B grew more bacteria than Oxoid reinforced clostridial medium; neither medium was improved by the addition of 0.1 % (w/v) maltose, cellobiose or 0.5 % (w/v) sodium lactate.

There was always a 24–36 hr. lag before there was visible bacterial growth on any

medium after inoculation from antibiotic-treated suspensions although rumen fluid inoculations grew overnight. All viable count cultures were incubated for 10 days because there was occasionally a 7-day lag before growth began.

Preparation of almost bacteria-free Entodinia

Table 1 shows that to obtain protozoa probably contaminated with less than 10 % of their number of bacteria it was necessary to incubate aerobically in the presence of penicillin + streptomycin + dihydrostreptomycin + neomycin. The addition of dried grass and yeast extract increased the number of surviving protozoa when penicillin (Coleman, 1960*b*) or penicillin + streptomycin were present, but with the four anti-

Table 1. *The effect of various antibiotics and of aerobic incubation conditions on protozoal survival and the removal of bacteria*

Experiment carried out either anaerobically in completely filled and sealed 7.5 ml. tubes or aerobically in cotton plugged 5 in. $\times$ $\frac{1}{2}$ in. test tubes containing 7.4 ml. liquid. The inoculum was prepared as described in the text and all tubes were incubated for 3 days. All the tubes contained salts, 0.03 % (w/v) L-cysteine, 0.15 % (w/v) NaHCO₃, 0.17 % (w/v) rice starch, 1400 units penicillin/ml. and 12 % (v/v) PARF. The numbers of viable bacteria are those that grew on media A, B and C respectively.

Aerobic or anaerobic	Additional antibiotics*	Protozoa/ml.	Bacteria/ml.
Initial		38,000	10 ⁶ , 10 ⁶ , 7 $\times$ 10 ⁶
Anaerobic	None	40,000	10 ⁵ , > 10 ⁶ , 10 $\times$ 10 ⁷
Anaerobic	Str	40,000	10 ⁴ , 10 ⁴ , 10 ⁶
Aerobic	Str	26,000	10 ³ , 10 ³ , 20 $\times$ 10 ⁵
Aerobic	Str, Dhs, Neo	12,000	10 ³ , 10 ³ , 1 $\times$ 10 ³
Anaerobic	Str, Dhs, Neo	21,000	10 ⁴ , < 10 ³ , 80 $\times$ 10 ³
Aerobic	Neo	17,800	10 ³ , 10 ³ , 3 $\times$ 10 ³
Aerobic	Str, Neo	16,500	10 ³ , 10 ³ , 3 $\times$ 10 ³
Aerobic	Dhs, Neo	15,000	10 ³ , 10 ³ , 4 $\times$ 10 ³
Aerobic	Str, Dhs	27,000	10 ³ , 10 ⁴ , 250 $\times$ 10 ³
Aerobic	No penicillin	10,000	> 10 ⁶ , 10 ³ , ?

* Str = 570 μ g. streptomycin sulphate/ml.; Dhs = 570 μ g. dihydrostreptomycin sulphate/ml.; Neo = 570 μ g. neomycin sulphate/ml.

biotics together they only increased the number of bacteria. The omission of the antibiotics singly or in pairs showed that penicillin + neomycin were almost as effective as the four antibiotics together (Table 1). Sodium propionate was added routinely to media (in salt solution B) to suppress the growth of moulds (Mrak & Phaff, 1948).

To ensure adequate survival of the protozoa it was essential to add 12 % (v/v) PARF to the incubation medium (Table 2). Other rumen fluid fractions and lower or higher concentrations of PARF were less effective in producing the largest possible number of 'bacteria-free' protozoa.

Figure 1 shows a time course for incubation under aerobic and anaerobic conditions. Although in about half the experiments the same final result was obtained under both conditions, the anaerobic incubation was not used routinely since an extra day was required and frequently the aerobic bacterial count (medium A) was too high.

Since the number of viable bacteria was determined on suspensions of intact protozoa it was possible for the results obtained to be low, since each protozoan that

contained one or more bacteria would give rise to only one colony in the assay. However, breakage of the 'bacteria free' protozoa in a Potter homogenizer before diluting the suspension to determine the number of viable bacteria did not increase the number of bacteria that grew on media A or C. This shows, within the limits of the method, that if a protozoan contained bacteria it contained only one bacterium.

Table 2. *The effect of various rumen fluid fractions on protozoal survival and the removal of bacteria*

Experiment carried out in cotton plugged 5 in. $\times$ $\frac{1}{2}$ in. test tubes containing 7.4 ml. medium incubated aerobically for 3 days. All tubes contained the standard incubation medium used for bacterial removal except that the PARF was replaced by various rumen fluid fractions as indicated below. The numbers of viable bacteria are those that grew on media A, B and C respectively. The initial number of protozoa was 40,000/ml.

Rumen fluid fraction	Protozoa/ml.	Bacteria/ml.
None	2,900	10^4 , 10^3 , 10^3
3% (v/v) PARF	11,600	10^4 , 10^3 , $< 10^3$
12% (v/v) PARF	15,400	10^3 , 10^3 , $< 10^3$
30% (v/v) PARF	19,400	10^3 , $< 10^3$, 150×10^3
12% (v/v) Seitz-filtered rumen fluid	9,500	10^3 , $< 10^3$, $< 10^3$
12% (v/v) ARF	8,700	10^3 , 10^3 , 1×10^3
12% (v/v) autoclaved supernatant rumen fluid	11,600	10^4 , 10^3 , $< 10^3$

Fig. 1

Fig. 2

Fig. 1. Effect of incubation time on the survival of protozoa and the disappearance of bacteria during the incubation used for bacterial removal. $\circ$ — $\circ$, Incubation under aerobic conditions; $\times$ — $\times$, incubation under anaerobic conditions. The figures at each point refer to the number of bacteria (in thousands) that grew on media A, B and C respectively.

Fig. 2. Effect of starch concentration on the survival of protozoa under otherwise standard conditions during the incubation used for bacterial removal. Duration of experiment was 3 days and the initial number of protozoa 60,000/ml. $\circ$ — $\circ$, Soluble starch; $\times$ — $\times$, rice starch grains.

Replacement of the rice starch grains by soluble starch

For these experiments protozoa, the gastric sacs of which contained only a few rice starch grains, were prepared by the addition of only 5 mg. rice starch to the inoculum cultures on the day before that on which the protozoa were required.

Figure 2 shows that in the absence of starch less than 5 % of the protozoa survived after 3 days and that the addition of up to 1.6 % soluble starch linearly increased the number of survivors. Although ten times more soluble starch than rice starch grains was required to obtain the same survival, the maximum number was double that in the presence of rice starch grains. Unfortunately in half the experiments 10^4 – 10^5 bacteria/ml. survived in the presence of soluble starch. In contrast to the protozoa incubated with 0.04–0.08 % (w/v) rice starch grains or 1.6–3.2 % (w/v) soluble starch, those which survived in the presence of 0–0.8 % (w/v) soluble starch contained no material which stained blue with iodine. These latter protozoa when incubated under the second incubation conditions in the absence of carbohydrate died more quickly than normal protozoa full of rice starch grains and the effect of carbohydrates on their survival could be studied.

The survival of bacteria-free Entodinia

It did not prove possible to maintain the normal 'bacteria-free' protozoa alive for longer than 3–4 days; the numbers steadily declined throughout this period. Nevertheless, various factors influenced the rate at which the organisms died. To obtain maximum survival of the protozoa it was necessary to decrease to a minimum the manipulations of the organisms after removal from the first incubation medium. The best results were obtained by sucking off the supernatant fluid from the first incubation and inoculating the residual protozoa directly into new medium. As the object of the work was to study the effect of various substances on the survival and metabolism of the protozoa, these were usually washed twice before inoculation. Under these conditions damage to the organisms, as measured by the decrease in the number of living protozoa over the first 6 hr., was 20 % as compared with 10 % for unwashed protozoa; subsequently the rate of decrease was the same for both cultures.

The lowest effective concentration of L-cysteine was 0.03 % (w/v). Omission of the L-cysteine decreased the number of survivors after 24 hr. by over 60 % and an increase in concentration to 0.15 % (w/v) had no additional effect.

Omission of the antibiotics singly or all together did not usually result in bacterial growth during the first 24 hr., but the number of protozoa decreased by 5–20 %. DL-*p*-Fluorophenylalanine (2.6×10^{-3} M) was routinely used in this second incubation medium to suppress the growth of bacteria which subsequently grew specifically on medium C. In the absence of *p*-fluorophenylalanine these bacteria were found in about half the experiments where nutrient materials were added in an attempt to stimulate the protozoa. *p*-Fluorophenylalanine in concentrations up to 0.005 M had no effect on the survival of the protozoa whether bacteria grew in its absence or not.

Effect of CO₂ and rumen fluid fractions. Seitz-filtered rumen fluid was more effective than other rumen fluid fractions tested in maintaining 'bacteria-free' protozoa alive (Table 3). The survival in the absence of Seitz-filtered rumen fluid was variable when experiments were carried out in sealed tubes or under 100 % N₂ but more consistent results were obtained with 5 % (v/v) CO₂ in the gas phase. In those experiments in sealed tubes with poor survival in the absence of Seitz-filtered rumen fluid, 5 % (v/v) CO₂ was stimulatory in the presence of 0–14 % (v/v) but not with 42 % (v/v) Seitz-filtered rumen fluid. An increase in the CO₂ to 100 % (plus

the addition of 0.4 % (w/v) Na_2CO_3 to maintain the pH value constant) had no further stimulatory effect (Table 3). The addition of 0.14 % (w/v) NaHCO_3 to the sealed tube experiments gave similar results to gassing the medium with 95 % (v/v) $\text{N}_2 + 5$ % (v/v) CO_2 .

Table 3. *The effect of carbon dioxide and rumen fluid fractions on protozoal survival during the second incubation*

Experiment carried out either anaerobically in 5 in. $\times$ $\frac{1}{2}$ in. test tubes containing 7.4 ml. medium under the appropriate gas or in completely filled and sealed tubes containing 7.5 ml. medium incubated for 24 hr. The standard inoculum and medium was used except where the carbon dioxide tension or rumen fluid fraction was varied. Where 100 % CO_2 was present, 0.4 % (w/v) Na_2CO_3 was added to the medium.

CO_2 added	Rumen fluid fraction	Number of protozoa relative to initial = 100
None*	None	20
None*	14 % (v/v) Seitz-filtered	36
None*	42 % (v/v) Seitz-filtered	50
5 %†	None	42
5 %†	14 % (v/v) Seitz-filtered	65
5 %†	42 % (v/v) Seitz-filtered	49
100 %	None	40
100 %	14 % (v/v) Seitz-filtered	68
5 %†	14 % (v/v) PARF	54
5 %†	14 % (v/v) ARF	52
5 %†	14 % (v/v) Autoclaved supernatant	56
5 %†	14 % (v/v) Autoclaved rumen bacteria	40

* Experiment carried out in completely filled and sealed tubes.

† +95 % (v/v) N_2 .

Although Abou Akkada & Howard (1960) found that salt solutions buffered only with phosphate were harmful to their *Entodinia* no such effect was noticed in the present studies. Under otherwise standard conditions salt solution B was 10–20 % more effective in the maintenance of the protozoa than was the same solution when the sodium propionate was omitted and the concentration of the other constituents increased to maintain the osmotic pressure, or when propionate was replaced by an equimolar amount of sodium acetate or by 0.1 % (w/v) NaHCO_3 . Similar results were obtained with growing protozoa (Coleman, 1958, 1960*a*) where sodium acetate could be omitted without harming the protozoa.

Effect of various additives on protozoal survival. None of the following substances at the concentrations indicated prolonged the life of the protozoa incubated under standard conditions: 0.03–0.15 % (v/v) agar (Davis); 0.0005 M- NH_4Cl ; 0.3 % (w/v) Bacto-peptone (Difco); 0.3 % (w/v) Bacto-tryptose; a mixture of 0.1–100 $\mu\text{g./ml.}$ each of *isobutyric* acid, *valeric* acid and *isovaleric* acid (found by Wegner & Foster, 1960, to stimulate certain rumen bacteria); 0.3 % (w/v) Casamino acids (Difco); 0.1 % (w/v) casein; 0.14 % (w/v) cellobiose; 0.001–0.01 % (w/v) cholesterol; 0.14 % (w/v) glucose; a mixture of eleven growth factors (0.1–0.5 $\mu\text{g./ml.}$ each of *p*-aminobenzoic acid, β -alanine, biotin, calcium pantothenate, folic acid, nicotinic acid, nicotinamide, pyridoxin, riboflavin, thiamine, vitamin B_{12}); 0.01 % (w/v) haematin; 5 % (v/v) horse serum; 0.005 M-DL-lactate; 0.3 % (w/v) malt extract;

0.14 % (w/v) maltose; 1 % (v/v) milk (fresh); 0.3 % Tryptone; 0.01–0.1 % (w/v) Tween 80; 7–20 % (v/v) tomato juice; 0.003–0.3 % (w/v) yeast extract (Difco or Oxoid).

Replacement of rice starch grains by other carbohydrates. For these experiments the protozoa were prepared under standard conditions except that on the day before

Table 4. *The effect of various carbohydrates on the survival of 'bacteria-free' Entodinia*

Experiment carried out in sealed 5 in. $\times$ $\frac{1}{2}$ in. test tubes under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in the presence of 13 % (v/v) Seitz-filtered rumen fluid. The protozoa differed from those used in other experiments in that they were rendered bacteria-free by incubation in a medium containing soluble starch instead of rice starch and contained no iodine-staining intracellular material. For exact conditions see text.

Carbohydrate	Number of survivors after 24 hr.*	Carbohydrate	Number of survivors after 24 hr.*
0.3–1.0 % Soluble starch	1.00	1.3 % Sorbose	0
0.08 % Rice starch	0.4–0.8	1.3 % Xylose	0.5
1.0 % Amylose	0.8	1.3 % Sorbitol	0.2
1.0 % Amylopectin	0.9	1.6 % Cellobiose	0.3
0.4 % Inulin	0.05	1.3 % Lactose	0.1
0.4 % Dextrin	0.5	1.6 % Maltose	0.7–1.0
1.3 % Arabinose	0.1	1.3 % Melibiose	0.5
1.6 % Fructose	0.2	1.6 % Sucrose	0.6
1.6 % Galactose	–0.6	1.3 % Salicin	0
1.6 % Glucose	0.5–0.9	1.3 % Raffinose	0.1
1.6 % Mannose	0.1		

* The results are quoted as (survivors in presence of carbohydrate – survivors in absence of carbohydrate)/(survivors in presence of soluble starch – survivors in absence of carbohydrate).

Fig. 3. Effect of various carbohydrates on the survival of starved 'bacteria-free' protozoa. Δ , no added carbohydrate; $\times$, 0.8 % (w/v) soluble starch; $\bullet$, 0.08 % (w/v) rice starch grains; $\circ$, 1.6 % (w/v) maltose; $\blacktriangle$, 1.6 % (w/v) glucose.

inoculation of the first incubation medium only 5 mg. rice starch was added to each inoculum culture and the rice starch grains in the first incubation were replaced by 0.2 g. soluble starch in each 15×2.2 cm. tube. These protozoa died at 2–10 times the usual rate when incubated in the absence of carbohydrate and contained no material which stained blue with iodine. The rate at which the protozoa died was decreased by adding several carbohydrates of which soluble starch was the most effective, followed by maltose and glucose; fructose and the fructose polysaccharide inulin were relatively ineffective (Table 4). There was considerable variation between experiments in the relative effectiveness of these various materials; the figures quoted are averages. Figure 3 shows that the stimulatory effect also varies with time.

DISCUSSION

As far as the author is aware the present work represents the first successful attempt to prepare bacteria-free *Entodinia* where several media designed to detect rumen bacteria have been used. There is disagreement over the best type of medium for the growth of rumen bacteria. Wilson & Briggs (1955) reported that a rich broth (reinforced clostridial medium) gave the best results of nine media tested, whereas Bryant & Burkey (1953) favoured a poor medium containing a high concentration of autoclaved rumen fluid. Media based on these two types have been tested and the high rumen fluid medium gave the highest results from antibiotic-treated suspensions; King & Smith (1955) obtained similar results with fresh rumen contents. In view of the present findings, it is considered that the two reports of bacteria-free *Entodinium* spp. in the literature are of doubtful validity. Abou Akkada & Howard (1960) treated *Entodinium caudatum* suspensions with chloramphenicol and found no bacteria able to grow in a semi-solid thioglycollate starch medium. This medium is similar, except that glucose was replaced by starch, to the fluid thioglycollate medium used in the present experiments, and in penicillin + streptomycin treated cultures counts of 10^6 bacteria/ml. were obtained on medium C and $0-10^2$ /ml. on the fluid thioglycollate medium. It is essential to carry out all tests for viable bacteria on at least one aerobic and one anaerobic medium and that various standard media should be tested to enable the best to be selected.

The absence of any stimulatory effect of ordinary culture medium constituents on 'bacteria-free' protozoa under standard conditions is in contrast to the results reported previously (Coleman, 1960*b*) where penicillin-treated *Entodinia* were stimulated by the addition of yeast extract and dried grass. These cultures are now known to have contained more bacteria (Table 1) than at first reported and it is likely that the effect of these additives was indirect and was the result of bacterial metabolism.

In previous experiments (Coleman, 1958, 1960*a*) carried out in the presence of bacteria the only source of carbohydrate that produced growth and division was intact rice starch grains. It is likely that only by the use of a solid food material, which was engulfed by the protozoa, was it possible to supply utilizable carbohydrate without extensive bacterial breakdown and acid production before assimilation by the protozoa. In the present experiments where starved 'bacteria-free' protozoa have been produced, an effect of soluble starch and, more important, of glucose and maltose on the survival of the protozoa was found for the first time. The apparent inability of *Entodinium* spp. to utilize soluble carbohydrates has for long

been in contrast to the rapid utilization of sugars, especially glucose, by Holotrich protozoa (Oxford, 1951, 1955).

I wish to thank Mr R. B. Taylor, who fistulated the sheep used in this work; Dr M. P. Bryant for giving me the details of his improved culture medium before publications, and Miss J. M. How and Miss J. G. Pearson for their valuable technical assistance.

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