Laboratory Cultivation of Some Human Parasitic Amoebae

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SUMMARY

Cultural conditions and supplementary substances necessary for the laboratory cultivation of some human parasitic amoebae were investigated by using a basal solution containing inorganic salts, citrate and lactate. Three supplementary components were found necessary: starch grains, animal protein (soluble or insoluble) and living bacteria. Restraint of bacterial growth by antibiotics, and of the development of an alkaline reaction (due to pellicle-forming aerobes) by carbon dioxide, improved the amoebic growth. Under these conditions, all the common parasitic amoebae examined, except *Iodamoeba butschlii*, were grown and maintained for long periods in laboratory culture.

INTRODUCTION

This work began with the observation of *Entamoeba histolytica* trophozoites in a culture after incubation for 24 hr at 37° of cyst-containing faeces suspended in a simple defined medium suitable for growing *Escherichia coli* (Robinson, 1951). A method for cultivating the parasitic amoebae was then developed in which a liquid culture of *E. coli* in this defined medium + starch + erythromycin was overlaid on a slope of saline agar in a quarter-ounce screw-capped bottle (gas-phase air) and inoculated with cyst-containing faeces (of normal bacterial content). By this process *E. histolytica* was grown in primary culture and in the first subculture, while other amoebic species occasionally appeared in recognizable numbers. But even when normal (i.e. amoeba-free) faeces was added, further amoebic subcultures did not grow. It was decided to examine the nature of the supplements which were needed to obtain continuous subculture of amoebae in *E. coli* cultures in the defined medium + starch + erythromycin overlaid on saline agar.

METHODS

Saline agar slopes. Agar 1.5% (w/v) + NaCl 0.7% (w/v) in water was distributed in 2.5 ml. volumes in quarter-ounce screw-capped glass bottles and sloped after autoclaving.

Antibiotics. Solutions of erythromycin, ristocetin and streptomycin (0.5%, w/v), chloramphenicol (0.2%, w/v) and polymyxin (5000 units/ml.) were used.

Rice starch. Rice powder (British Drug Houses Ltd.) after drying was dry-sterilized in partly filled quarter-ounce bottles with caps screwed tight.

Phthalate (0.05 M) diluent. Potassium hydrogen phthalate 10.02 g. and sodium hydroxide 2 g. were dissolved in 1 l. water, adjusted to pH 6.5 and the solution autoclaved.

Defined medium R for growing *Escherichia coli*. Concentrated stock solution consisted of 125 g. sodium chloride, 50 g. citric acid monohydrate, 12.5 g. potassium

dihydrogen phosphate, 25 g. ammonium sulphate, 1.25 g. magnesium sulphate heptahydrate and 100 ml. lactic acid (British Drug Houses Ltd., 90.08 %) in 2.5 l. water. For R medium, one volume of concentrated stock solution was diluted with nine volumes of 0.33 % (w/v) sodium hydroxide, adjusted to pH 7 and autoclaved. Stock more than 4 weeks old was used to avoid pH change on autoclaving.

Basal amoebic medium BR. *Escherichia coli* strain B was incubated for 48 hr at 37° in shallow layers of medium R in sealed flat bottles; this living culture was the basal medium BR.

Supplemented media for amoebic growth. The general method of preparing supplemented media was to boil a proposed supplement in medium R, filter through Whatman no. 1 paper, autoclave the filtrate at pH 7, inoculate with *Escherichia coli* B, incubate at 37° in shallow layers for 48 hr and store the living culture at room temperature until required. Media with 70 different complex supplements were thus prepared; the main supplements used are indicated in Table 1; the preparation of four are described below:

Medium BRS (serum added). Equal volumes of medium BR and Seitz-filtered heated (56°) serum (sheep) were mixed and incubated for 48 hr at 37°. Sheep serum was better than any other.

Medium BRMt (serum metaprotein added). Metaprotein (Cole, 1919) was prepared to exclude soluble (at pH 7) material, by sulphosalicylic acid precipitation of human serum. The precipitate was washed with water, the suspension boiled, dissolved in NaOH, filtered, re-precipitated by acetic acid and the precipitate washed for 4 days, and autoclaved as an aqueous cream. For use, 5 ml. was added to 100 ml. medium R, (adjusted to pH 7) steamed, inoculated with *Escherichia coli* B and incubated.

Medium BRT (tripe extract added). Tripe (500 g.) was sliced into 1 cm. cubes, boiled with 1 l. medium R for 4 hr on 3 successive days, filtered through lint, the extract autoclaved, inoculated with *Escherichia coli* B and incubated.

Medium BRG (gastric mucin added). Gastric mucin (Armour) was triturated in medium R to give 1 or 2 % (w/v), autoclaved, inoculated with *Escherichia coli* B and incubated.

The inocula of amoebae used and the maintenance of the amoebic cultures. Except for two strains of *Entamoeba histolytica*, all the amoebae used were derived from the faeces of patients and were subcultured two or three times weekly in phthalate dilutions (1/2 to 1/10) of medium BRS and medium BRT, containing antibiotic (usually erythromycin) and starch, as a liquid overlay on the saline agar slopes. Bactopeptone was often added to about 0.2 %. All cultures were cleared of *Blastocystis* and in later work *Pseudomonas aeruginosa*.

Method for removal of Blastocystis. Culture sediment (0.4 ml.) was allowed to settle for 2 min. in 4 ml. of 0.1 N-HCl, the fluid removed and the remaining settled fragments taken up in a few ml. of liquid overlay from the culture to be inoculated (Smedley, 1956).

Methods for antagonizing bacteria in amoebic cultures. These methods were required for two purposes: (a) the elimination of bacterial species, (b) the slowing of multiplication of a mixed flora on which the present medium is based. The interactions of the bacterial species in these amoebic cultures were too complex for their control to be anything but empirical; no transfer of trophozoites (as opposed to cysts) was possible in the absence of living bacteria in the inoculum (apart from the presence of *Escherichia coli* in the culture medium). For purpose (a) four or five successive amoebic sub-

cultures were made in medium diluted to the maximum degree (by medium R or phthalate solution) which permitted the amoebae to grow at all, and containing the appropriate antibiotic. For total elimination of bacteria a prolonged series of tests on successive amoebic subcultures was needed to be sure that an apparently eliminated bacterial species had not become latent. For purpose (b) continuous dosage with antibiotic (usually erythromycin 10–200 $\mu\text{g./ml.}$) was added to the medium. When bacterial aerobes increased, to the detriment of amoebic growth (see later), a stream of carbon dioxide was used to displace most of the air above the culture for a few seconds before screwing up the cap of a bottle (this process was also used with polymyxin for elimination of *Pseudomonas aeruginosa*). Escape from antibiotic control, i.e. development of resistance in the bacterial flora of amoebic cultures, was the commonest cause of the dying out of amoebae kept for long periods as stock strains. This occurred more commonly when chloramphenicol or penicillin was used than with erythromycin. It was corrected by dispersion of whole sediment from an amoebic culture in saline medium containing several antibiotics in high dosage. After settling for 30 min. the saline solution was replaced by amoebic culture medium + starch, the whole process (growth + treatment) being done, as often as necessary, in 9 ml. volume in a screw-capped one-ounce bottle without a saline agar slope.

Testing for amoebae in supplemented media. The various supplemented media (after *Escherichia coli* B had been grown in them) were diluted 1/2 to 1/10, usually with phthalate diluent, occasionally with medium R or medium BR, + starch + antibiotic solution, and overlaid on saline agar slopes at the time of inoculation with amoebae. Sediment from the first bottle was then transferred, after 1–4 days at 37°, to a second similar bottle, and so on, until a sufficient number of subcultures showed that the amoebae grew continuously in the given medium. Microscopic examination of wet preparations in iodine solution was the means of assessment; this gave information about the state of the starch, the bacteria, the degree of protein coagulation and the carbohydrate metabolism (colour in iodine) of the trophozoites, as well as their number, size and disposition (clumped or diffuse). Generally, number and size of trophozoites, indicating satisfactory growth, ran parallel (in small as well as large species) to iodine-staining of the cytoplasm, which itself constituted a criterion of good growth. From this information, variations and replications were made as the amoebic subculture series proceeded, to prolong it as far as possible.

CONCLUSIONS

Growth maxima from the main groups of supplemented media are shown in Table 1. Because different strains of the same amoebic species did not always give the same results on the same medium it was concluded that the effect of the supplement on the amoebic growth was because of the accompanying bacteria. This was supported by the observed lag in amoebic growth on first transferring a species from one supplemented medium to another, which suggested that adaptation of the bacterial flora was involved. It also accorded with the fact that continual adjustment of concentration and type of antibiotic was one of the principal means by which prolonged growth of the protozoal series was obtained with the less easily grown amoebic species and less effective supplemented media. Another means of affecting the bacterial flora, and so prolonging a series of amoebic subcultures, was by addition of some of the substances (particularly

Bacto-peptone and Lab-Lemco) shown at the end of Table 1 as incapable of supporting amoebic growth by themselves alone.

For *Entamoeba coli*, *Entamoeba hartmanni* and *Endolimax nana*, a single strongly growing strain of each was selected for the experiments. For *Dientamoeba fragilis*, two strains were used which, though mainly similar in behaviour on the various supplements, were different on medium BRG ('Newman' died after 53; 'Raja' after 17 subcultures) and with a mucoid material prepared from human colon (Newman 2, Raja 21 subcultures). For *Entamoeba histolytica*, 8 strains were used and discrepancies

Table 1. *Maximum numbers of positive serial subcultures obtained with different species of human parasitic amoebae with various supplements*

Figures with + indicate that amoebae were still growing when discarded;
figures without + indicate that amoebae died at this stage.

Source	Media and supplements, etc.	Protozoa				
		<i>Entamoeba histolytica</i>	<i>Ent. coli</i>	<i>Ent. hartmanni</i>	<i>Endolimax nana</i>	<i>Dientamoeba fragilis</i>
Serum	BRS; basal medium + serum (see Methods)	100 +	100 +	100 +	100 +	100 +
	BRMt; basal medium + metaprotein (see Methods)	57 +	40 +	20 +	63 +	11 +
	Extract of heat-coagulated human serum	30 +	14 +	16	14 +	14 +
	Same, Seitz-filtered	15 +	3	13	2	0
	Inspissated serum slope + phthalate extract overlay after BR	18 +	20 +	8 +	28 +	8 +
	Seromucoid (Winzler <i>et al.</i> 1948)	16 +	9 +	16 +	8 +	2
Colon	Human colonic mucoid purified (Hawk, 1923)	12 +	27	8	2	21
Tendon	Ox tendo-mucoid (Hawk, 1923)	10 +	5 +	2	5	0
Stomach	BRT (see Methods)	64 +	78 +	16 +	112 +	63 +
	Tripe mucoid (Hawk, 1923)	13 +	4	16	22 +	10
	BRG (see Methods)	50 +	49	40	41	53
Muscle	Extract of horse muscle	22 +	2	0	0	2
	NaOH extract of ox heart, pptd. by HCl, washed, used solid	15 +	1	22 +	4	13
	Filtrate from previous	2	0	1	1	1
Liver	Extract of human liver	14	1	2	2	4
Fish	Extract of tinned salmon	25 +	2	0	5	8
	Extract of fresh plaice	10	1	0	0	0
Hen eggs	Extract of hard-boiled eggs	16 +	12 +	12 +	4	17 +
Gelatin	Steamed at 2 % (w/v) in R	3	3	2	2	2
	As previous, but incubated with <i>Proteus mirabilis</i> (instead of <i>Escherichia coli</i>)	5	11	3	2	1
Yeastrel, Marmite, Lab-Lemco, Bacto-peptone, Proteose peptone, Bacto-casitone, Bacto-casamino-acids, asparagine, glucosamine, cholesterol, fish solubles, distiller's solubles, vegetable mucoid from <i>Plantago coronopus</i> , faeces and controls of BR without supplement.		< 3	< 3	< 3	< 3	< 3

occurred from the maxima recorded in Table 1: one died after 8 subcultures on inspissated serum, two after 3 with fibrinogen extract, one after 4 with horse-meat extract and two after 3 with plaice extract. Differences observed between species (e.g. *E. hartmanni* required more concentrated supplement and more varied antibiotic control of bacteria, than *E. histolytica*) were like those found between more and less fastidious strains of one amoebic species. This suggested that the nutritional requirements of the various parasitic amoebae differed in degree but not in kind. If it be assumed that the maximum amoebic survival (owing to traces of growth factor in the inoculum and in some of the cultures themselves) is three subcultures, then the results of Table 1 indicate that many bacterial nutrients (final group in Table 1) were incapable of supporting amoebic growth, but that protein supplements were able to do so. When protein was completely removed from any of the supplements amoebic growth did not occur. For example, fibrinogen was removed from solution as coagulum on autoclaving; the resulting clear solution did not support amoebic growth. But a cloudy suspension of fibrinogen, prepared by repeated heating, trituration and lint filtration, gave the amoebic growth maxima shown in Table 1. The clearest extracts shown in Table 1 were those from human liver, plaice and horse meat, which give poor amoebic growth maxima. The point was confirmed by showing that the removal of 'cloud' by the Seitz filter removed also the property of supporting amoebic growth, e.g. from medium BRG in Table 1, except in the case of seromucoid, which (unlike the tissue mucoids) passed the Seitz filter and was not coagulated by heat.

Table 2. *Insufficiency of either phosphotungstic precipitate (X: 1 ml.) or filtrate (Y: 1 ml.) of deproteinized mucoid-containing serum filtrate to support two successive cultures (a) and (b) of Entamoeba histolytica and Entamoeba coli, unless reinforced by extract (Z: 1 ml.) of heat-coagulated whole serum in R medium.*

Defined medium = R. Culture of *Escherichia coli* B in R = BR.

Bottle	Composition of 3 ml. overlay, with starch grains + erythromycin 20 µg./ml. on saline agar slope			Trophozoites/ml. after 3 days incubation			
				<i>Entamoeba histolytica</i>		<i>Entamoeba coli</i>	
				(a)	(b)	(a)	(b)
1	X	BR	R	10,000	0	0	0
2	Y	BR	R	0	0	0	0
3	X	Y	R	3,000	0	1,000	0
4	X	BR	Z	50,000	26,000	35,000	31,000
5	Y	BR	Z	75,000	16,000	30,000	13,000

An experiment with one strain of *Entamoeba histolytica* and one of *Entamoeba coli* showed that the efficiency of serum as growth factor was not simply due to its seromucoid content: when deproteinized serum filtrate containing seromucoid was separated by phosphotungstic acid into mucoid-containing precipitate incorporated in medium BR at about 0.1 % (w/v), as in serum, and into mucoid-free filtrate similarly incorporated (after dialysis) in medium BR, both supplements needed addition of cloudy filtrate of heat-coagulated whole serum boiled in medium R to restore amoebic growth in subculture (Table 2).

The suitability of mucoid supplement as amoebic growth supplement led to a test of

gastric mucin (Armour and Co.), with which (alone of the manufactured products tested) long runs of subcultures sometimes resulted. Continuously viable cultures of *Entamoeba histolytica* with centrifuged 0.05 % (w/v) commercial gastric mucin in saline were reported by Dolkart & Halpern (1958) who stated that their results were improved by adding small amounts of egg medium.

Once the need for protein for amoebic growth became clear, the question was: were proteolytic bacteria necessary to split it? Accordingly, the bacteria accompanying some of the *Entamoeba histolytica* strains were examined after the amoebae had grown. Anaerobic plates showed predominance of *Escherichia coli* but aerobically heavy growths of *Aerobacter*, *Achromobacter*, *Proteus* and *Pseudomonas* species occurred; *Streptococcus faecalis* was always present and *Clostridium* species absent. After elimination of the proteolytic *Proteus*, *Pseudomonas* and *Bacillus* species by the technique given above, serial amoebic subcultures were made on medium BRMt with parallel inoculations into nutrient gelatin at each transfer. Of 36 such cultures, most showed gelatin liquefaction after 3 days and all but two after 14 days. The organism responsible was found to be a proteolytic variety of *S. faecalis* which was impossible to eliminate; although it could be decreased apparently to zero for a time, it always reappeared in the cultures. Two *Entamoeba histolytica* strains were isolated monoxenically from faecal cysts by the method of Singh, Das & Saxena (1963) by using 24 hr instead of the recommended 48 hr in dilute HCl suspension and inoculation into overlays of BRS and BRT media with starch and erythromycin (10 µg./ml). Blastocystis (one strain) grew but was removed by sedimentation in HCl solution (above). Both these strains were successfully transferred to medium BRMt and gave runs of more than 8 serial subcultures, tested by parallel inoculations of aerobic and anaerobic plates at 37° and 25°, as well as by inoculation on nutrient gelatin. The plates showed *Escherichia coli* to be the only organism accompanying the amoebae; 30 gelatin controls did not liquefy in 14 days.

The finding that proteolytic bacteria were not essential for *Entamoeba histolytica* and, by inference, for the other strains used, supported the idea that no liquid amoebic growth factor was liberated by bacteria from protein. Attempts to grow amoebae by the diffusion of growth factor through agar layers or viscose sacs always failed.

At this point the microscopical appearance of the cultures became relevant, for it had been noticed that the growth of all amoebic species tended to be in foci, the individuals being clustered upon bacterial colonies. Plate 1, fig. 1, 2 give a cross-section of the trophozoites in such clusters. Over 100 *Endolimax nana* individuals were calculated to be present in the bunch shown in Pl. 1, fig. 2. The failure to find a soluble metabolite suggested that the trophozoites might be ingesting protein fragments directly, hence they appeared to be clumped on the bacterial colonies, but were really on the protein beneath. Microscopic study of the sediments from BRMt cultures left no doubt that this was not so: the trophozoites could be seen in contact with bacterial networks which had often formed around protein fragments; but to these fragments themselves no trophozoites were ever seen to be attached. Plate 1, fig 3 shows such an arrangement in the case of *Entamoeba coli*. Because the outline of the protein fragment was sacrificed in focusing the amoebae, a diagram of the positions in the microphotograph is given beside it. The conclusion of this part of the work was therefore that 'amoebic growth factor' was ingested by trophozoites in the form of protein-nourished bacterial bodies.

The gas phase in amoebic cultures

Experiment 1. For growth of my amoebae in the manner described above, it was not necessary to tighten the cap of the bottle; but growth did not occur in a shallow layer of supplemented BR medium + starch + antibiotic in an open container without a saline agar slope. This observation was investigated by serial subcultures of *Entamoeba histolytica* under varied physical conditions in 0.8 ml. volumes of medium BRS and medium BRG in 70 × 11 mm. tubes in a moist incubator. Figure 1 gives as a diagram the conditions and amoebic counts (at peak) with medium BRG. The results showed that continuous serial subcultures were possible under these conditions: (a) with 1.5 ml. saline agar slope; (b) with slope replaced by a glass rod of equal volume; (c) with bung in tube, but not under conditions (d) with open tube in standard bacteriological anaerobic jar (pure hydrogen); (e) with open tube in air. When the height of the liquid

Fig. 1. Growth of *Entamoeba histolytica* under five physical conditions in 70 × 11 mm. tubes, containing starch and 0.8 ml. volumes of medium BRG diluted 1/5 in R with erythromycin 20 µg./ml. The base line represents zero, the three dotted lines 100, 200 and 300 thousand trophozoites/ml. The vertical lines represent the trophozoite counts in successive cultures. Conditions: (a) with 1.5 ml. saline agar slope; (b) with 1.5 ml. glass rod; (c) with bung in tube; (d) with tube in hydrogen atmosphere of anaerobic jar; (e) with tube open to air.

column was increased in (d) and (e) to that of (a) and (b) by increasing the volume, growth still failed after a few subcultures in hydrogen (d), but succeeded in the tube open to air (e). Therefore, when open to air, the cultures showed a critical surface: height ratio, although they were not inhibited by air in the bunged tube (c). This finding was confirmed many times with various media in various vessels, and with various species of amoebae.

Experiment 2. Drops of amoebic cultures mixed on a tile with pH indicators showed very rapid alkaline increase suggestive of loss of CO₂. On releasing the bottle caps, bubbles rose from below the base of the slope, showing that gas had been retained under pressure. Abundance of CO₂ was shown in the cultures by passing a stream of hydrogen into lime water. For rough estimations, overlays from cultures in medium BRS were pooled, and 10 ml. titrated with 0.05 N-Na₂CO₃ from pH 7 to maximum colour with phenolphthalein (corresponding to excess Na₂CO₃); this took 23 ml.; a blank titration with medium BRS from pH 7 took 6 ml. Since 1 ml. of 0.05 N-Na₂CO₃ absorbs 0.0011 g. CO₂, this gives 1.87 g. CO₂/l. culture, if all the acidity in this range were due to CO₂.

Experiment 3. Other gas-phase experiments were done with liquid BRS + starch cultures in 8 × 2.5 cm. one-ounce glass bottles closed by screw caps when required (incubation in a water bath at 37°). Bubbling gases through these cultures was un-

successful, and gas was therefore passed through a reservoir to lessen pressure changes, to emerge from a nozzle 2 cm. above the level of medium in an uncapped bottle. Each run of 4 days comprised four bottles containing equal amounts of medium, starch, antibiotic when needed, and inoculum. Two bottles received gases; the third was capped and the fourth uncapped as control. Amoebae were counted daily in a haemocytometer. The gas flow was kept at about 1 l./hr. Culture volumes were restored daily by adding water. These experiments were to compare the effects of carbon dioxide and of oxygen as shown in Fig. 2. The following results were obtained with three runs.

Fig. 2. Diagram of Expt. 3. Passage of gas from cylinder through jar with manometer tube to issue above liquid culture in bottle placed in water bath at 37°. Each run comprised two such arrangements (for comparison of two gases), accompanied by control cultures in capped and uncapped universals.

(1) Four 9 ml. volumes of medium BRS 1/5 in medium R + erythromycin 7 µg./ml. + starch were inoculated with *Entamoeba histolytica*. Amoebic counts (48 hr) were 27,500/ml. with 5% (v/v) CO₂ in oxygen, nil with oxygen, nil in uncapped control and 250,000/ml. in the capped control. (2) With the same amoebic strain and the gases as above, with 9 ml. volumes of undiluted medium BRS containing erythromycin 70 µg./ml. + starch, the amoebae/ml. (maximum at 48 hr) were: nil for the uncapped control; 20,000 for O₂; 360,000 for the O₂ + CO₂; 570,000 for the capped control. (3) With *Endolimax nana*, 3.5 ml. volumes of undiluted medium BRS with erythromycin 33 µg./ml. + starch, and gases O₂ and CO₂, the amoebae/ml. (maximum at 72 hr) were: 20,000 in uncapped control, 20,000 in O₂; 110,000 in CO₂; 170,000 in the capped control.

Nelson & Jones (1964) recorded improvement in amoebic growth by the use of CO₂ + bicarbonate buffer. From the present experiments it was at first concluded that CO₂ was necessary for amoebic growth; but it became clear as the work proceeded that inhibition of amoebic growth under these experimental conditions was due to the culture becoming alkaline because of the growth of a pellicle of aerobic bacteria. The pellicles on the cultures of Expt. 3 incubated in a mixture of CO₂ and O₂ were usually just detectable as very thin transparent skins, while the thick white rings of those without CO₂ were striking, and when bromthymol blue was added, the downward spread of an alkaline reaction was seen. The phenomenon was like that on which Simmons (1926) based his citrate + salts + bromthymol blue agar test for distinguishing *Aerobacter aerogenes* by the development of a blue colour, which did not occur when the culture was sealed; that is when CO₂ was retained above the surface of the medium; the development of carbonate from organic acids (Ayers & Rupp, 1918) in presence of abundant oxygen was inhibited by relatively small amounts of CO₂.

Experiment 4. Serial cultivation of amoebae on BR-based medium in the anaerobic jar gave variable numbers of positive subcultures on different occasions, but always

failed eventually. This was apparently due to progressive diminution of the bacterial flora because of oxygen lack. It also occurred when the jar was filled with CO₂ instead of H₂, and when the hydrogen-filled jar contained a CO₂ absorbent. Thus absence of O₂ from the gas phase prevented the growth of aerobic bacteria which promoted amoebic growth, provided that excessive alkali production did not occur. This was corroborated by the parallel effect on amoebic growth observed throughout this work of anaerobiosis or (a) partial CO₂ atmosphere, and (b) chemical antibiotics; both factors increased amoebic growth by restraining bacterial growth to a point beyond which they lessened it. Also, because of this effect, optimal concentrations of antibiotic were lower when CO₂ was added to the gas phase above the culture.

Experiment 5. Since the above experiments made it likely that the effect of the agar slope in the amoebic cultures was to establish a pH gradient by retaining CO₂, thus preventing alkalization of the culture sediment from above while permitting aerobic bacterial growth at the surface, it seemed that the slope should be replaceable for long-term maintenance, by the alternate stimulation and repression of the growth of the aerobic bacteria. An experiment was made in which a strain of *Entamoeba histolytica* was maintained for 3 months without recourse to a saline agar slope by changing from shallow to deep conditions and vice versa. The rubber-capped 120 × 14 mm. tubes from (Capon & Heaton) transfusion sets were used with 9 ml. volumes of liquid medium for the deep conditions, which proved necessary once for every three shallow sub-cultures of 3 ml. volumes in one-ounce bottles.

The necessity for starch grains. Media were developed in which other components, e.g. living *Trypanosoma cruzi* in the medium of Phillips (1962), replaced living bacteria; in such media, starch particles appear to be unnecessary. But in the medium described above, no amoebic growth was ever obtained without particulate starch. This was tested for all the parasitic amoebae: the substitution of sugars, dextrin or soluble starch was ineffective in promoting the growth of any species in the absence of starch grains. In confirmation, an experiment with *Entamoeba histolytica* may be quoted. Trophozoites in a faecal specimen were observed to ingest a yeast. These trophozoites were isolated, grown separately and added to cultures of the amoeba in presence and in absence of starch. Without starch, the yeast was not ingested and the amoebae died; with starch, the yeast was ingested *in vitro* as it had been *in vivo*.

DISCUSSION

I have found only one report of previous work aimed to find an 'amoebic growth factor': Nakayama (1958) used a method, like that described here, of adding organic supplements to citrate + Ringer solution overlaying an agar slope. His conclusion is opposed to mine, since he found that protein preparations could be replaced by a variety of soluble protein breakdown products (e.g. proteose, glucosamine). It could be argued from the present results that particulate as opposed to soluble protein is required for amoebic nutrition. Even in 'clear liquid serum' medium a little coagulum was always produced.

Haematoxylin stains commonly show bacteria to be adherent to trophozoites from any source, and the probable ingestion of bacteria by *Entamoeba histolytica* has been confirmed by electron micrographs of bacteria in food vacuoles (Fletcher, Maegraith & Jarumilinta, 1962). 'Nests' (Balamuth & Howard, 1946), and 'crowding' of tropho-

zoites (Everitt, 1950), were terms used by these workers, without explanation. In the medium described in the present paper amoebic bunches (such as those shown in Pl. 1, figs. 1-3) were seen to be satellites upon bacterial colonies, parts of which often persisted as wisps, binding two or three trophozoites together after separation from the bunch. The siting of the amoebae thus followed that of the accompanying bacteria; the prominence of bunching seen in the present work was possibly due to the favouring of bacterial growth in coherent networks (see Pl. 1, fig. 3). Thus a scheme for the processes of growth in the type of amoebic culture described here might be formulated as follows. (1) At some points in the sediment there is focal decrease of pH value (perhaps due to liberated CO_2 , held under the butt of the agar slope) sufficient to allow bacterial attack on starch grains. (2) Enough acid is formed from the starch to permit the coating of growing bacteria by protein (analogous to the coating of red blood cells for phagocytosis), from which trophozoites, by ingestion of such bacteria, obtain the ability ('growth factor') to ingest and metabolize grains of starch, which iodine staining shows to be important in their metabolism on the present medium. (3) The amoebae congregate and reproduce on the bacterial colonies, ingesting starch rapidly and possibly stimulating further bacterial growth in their neighbourhood by dextrin formation. (4) The break up of the amoebic clusters follows that of the bacterial colonies on which the amoebae are sited; this seems to be associated with further acid production. This scheme suggests that 'protein-conditioning' of bacteria for ingestion constitutes a difference between parasitic amoebae which require it, and free-living amoebae, which do not.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

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(Facing p. 79)

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EXPLANATION OF PLATE

- Fig. 1. *Entamoeba histolytica* (12353/60) culture. Trophozoites clustered on bacterial colony. Wet preparation in iodine. One scale division = 12·2 μ .
- Fig. 2. *Endolimax nana* (2687/62) culture. Trophozoites clustered on bacterial colony. Wet preparation in iodine. One scale division = 3·4 μ .
- Fig. 3. *Entamoeba coli* (10390/61) culture. Five trophozoites in bacterial network formed around (denser) fragment of metaprotein. Wet preparation in iodine. One scale division = 3·4 μ .
- Fig. 4. Diagram of Fig. 3. Bacterial network striated, protein fragment shaded and *Entamoeba coli* numbered.