THE RÔLE OF CULTURE MEDIUM AND ITS CONSTITUENTS ON THE GROWTH AND VIABILITY OF *ENTAMOEBA HISTOLYTICA*

by A. K. MUKHERJEA, *Research Fellow, N.I.S.I., Department of Protozoology, Indian Institute for Medical Research, Calcutta*

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INTRODUCTION

Attempts have been made in the past by several workers to prepare a medium which would encourage the growth of *Entamoeba histolytica* free from bacteria, so that a pure line strain of this protozoon may conveniently be used for several purposes, particularly for screening antiamoebic drugs, and for preparing specific antigens for the diagnosis of amoebiasis. The progress of our work in this direction and the results obtained have already been reported (Mukherjea, 1951: 9, 35 and 47).

The present work which is a continuation of the past investigation already referred to, has been undertaken to determine the rôle of the culture medium and its constituents on the growth and viability of this protozoal parasite. This has been described under the following headings: (1) Isolation of *E. histolytica*, (2) Isolation of bacteria; (3) Growth of *E. histolytica* under conditions of changed composition of the medium; (4) The slant in relation to the growth of *E. histolytica*; (5) The activation of bacteria and its effect on the constituents of the medium; (6) The constituents of a medium and reduction of the substrates.

MATERIALS AND METHODS

1. *Isolation of* E. Histolytica

All the following experiments were carried out with strains of *E. histolytica* isolated from stools of persons suffering from amoebiasis. For isolation of the amoeba normal saline emulsion of fresh stool, after separation of the coarse faecal particles by standing, was used for inoculating the culture medium. It was incubated at 37°C. and examined microscopically after 48 hours and thereafter.

E. histolytica strains no. 4, 5 and 6: In case of 4 and 6 Dobell and Laidlaw's medium (hereafter referred to as D.L. medium) was used. After 48 hours' incubation the growth in the primary cultures was scanty and did not live longer than 48 hours. There was no growth on subculture. In isolating the amoeba from strain no. 5, two separate cultures were made, one in pure D.L. medium and the other in the same medium to which acriflavine in 1 : 40,000 had been added. Growth occurred in both the culture tubes. It persisted for 48 hours in the tube without acriflavine whereas for 72 hours in the tube with acriflavine. Subcultures from these tubes produced no growth.

E. histolytica strain no. 8: Cultures were made in several E_8 media whose composition is given later. A fair growth was noticed. The ameobae, however, disappeared after 72 hours. This strain perished after the second subculture.

*E. histolytica strain no. 7**: This strain was isolated from the stool of a patient suffering from amoebiasis together with ariboflavinosis. As usual the primary

* With a view to determining the pathogenicity of the amoebae, intrarectal inoculation in two kittens, led to the appearance of motile trophozoites in the stool. The kittens died within 72 to 96 hours. No signs of ulceration were, however, detected in the large intestine.

culture was made in D.L. medium. After 48 hours, growth occurred in the primary culture; it persisted up to 72 hours. Serial subcultures in D.L. medium at 48 hours' intervals were positive although the growth was always feeble.

2. *Isolation of Bacteria*

Bacteria accompanying E. histolytica strain no. 7: These were isolated by culturing a loopful of fluid from an amoebic culture tube on a McConkey's plate. After 48 hours' incubation at 37°C. two types of lactose-fermenting colonies were seen, *viz.* (i) deep pink and (ii) of a lighter colour. Two colonies from (i) were grown separately on nutrient agar, and labelled as *A* and *B*. Two light pink colonies from (ii) were similarly grown on nutrient agar, and labelled as *C* and *D*. The morphology, growth and biochemical reactions of these four cultures were studied and the results are given below:

(1) *On nutrient agar*:

(*a*) *A and B*: Small, circular and convex colonies; Gram-negative bacilli with well developed capsules; no spores.

(*b*) *C and D*: Large, circular, convex and mucoid colonies; Gram-negative bacilli with well developed capsules; non-sporing.

(2) *In nutrient broth*:

(*a*) *A and B*: Uniform turbidity with deposits at the bottom. No pellicle on the surface. Non-motile.

(*b*) *C and D*: Uniform turbidity with deposits at the bottom. Surface-pellicle observed. Non-motile.

(3) *In litmus milk medium*:

(*a*) *A and B*: Acid but no clotting.

(*b*) *C and D*: No acid and no clotting, but after 48 hours, acid and clotting were observed.

(4) *In Koser's and uric acid media*:

(*a*) *A and B*: No growth in either medium.

(*b*) *C and D*: Growth in both the media.

(5) *Sugar fermentation*:

A, *B* also *C* and *D* were inoculated separately into four sets of sugar tubes each containing eleven different sugars. Fermentation reactions, as noticed after 24 hours' incubation at 37°C., are tabulated in Table I.

(6) *Voges-Proskauer reaction* (*V.P.*):

A and *B*, also *C* were V.P. negative, but *D* was V.P. positive.

(7) *Methyl-red reaction* (*M.R.*):

A and *B* gave positive, but *C* and *D* negative reactions.

The important features which emerged from the several tests, stated above, are tabulated in Table II.

TABLE I

Sugar fermentation reactions with bacteria from cultures in A, B and C and D tubes

Culture in tube.	Glucose.	Lactose.	Mannite.	Saccharose.	Arabinose.	Xylose.	Rhamnose.	Inositol.	Raffinose.	Dulcite.	Maltose.	Remarks.
A	A.G.	A.G.	A.G.	Nil	A.G.	A.G.	A.G.	Nil	Nil	Nil	A.G.	Uniform reactions in both the tubes *A* and *B*.
B	A.G.	A.G.	A.G.	Nil	A.G.	A.G.	A.G.	Nil	Nil	Nil	A.G.	
C	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	Uniform reactions in both the tubes *C* and *D*.
D	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	

A.G. = Acid and gas.

TABLE II

Showing the characteristic reactions of the bacteria in cultures A, B and C and D

Culture in tube.	Motility.	Capsule.	Saccharose.	Dulcite.	M.R.	V.P.	Growth in Koser's and uric acid media.	Litmus milk.	Remarks.
A	−	+	−	−	+	−	−	Acid only.	Uniform reactions in both the tubes *A* and *B*.
B	−	+	−	−	+	−	−	Acid only.	
C	−	+	A.G.	A.G.	−	−	+	No acid and no clotting in 24 hours.	Reactions slightly differ in the two tubes *C* and *D*.
D	−	+	A.G.	A.G.	−	+	+	No acid and no clotting in 24 hours.	

From the morphology, growth in Koser's and uric acid media, sugar and other biochemical reactions (*vide* Table above), it is clear that *A* and *B* were one and the same organism, whereas *C* and *D* included two different types. *A* and *B* were, therefore, combined and labelled as bacterial strain no. 2, *C* as bacterial strain no. 1 and *D* as strain no. 3. Strains no. 1 and 3, as Table II shows, belonged to the saprophytic type of *B. coli*, whereas no. 2 was of intestinal origin. As very little difference was noticed in bacteria in strains no. 1 and 3 and since both appeared as saprophytic they will henceforth be referred to as strain no. (1+3).

Bacteria growing with E. histolytica strains no. 4, 5, 6 and 8 (see page 437). All these were noticed to be pathogenic strains of intestinal *B. coli*.

3. *Growth of* E. histolytica *under conditions of changed composition of the medium*

(*A*)* As has been mentioned previously the growth of *E. histolytica* (strain no. 7) was always feeble in D.L. medium and continuous subcultures in the same medium further deteriorated the growth and viability. Attempts were, therefore, made to improve the growth by changing the composition of the medium. With this object in view and also for the purpose of studying which constituents (*e.g.* serum, peptone, meat extract, etc.) when added to the 'basal medium' (see combination *A* in Table III) were responsible for stimulating the growth, the following investigations were carried out.

In the beginning, a simple type of slant and the fluid overlay were prepared as follows. The solid slant was made of 2½% agar in 0·5% saline solution. The overlay fluid was made by dissolving egg albumin in sterile salt solution having the composition: sodium chloride—0·85 gm.; potassium chloride—0·02 gm.; calcium chloride—0·02 gm.; distilled water—100 c.c. The white portion of two hen's eggs was mixed with 250 c.c. of this salt solution which had been previously autoclaved. Starting with such a plain agar slant, its composition was progressively altered by adding other substances such as peptone, meat extract, etc. as is shown below. Likewise, the composition of the fluid portion was changed.

Slant:

(*a*) Plain agar slant.
(*b*) Plain agar+1% peptone+meat extract (50 gm. ox-heart extracted in 100 c.c. water) = nutrient agar slant.
(*c*) Nutrient agar+horse serum (10%) = serum agar slant.
(*d*) Serum agar+haemoglobin (5% rabbit's blood) = blood agar slant.
(*e*) Blood agar: heated to 80°C. for 10 minutes = altered blood agar slant.
(*f*) Horse serum: this was inspissated into a slant = Dobell and Laidlaw's slant.

Fluid overlay:

(*a*) Egg albumin in salt solution.
(*b*) Egg albumin in salt solution+horse serum (9 : 1).
(*c*) Egg albumin in salt solution+serum+acriflavine (1 : 40,000).
(*d*) Egg albumin in salt solution+1% peptone water (3 : 1).
(*e*) Egg albumin in salt solution+meat extract (3 : 1).
(*f*) Egg albumin in salt solution+nutrient broth (3 : 1).
(*g*) Serum in normal saline solution (1 : 9).
(*h*) Serum in normal saline solution+haemoglobin solution† (2 : 5).

The peptone used in these experiments was prepared by digesting meat with pancreas. In making nutrient agar or broth this impure peptone was employed. Apart from peptones, the pancreatic meat digest also contained polypeptides, amino acids, reducing substances, etc. as impurities.

To understand in what ways the different components of the slant and also of the fluid influenced the growth and viability of the amoebae, the slants and the fluid overlays were differently combined. Before inoculating the trophozoites, starch was added in every tube. The results have been shown in Table III.

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* For (*B*), see page 443.

† In preparing haemoglobin solution 10 c.c. of rabbit's blood was at first laked in 90 c.c. of sterile distilled water which was then made isotonic by mixing with sterile 1·2% sodium chloride solution in the proportion of 2 : 4.

TABLE III

Showing the growth and viability of the amoebae in various combinations of slants and fluid overlays

Group.	Combinations of slants + fluids.	Growth after 48 hours.	Viability.
A	*1. Slant (*a*) + Fluid (*a*)	+	48 hours.
	2. Slant (*a*) + Fluid (*d*)	+ + +	72 hours.
	3. Slant (*a*) + Fluid (*e*)	+ + +	72 hours.
	4. Slant (*a*) + Fluid (*f*)	+ + +	72 hours.
	5. Slant (*b*) + Fluid (*a*)	+ + +	72 hours.
B	1. Slant (*a*) + Fluid (*b*)	+ + +	96 hours.
	2. Slant (*f*) + Fluid (*a*)	+	72 hours.
C	1. Slant (*c*) + Fluid (*a*)	+ + +	96 hours.
	2. Slant (*d*) + Fluid (*a*)	+ + +	96 hours.
	3. Slant (*e*) + Fluid (*a*)	+ + +	96 hours.
D	1. Slant (*c*) + Fluid (*b*)	+ + +	96 hours.
	2. Slant (*d*) + Fluid (*b*)	+ + + +	96 hours.
	3. Slant (*e*) + Fluid (*b*)	+ + + +	96 hours.
E	1. Slant (*c*) + Fluid (*c*)	+ + + +	120 hours.
	2. Slant (*d*) + Fluid (*c*)	+ + + + +	144 hours.
	3. Slant (*e*) + Fluid (*c*)	+ + + + +	144 hours.
	4. Slant (*f*) + Fluid (*c*)	+ + +	144 hours.
F	1. Nil + Fluid (*g*)	+	48 hours.
	2. Nil + Fluid (*g*) + Hb. ..	+ + +	120 hours.
	3. Slant + Fluid (*g*) + Hb. ..	+ + + +	96 hours.
G	1. Slant (*a*) + Fluid (*a*) + acriflavine ..	+	48 hours.

From the above table the following conclusions are made:—

1. The basal medium was very feeble as regards its power to support the growth of the amoebae.

2. Peptone and meat extract used either separately (A_2, A_3), or in combination (A_4) activated the growth of the amoebae in the basal medium. No difference could be discovered when they were added to the fluid (A_2, A_3, A_4) or incorporated in the slant (A_5).

3. The addition of serum to either the fluid overlay (B_1) or the slant (C_1) had a marked effect on the growth and viability of the amoebae which, however, did not improve on the addition of serum to both the fluid and the slant (D_1).

4. Acriflavine † when mixed with the basal medium did not lead to any improvement of the growth of the amoebae (G_1), but when added to an enriched medium containing serum, peptone or meat extract, it caused a very marked stimulation of the growth and an increase in the viability of the organisms (E_1, E_2, E_3 and E_4).

5. Though serum solution itself (F_1) failed to stimulate the growth of the amoebae, addition of haemoglobin (F_2) always encouraged the growth which further improved when serum solution containing haemoglobin was put as overlay (F_3). There was no appreciable increase in the growth of the amoebae when haemoglobin was included in the slant of an enriched medium.

6. The growth and viability of the trophozoites were diminished when the serum was inspissated (B_2).

7. Heating the haemoglobin made no difference in the growth of the amoebae.

* The combination in A_1 constitutes the '*basal medium*'.

† Details of the action of dyes and other bacteriostatic substances will be reported in a later communication.

From the above it will be seen that the combinations as stated in E_1, E_2 and E_3 were most conducive to the growth and viability of the amoebae. Due to this the combinations in E_1, E_2 and E_3 were chosen as the 'preserving media'.

It may be noted that when serum was added to the basal medium, there was an appreciable improvement of the growth and viability of the amoebae. Such improvements were also noticed when stock peptone, meat extract and nutrient broth were used. These observations, therefore, led us to undertake studies to explain in what different ways serum, stock peptone, meat extract and nutrient broth influenced the growth of *E. histolytica* (strain no. 7).

Improved growth of the amoebae on addition of serum to the basal medium may be regarded to have been effected by some of its constituents such as, albumin, globulins, urea, uric acid, creatine, creatinine, other undetermined nitrogen, amino acids, fatty acids, cholesterol, inorganic salts, glucose, vitamins, etc. or by some unknown factors. In order to determine the rôle of these substances on the growth of the amoebae, the different constituents of the serum have been placed in three groups:

(*a*) Urea, uric acid, etc. and amino acids—as 'alternate sources of nitrogen'.
(*b*) Sterols, *e.g.* cholesterol—as food for the amoebae.
(*c*) Vitamins, especially B-complex group—as accessory growth substances.

It has been observed that the behaviour of the amoebae in solution of albumin and globulins of serum is the same as when they are grown in that of pure egg albumin. The growth of amoebae in presence of serum may thus be directly due to the introduction of either of the following substances: (1) alternate sources of nitrogen, (2) sterols and (3) accessory growth substances. Tests in proving these points were therefore undertaken.

Stock preparations of the above substances were made as follows:

(1) *Alternate sources of nitrogen*: Urea and uric acid were used in the same proportions as they are present in the serum. Ammonium chloride was used in trace as substitute for creatine, creatinine and other undetermined nitrogen of serum. The composition thus stands as: urea—0·20 gm.; uric acid—0·12 gm.; ammonium chloride—0·24 gm.; salt solution (stock)—1,000 c.c. After sterilisation the white portion of two hen's eggs was mixed with 250 c.c. of the solution. The composition of this fluid is now identical with that of the fluid portion of the basal medium after the addition of urea, etc. in the same proportions as they exist in the serum.

(2) *Cholesterol*: Sterile cholesterol powder+sterile starch (1 : 5). One loop of this mixture was used for each culture tube.

(3) *Amino acid solution*: 50 mgm. each of glycine, alanine, cystine, leucine, tryptophane and aspartic acid were mixed in 10 c.c. of normal saline and sterilised. 0·2 c.c. of this solution was used for each culture tube.

(4) *Vitamin B-complex solution*: This was prepared by dissolving the contents of one 'Betalin compound' capsule (Lilly) in 50 c.c. normal saline. After sterilisation, 8 to 10 drops were used for each culture tube.

The effect of the different constituents of the serum on the growth of *E. histolytica* (strain no. 7) is shown in Table IV.

An analysis of the effects of different constituents of the serum indicates that vitamin B-complex with the addition of cholesterol and amino acids influenced the nutrition of the amoeba. However, a closer study led us to believe that amongst the substances tested vitamin B-complex was essentially the most important factor which brought about an increase in growth of the amoeba together with augmentation of its viability.

While vitamin B-complex when added to the basal medium no doubt stimulated the growth of the amoebae, though feebly, it exercised profound influence on

TABLE IV

Showing the effects of the different constituents of serum on the growth of E. histolytica

Substances added to the basal medium.	Growth after 48 hours.	Viability.
Alternate sources of nitrogen	+	48 hours.
Alternate sources of nitrogen + cholesterol	+	48 hours.
Alternate sources of nitrogen + cholesterol + amino acids ..	+	48 hours.
Alternate sources of nitrogen + cholesterol + amino acids + vitamin B-complex	+	72 hours.
Cholesterol	+	48 hours.
Vitamin B-complex	+ to ++	72 hours.
Amino acids	+	48 hours.
Cholesterol + amino acids + vitamin B-complex	+ to ++	72 hours.

their growth when mixed with an enriched medium containing serum, peptone and meat extract (*e.g.* E_3 medium). This is manifest in the results given in Table V.

TABLE V

Showing the effects of vitamin B-complex on the growth of amoebae when mixed with enriched medium

Medium.	Growth after 48 hrs.	Growth after 72 hrs.	Growth after 96 hrs.	Remarks.
E_3 ..	++	+	−	The growth and also the viability are distinctly feeble in all the tubes.
E_3 ..	+	+	−	
E_3 ..	++	+	−	
E_3 + vitamin B-complex ..	+++++	+++	+	The growth is profuse and the viability prolonged.
E_3 + vitamin B-complex ..	++++	+	−	
E_3 + vitamin B-complex ..	+++++	++	+/2	

+/2 indicates five or less than five trophozoites per field as observed under low power.

(*B*) The physical and chemical changes which directly lead to increased multiplication of *E. histolytica*, and whether they are due to putrefactive reaction or to reaction of fermentation, have been discussed in a previous paper (Mukherjea, 1951 : 9). Indeed, such changes are due to the presence of different types of bacteria which are found in association with the amoebae.

While the bacteria are responsible for the type of reaction that is taking place in the culture medium, the constituents of the medium themselves, on the other hand, can modify the intensity of the reaction. The latter has now been observed by culturing *E. histolytica* (strain no. 7) in B_2, D_1, D_2 and D_3 and also in E_1, E_2, E_3 and E_4 media. After 48 hours' incubation, observations were made for signs of reactions such as smell and changes of *p*H. The details of these observations are given in Table VI.

TABLE VI

Showing the relation between composition of the medium, reactions taking place as a result of bacterial growth and growth of the amoebae

Medium.	Smell (foetid).	*p*H.	Growth after 48 hours.
B_2 (= basal medium)	Nil	4·8 to 5·0	+
D_1 (= B_2 + peptone + meat extract + serum) ..	+	5·4 to 5·6	++
D_2 (= D_1 + blood)	+	5·8 to 6·0	++
D_3 (= D_1 + heated blood)	+	5·6 to 5·8	++
E_1 (= B_2 + peptone + meat extract + serum + acriflavine)	+++	6·6 to 6·8	+++++
E_2 (= E_1 + blood in the slant)	+++	6·4 to 6·8	+++++
E_3 (= E_1 + heated blood in the slant)	+++	6·4 to 6·8	++++
E_4 (= D.L. medium + serum and acriflavine) ..	++	5·6 to 6·4	+++

The table shows that:

(1) The sign of putrefaction such as foetid smell, was absent in B_2. The *p*H was below 5·0 and the growth of the amoebae was feeble (+).

(2) Putrefaction was apparent in D_1, D_2 and D_3. The *p*H remained above 5·4 and the growth of the amoebae was from fair to moderate (++).

(3) The putrefying reactions were intense in E_1, E_2 and E_3. The *p*H remained close to 6·8. The growth of the amoebae was profuse (+++++).

The following conclusions are thus made from the above observations:—

(i) Substances such as peptone, meat extract and serum are responsible for stimulating the putrefaction (D_1, D_2 and D_3).

(ii) Acriflavine further increases putrefaction (E_1, E_2 and E_3).

(iii) With increase in the intensity of putrefaction the growth and viability of the amoebae also increase.

(iv) Inspissation of serum leads to the depression of the growth-promoting properties of the serum slant.

4. *The slant in relation to the growth of* E. histolytica

It has already been reported in this paper that a slant containing agar and salt (*i.e.* the 'inert' slant) does not provide any nourishment to the amoebae. On the other hand, slants in A_5, C_1, C_2 and C_3 media (*vide* Table III) contain substances which directly go to nourish them. In order to study in what other ways a slant may directly encourage the growth of the amoebae, an 'inert' slant was overlaid by solution of egg albumin and horse serum. In actual experiments, the height of the fluid in all the tubes, whether with the slant or without it, was the same. The results are noted in Table VII.

As has previously been mentioned, a slant of agar and salt in presence of an 'inert' fluid consisting of egg albumin in normal saline acts in an inert manner as it does not stimulate the amoebae to grow. On the other hand, when the slant is overlaid by the same fluid enriched by the addition of horse serum there is an abundant growth (*vide* tubes no. 1, 2 and 3 in Table VII). In absence of the 'inert' slant (agar + salt), the fluid itself is unable to encourage the multiplication of the amoebae (*vide* tubes no. 4 and 5 in Table VII). This indicates that in some unaccountable ways the 'inert' slant under certain conditions can activate the growth of the amoebae.

TABLE VII

Showing the effects of 'inert' slant on the growth of E. histolytica

Tube No.	Medium.	Growth after				Remarks.
		48 hrs.	72 hrs.	96 hrs.	120 hrs.	
1.	Egg albumin and serum solution + inert slant ..	+++	+	−	−	The growth and the viability are increased.
2.	Egg albumin and serum solution + inert slant ..	++	+++	+	−	
3.	Egg albumin and serum solution + inert slant ..	++++	++++	++	−	
4.	Egg albumin and serum solution	+	−	−	−	The growth is uniformly feeble in all the tubes.
5.	Egg albumin and serum solution	++	+/2	−	−	

+/2 indicates five or less than five trophozoites per field as observed under low power.

It was further observed that while combination of the 'inert' slant and the fluid containing egg albumin and horse serum was more effective than the fluid itself, the growth of the amoebae was noticed to be definitely feeble when the same fluid, but without the slant, was mixed with agar to make it viscous. In actual experiments this semisolid medium was prepared by mixing 5 c.c. of a 3% agar solution with 28 c.c. of the fluid. It may be stated that the slant is made mainly of agar. When the slant is withdrawn and agar is incorporated in the albuminous fluid enriched by the addition of horse serum, this semisolid or viscous material behaves in a remarkably different manner than when the agar in the form of slant is used.

5. *The activation of bacteria and its effect on the constituents of the medium*

In stimulating the growth and viability of the amoebae the associated bacteria and the individual constituents of the medium are equally important. The conditions leading to the growth of the amoebae develop as a result of their mutual actions. This is explained by stating that while some constituents of the medium, *e.g.* peptone, help the growth and rapid multiplication of the bacteria, others, such as egg albumin, are unable to perform the same function. After bacteria have been activated by growing in a medium containing peptone, they are able to attack the albumin and break it into simpler constituents. Without peptone, as has been stated, the same bacteria cannot hydrolyse albumin or any other proteins. These observations were the results of the experiments described below.

Bacteria, strains no. 2 and (1+3), were cultivated in several liquid media, *e.g.* (*a*) egg albumin solution; (*b*) egg albumin solution+peptone; (*c*) egg albumin solution+horse serum; (*d*) nutrient broth; and (*e*) glucose broth. At the end of 24 and 48 hours' incubation the growth of the bacteria and the *p*H of the culture fluid were noted. At the end of 48 hours, tests were made for proteoses and peptones in culture fluids (*a*) and (*c*). The results have been incorporated in Table VIII.

It will thus be seen that:

1. (*a*) In all the above experiments in the case of strain no. 2, irrespective of the growth of the bacteria, starch was not affected in any way.

TABLE VIII

Showing the action of bacterial strains no. 2 and (1+3) when grown in different fluid media

Serial no.	Medium.	Bacterial strain no. 2.	Bacterial strain no. (1+3).
(*a*)	Egg albumin soln.+ starch (*p*H 6·8).	*p*H after 24 hrs. : 6·8 *p*H after 48 hrs. : 6·8 Growth after 24 hrs. Nil Growth after 48 hrs. + Proteoses+peptones: Nil	*p*H after 24 hrs. : 6·8 *p*H after 48 hrs. : 6·8 Growth after 24 hrs. + Growth after 48 hrs. ++ Proteoses+peptones: +
(*b*)	Egg albumin soln.+ peptone+starch (*p*H 6·8).	*p*H after 24 hrs. : 6·4 to 6·6 Growth after 24 hrs. ++++	*p*H after 24 hrs. : 6·8 Growth after 24 hrs. ++++
(*c*)	Egg albumin soln.+ horse serum+starch (*p*H 6·8).	*p*H after 24 hrs. : 6·8 Growth after 24 hrs. ++++ Proteoses+peptones: +++	*p*H after 24 hrs. : 6·8 Growth after 24 hrs. ++++ Proteoses+peptones ++++
(*d*)	Nutrient broth+ starch (*p*H 6·8).	*p*H after 24 hrs. : 6·4 to 6·6 Growth after 24 hrs. ++++	*p*H after 24 hrs. : 6·8 Growth after 24 hrs. ++++
(*e*)	Glucose broth (*p*H 6·8).	*p*H after 24 hrs. : 4·4 to 4·6 Growth after 24 hrs. ++++	*p*H after 24 hrs. : 5·4 to 5·8 *p*H after 48 hrs. : 6·2 to 6·6 Growth after 24 hrs. ++++

(*b*) Although there was no growth of the bacteria in egg albumin solution up to 24 hours, a feeble growth was noticed after 48 hours. There was no hydrolysis of egg albumin (*vide* no. (*a*) in Table VIII).

(*c*) The same strain of bacteria showed a profuse growth in a medium containing a small quantity of peptone or horse serum in addition to egg albumin. This was accompanied by hydrolysis of egg albumin (*vide* nos. (*b*) and (*c*)).

(*d*) In nutrient broth (peptone+meat extract), and glucose broth (glucose+ nutrient broth) growth was profuse but acid was produced in the latter medium only (*vide* nos. (*d*) and (*e*)).

2. (*a*) In the case of strain no. (1+3), as with strain no. 2, starch was not affected.

(*b*) There was feeble growth in egg albumin up to 24 hours, thereafter the growth increased. There was slight hydrolysis of egg albumin (*vide* no. (*a*)).

(*c*) The growth of the bacteria was profuse in a medium containing a small quantity of peptone or horse serum in addition to egg albumin; there was also hydrolysis of egg albumin (*vide* nos. (*b*) and (*c*)).

(*d*) In nutrient broth and glucose broth, growth was likewise profuse. In case of glucose broth there was slight acidity at first which became neutral after 48 hours (*vide* nos. (*d*) and (*e*)).

6. *The constituents of a medium and reduction of the substrates*

As amoebae are obligatory anaerobes, for anaerobic respiration they require a medium which has already undergone reduction. In absence of chemical substances such as cysteine, glutathione, etc., which when mixed with the culture fluid

are themselves able to reduce the medium, the lowering of the oxidation-reduction potential of the medium is accomplished by the bacteria. Some common constituents of the medium such as peptone, meat extract, etc., also contribute to some extent in bringing about reduction for the purpose of respiration of the amoebae. The constituents, however, do not act independently in the same way as the

TABLE IX

Showing how the different constituents of the medium such as serum, egg albumin, etc. react with Bacterial Strain no. (1+3) *causing reduction of methylene blue*

Substrates.		Decolouration of methylene blue after an interval of								Remarks.
		10 mts.		10 mts.		20 mts.		18 hours.		
Serum solution	Emulsion of bacteria from strain no. (1+3) —0·3 c.c. put into the tubes.	Methylene blue solution—2 drops put into the tubes.	40%	2 more drops of methylene blue solution added.	10%	Further addition of 2 drops of methylene blue solution.	Nil	Additional 10 drops of methylene blue solution.	80%	Reactions are delayed, weak and unstable in both the tubes.
Egg albumin solution.			40%		10%		Nil		60%	
Peptone water			100%		100%		80%		100%	Reactions are quick, strong and stable in all the tubes.
Meat extract			100%		100%		100%		100%	
Nutrient broth			100% in 3 mts.		100% in 3 mts.		100% in 5 mts.		100%	

TABLE X

Showing how the different constituents of the medium such as serum, egg albumin, etc. react with Bacterial Strain no. 2 *causing reduction of methylene blue*

Substrates.		Decolouration of methylene blue after an interval of								Remarks.
		10 mts.		10 mts.		20 mts.		18 hours.		
Serum solution	Emulsion of bacteria from strain no. 2—0·3 c.c. put into the tubes.	Methylene blue solution—2 drops put into the tubes.	10%	2 more drops of methylene blue solution added.	Nil	Further addition of 2 drops of methylene blue solution.	Nil	Additional 10 drops of methylene blue solution.	50%	Reactions are extremely feeble and also unstable in both the tubes.
Egg albumin solution.			10%		Nil		Nil		20%	
Peptone water			50%		20%		20%		100%	Reactions are delayed, weak and unstable in all the tubes.
Meat extract			10%		10%		Nil		100%	
Nutrient broth			60%		40%		20%		100%	

chemical reducing agents but act conjointly with the bacteria. This was proved by taking several test tubes each containing 4 c.c. of the fluid. The different substrates used were horse serum, egg albumin, peptone, meat extract and nutrient broth. 0·3 c.c. of bacterial emulsion from strain no. (1+3) and a few drops of a buffered methylene blue solution (*p*H 7·2) were added gradually in all the tubes as shown in Table IX. The degree of reduction was judged by the extent of decolouration of the dye. At every step care was taken to prevent bacterial contamination. Similar experiments were carried out with bacterial strain no. 2 in place of strain no. (1+3). The results are noted in Table X.

It will thus be seen from Tables IX and X that the reactions induced by the bacteria varied not only with the constituents but also with the type of bacteria employed.

Discussion

In order to keep *E. histolytica* alive and for its growth and reproduction certain requirements must be fulfilled. These are:

(1) The proper type of the culture medium. Though different types of media are recommended for its cultivation, the results are never satisfactory in all cases. It is likely that the necessary conditions and substances favouring their growth are not present in the same manner in all these different media.

(2) Suitable types of bacteria growing with the amoebae; and

(3) Optimum temperatures for the growth and its viability.

The preparation of a medium containing proportionate amounts of nutrient materials for the amoebae is, therefore, of fundamental importance. The culture medium in addition to containing all the elements necessary for the growth and viability of the amoebae should also be properly constructed. How the construction of a medium influences the growth of the amoebae can be demonstrated by culturing them in the following media:

(*a*) in a fluid medium but without a slant. The growth is markedly feeble (Table VII).

(*b*) in the same medium with an 'inert' agar slant. Growth profuse (Table VII).

(*c*) in the same fluid when mixed with agar making it viscous. Growth feeble.

It follows that besides the constituents of a medium its construction is also important. It also appears that though the fluid itself can sustain the amoebae, the presence of a slant leads to a remarkable increase in their growth. In order to study in what ways a slant may encourage the growth of the protozoa, investigations were undertaken using slants having different compositions. While slants in A_5, C_1, C_2 and C_3 combinations (Table III) caused a distinct stimulation of the growth by providing the necessary nutritive factors, the 'inert' agar slant, on the other hand, which as a rule does not contain any growth-promoting or nutritive substances, also produced a noticeable effect on the growth of the amœbae (Table VII). In what way the 'inert' agar slant itself influences the physiological activities of the amoebae is difficult to explain.

Composition of the medium: To determine how the composition of the medium influences the growth of the amoebae, the various constituents which have been used in our work, are divided into three groups, namely:

(1) *basic substances*, *e.g.* starch, egg albumin; serum proteins, meat extract and peptone.

(2) *accessory growth substances*, *e.g.* haemoglobin and vitamin B-complex.

(3) *salts*, including the buffers, *e.g.* NaCl, KCl, $CaCl_2$, Na_2HPO_4, KH_2PO_4.

Basic substances: The basic substances, enumerated above, are essential for the growth of the amoebae. They encourage the growth and also prolong the viability of the parasites by the following ways:

(i) Serving as food materials, *e.g.* starch, coagulated egg and serum albumins.

(ii) *Promoting bacterial growth and metabolism*: It is known that *E. histolytica* cannot thrive in the absence of bacteria. In what way bacteria encourage growth of the amoebae will be mentioned in a subsequent paper; it may, however, be stated now that they do so by decomposing proteins. It is interesting to note that while the growth of the amoebae is stimulated by the products resulting from the action of the bacteria on egg albumin and serum proteins, the latter substances alone are unable to support the growth of the bacteria. To accomplish the growth of the bacteria in egg albumin or serum protein solution, addition of small quantities of peptone, meat extract, nutrient broth or even serum will be needed (*vide* Table VIII). This indicates that peptone, meat extract and serum contain some substance or substances which are essential not only for stimulating the metabolic activities of the bacteria, but also for inducing the bacteria to hydrolyse complex proteins such as egg albumin and serum proteins. The increase of foetid smell and the growth of the amoebae, noticed on the addition of peptone, meat extract and serum to the basal medium (*vide* Table VI), strongly suggest that these are due to stimulation of the putrefying reactions of the bacteria.

(iii) *Lowering the E_h of the medium*: *E. histolytica* being an obligatory anaerobe, requires a strongly reduced medium for respiration. In reducing the medium, apart from the bacteria growing in it, some of its basic constituents also contribute to a great extent. This will be evident from the results noted in Tables IX and X. The tables show that while in the case of peptone, meat extract and nutrient broth the reduction was quick and strong, on the other hand, it was slow and feeble in the case of egg albumin and serum. The tables also show that the extent of reduction and the time required for this to develop varied not only with the constituents but also with the type of the bacteria employed. When the reduction is quick, strong and stable, trophozoites after inoculation in such a medium are able to thrive and multiply. In the case of egg albumin, as has been reported, the reaction is delayed, weak and unstable and as a result of this many of the trophozoites tend to succumb.

When the growth and viability of the amoebae were noticed to increase following addition of serum to the basal medium containing egg albumin as the only protein constituent, it was surmised that the growth and viability were directly the result of the action of urea, uric acid, creatinine, etc. present in the serum. It may be stated in this connexion that egg albumin alone is unable to stimulate the growth of the amoebae. As observed by Bainbridge (1911) and confirmed by us and reported in this paper (*vide* Table VIII), the proteolytic bacteria always require an 'alternate sources of nitrogen' for hydrolysing egg albumin. The urea, uric acid, etc. of the serum may perhaps serve the same purpose, *i.e.* they function as the 'alternate sources of nitrogen' and thus encourage the growth of the amoebae. This is done by stimulating the bacteria enabling them to hydrolyse egg albumin. However, in actual experiments it became manifest that urea, uric acid, etc. in the proportions as these exist in the serum are incapable of serving as the 'alternate sources of nitrogen'. This was proved by adding urea, uric acid and ammonium chloride to the basal medium.

The other explanation which may be put forward to account for the growth-promoting properties of the serum itself is by its power to reduce the substrate. Tables IX and X show that unlike peptone, meat extract, etc. serum is not a powerful agent capable of reducing the substrate. Therefore, the idea that the serum itself improves the growth of the amoebae by supplying the reducing substances, cannot be entertained.

It is difficult to explain why amino acids and cholesterol either separately or in combination with other constituents of the serum, *e.g.* urea, uric acid, etc. fail to stimulate the growth of the amoebae. It may be mentioned that it has not been possible to study the rôle of the fatty acids on the growth of the amoebae and hence whether their growth is due to the fatty acids or some unknown substances present in the serum is also difficult to say.

Accessory growth substances: It has been demonstrated that the addition either of vitamin B-complex or haemoglobin (*vide* Tables V and III) leads to a remarkable increase in the growth of the amoebae in an enriched medium.

It is known that vitamins, especially the B-complex, are synthesised by bacteria. Continuous cultivation of the amoebae in any medium in course of time affects their growth and viability. Addition of a few drops of vitamin B-complex solution at this stage always profoundly increases the growth and viability (*vide* Table V). This leads us to conclude that the growing amoebae are in need of vitamin B-complex and that the bacteria being devitalised due to prolonged subcultures fail to produce this essential requirement.

It is not possible to advance the correct explanation on the mode of action of haemoglobin in stimulating the growth of the amoebae, but it seems probable that it does so by supplying the amoebae with respiratory enzymes.

Salts and buffers: The inorganic salts are mainly responsible for the maintenance of the osmotic pressure of the culture fluid. A change in the proportion of sodium chloride from 0·9% to 0·5% does not seem to appreciably affect the growth of the amoebae. This has been reported by Mukherjea (1951 : 9) while cultivating *E. histolytica* (strain no. 1). The present observations suggest that the amoebae are normally provided with the required amounts of $CaCl_2$ and KCl in the culture medium, these being met from such substances as serum, meat extract and peptone. Addition of any further quantity of these salts to the medium does not serve any additional advantage.

To counteract the fall in *p*H, buffers are used. This is not necessary in case where the proper type of bacteria is present. When grown in E_3 medium with or without the addition of a buffer, the amoebae (strain no. 7) showed no change in their growth and viability. However, the incorporation of buffer salts would be a safeguard against changes in reaction in the event of an unfavourable type of bacteria growing with the amoebae.

Summary

The important features that have emerged from the present investigations are:

(1) Two types of *B. coli*, one saprophytic, the other intestinal, were seen growing with *E. histolytica* strain no. 7 in the culture media.

(2) In stimulating the growth of the amoeba the constituents of a medium and its construction are equally important.

(3) Changes in the composition of the slant and also of the fluid have profound influences on the growth and viability of the amoeba.

(4) Some of the constituents of serum such as urea, uric acid, cholesterol, etc., fail to stimulate the growth of the amoeba.

(5) The basic constituents of a medium such as egg albumin, peptone, meat extract, serum, etc., supply the nutritional requirements of the amoeba. They also stimulate the growth and metabolism of the bacteria and supply the reducing substances necessary for the growth of the amoeba.

(6) Accessory growth substances such as haemoglobin, vitamin B-complex, etc., serve merely as stimulants for activating the basic constituents of the medium.

(7) External buffers are not required if the bacteria are of the putrefactive type.

(8) Addition of such substances as impure peptone, meat extract and serum, helps the growth and viability of the amoeba by stimulating the putrefactive process by the bacteria.

(9) The slant of a medium serves the function of providing nutrition to the amoeba and probably increases the E_h of the medium.

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