MATERIALS AND METHODS
The following modification of Boeck and Drbohlav medium (Dutta, 1961) was used for maintaining stock cultures of *Entamoeba histolytica*, *E. ranarum*, *E. invadens* and *E. moshkovskii*:

1. Eight hen's eggs are washed with soap and their shells are then sterilised by 70% alcohol.

2. The contents of these eggs are poured aseptically into a sterile wide-mouth glass stoppered bottle containing 100 ml of 0.85% sodium chloride and sterile glass beads.

3. The bottle is vigorously shaken to mix the eggs into an emulsion, which is passed through a sterile muslin.

4. Nearly 4-6 ml of egg emulsion is poured into cotton plugged sterile test tubes (1.5 x 15 cm), and the tubes are slanted inside the hot air oven.

5. The egg slants are heated to 80°C for 1 hr on three successive days.

6. In order to prepare the overlay, the buffalo blood is collected in large bulk from the slaughter house. After the blood clots, serum is separated and stored at 4°C. The serum is then centrifuged at 2500 rpm for 10 min to settle any red cells and particulate matter, and the clear opalescent serum is separated and passed through Seitz filter and dispensed into
flasks in small volumes. The serum is inactivated at 56°C for half an hour and then kept at 37°C for 48 hr to test the sterility of the batch. The sterile bovine serum prepared in this way can be stored for months at 4°C. The serum is finally diluted in the ratio 1:7 with sterile M/40 phosphate buffer saline (pH 7.0) and this diluted serum is then dispensed as overlay into the tubes containing egg slants. These tubes are then tested for the sterility and stored at 4°C.

7. Rice starch (BDH) sterilized by dry heat at 160°C for 1 hr is added to the final medium before use.

Brewer's (1942) anaerobic petri dishes made of glass, manufactured by Arthur Thomas Company, Philadelphia, U.S.A. (Fig. 1 & 2) were used for the culture and isolating of anaerobic amoebae. A dish consists of a groove with a raised portion and a cover. Two very small circular holes were made at two opposite ends of the cover to pass nitrogen. The holes were sealed with small pieces of celophane tape before sterilizing the dishes in an oven by dry heat at 160°C for one hour.
Preparation of non-nutrient agar for cultivation of amoebae

In order to make 1.5 or 2.0% agar (Difco), (Oxoid No.3, U.K.) it was dissolved in M/40 phosphate buffer, pH 6.5, containing 0.85% NaCl and 0.1% cysteine HCl in a water bath. The pH of the agar was again adjusted to 6.5 with N/10 NaOH. About 20-22 ml of the agar was poured into screw-capped tubes and the agar in the tubes was sterilized by autoclaving at 15 lb. /sq. in. pressure for 15 minutes. Indigo carmine, used as redox indicator dye for roughly determining the O-R potential in the agar, was dissolved in double glass distilled water and sterilized in test tubes plugged with cotton wool. When the temperature of the agar was about 50°C, indigo carmine solution was added to each tube to make its concentration about 0.01% in the agar, and the tubes after mixing the dye in agar, were stored at 4°C. Agar tubes kept for only upto 7-10 days were employed in the experiments.

Sterilization of rice starch

About 2 g of dry starch having fine particles (B.D.H., U.K.) was put in each small tube and the tubes were plugged with cotton wool. The tubes were heated at 180°C by dry heat for one hr. This does not char, disintegrate or render the starch soluble.
Maintenance of different species of bacteria

The bacteria used in this work are:

Salmonella paratyphosa
Salmonella gallinarum
Salmonella holtmulleri
Bacillus cereus
Mycobacterium smegmatis
Mycobacterium phlei
Agrobacterium chroococcum
Streptococcus pyogenes
Bacillus mycoides
Erwinia corotovora
Agrobacterium tumefaciens
Pseudomonas aeruginosa
Corynebacterium tritici
Escherichia coli
Aerobacter aerogenes
Staphylococcus albus
Sarcina lutea
Bacillus subtilis

They were maintained on nutrient agar slopes by sub-culturing. Young cultures, 2-3 days old, were always employed.

Preparation of plates

Agar in tubes was melted in a water bath. At about 50°C, agar from a tube was poured into the cover of a dish and covered with a sterile cover of an ordinary Petri dish.
This prevents condensation of water on the agar surface. After the agar had solidified, 2-3 loopfuls of *Escherichia coli* or *Aerobacter aerogenes* or any other bacterium, growing on nutrient agar, was spread as a thick circular patch 'bacterial patch' about 2.5 cm in diameter on the agar surface in the centre. One loopful of rice starch (BDH) was thoroughly mixed with the bacterial patch. Then a thick paste of lead acetate made with sterile distilled water was spread all round the raised portion of anaerobic dish (Fig. 2) in order to absorb H$_2$S produced by the break-down of cysteine by the resting bacteria incorporated in agar. The cover of the dish containing agar, bacteria and rice starch was put face downwards into the groove of the dish containing about 5 ml of liquid paraffin sterilized by dry heat at 160°C for one hour in order to prevent air entering the dish. The celophane tape put on the holes of the cover of the dish was removed and purified nitrogen gas, prepared by the method given below, was passed for 2 minutes through one hole of the cover and allowed to escape through the other. This replaced the air, trapped between the dish and the cover with nitrogen gas. The holes were then quickly sealed with plasticine to prevent air entering the dish. The plates were incubated at room temperature (about 20° to 25°C) for 18-20 hours or longer for cysteine to absorb oxygen from the agar. According to Reed and Orr (1943), cysteine at 0.1% produced an O-R potential of about -175 mv. The cover of the dish was then removed.
and anaerobic amoebae, growing with mixed bacteria + rice starch in modified Boeck and Drbohlav medium (M/40 phosphate buffer in 0.85% NaCl, pH 7.0, (8 parts) mixed with one part inactivated bovine serum Dutta and Mohan Rao, 1966), were inoculated in the centre of \textit{E. coli} circle. The cover was quickly sealed in liquid paraffin and nitrogen gas was passed again. Human and animal faecal samples infected with anaerobic amoebae were also inoculated in the centre of the bacterial patch for the isolation of these amoebae.

For the growth of anaerobic amoebae, the plates were incubated either at 25°C for culturing \textit{E. ranarum} and \textit{E. moshkovskii} or at 36° - 37°C for \textit{E. histolytica} and other intestinal amoebae.

**Method for the purification of commercial nitrogen gas**

Commercial nitrogen gas was passed through red hot copper (AR, BDH) and then through two traps of alkaline pyrogallol (10g pyrogallol in 100 ml of 40% KOH). This rendered the gas free of oxygen. It was then passed through one hole in the lid of the Petri dish and allowed to escape through the other (Fig. 1).

**Method of sub-culturing aerobic amoebae**

A piece of agar about one cm square, containing large numbers of amoebae growing in anaerobic dish, was cut and placed face downwards in the centre of a new bacterial patch in the Brewer's Petri dish and nitrogen gas was passed.
The amoebae moved out of the agar piece, fed on bacteria and rice starch and multiplied. This method of sub-culturing is very important because it prevents them from coming in contact with traces of air that may be present between the dish and the cover.

**Reducing agents**

Several reducing agents, producing different negative O-R potentials, have been used for the culture and isolation of anaerobic amoebae in conjunction with indigo carmine. A reduction in blue colour of the dye by 14% is supposed to give an O-R potential of about -100 mv, 86% reduction in colour about -150 mv and complete reduction in colour about -180 mv.

**Antibiotics**

Freshly prepared antibiotics were incorporated in the agar at 50°C to suppress the growth of Gram-positive and Gram negative bacteria coming with the inoculum of amoebae from modified Boeck and Drbohlav medium or from faecal samples infected with anaerobic amoebae.

**Acriflavine and gentian violet**

These dyes were dissolved separately in distilled water and sterilized by autoclaving in tubes plugged with cotton wool. They were incorporated in agar at 50°C-60°C before pouring the plates. Acriflavine is known to suppress the growth of starch-splitting bacteria and thus preserving starch needed for the rapid growth of anaerobic amoebae.
Since Blastocystis sp. does not thrive in the presence of starch, the removal of starch-splitting bacteria helps in the eradication of Blastocystis sp. Gentian violet has been found by Das and Singh (1970) to eradicate flagellates coming from faecal samples during the cultivation of Entamoeba histolytica.
Preparation of nutrient agar for cultivation of Amoebae

The following media were employed:

1. **Glucose and yeast extract**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.2%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.2%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.85%</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0%</td>
</tr>
</tbody>
</table>

   The medium was prepared in phosphate buffer, pH 7.0, and Seitz filtered glucose and 0.01% sterile solution of indigo carmine were added after autoclaving the medium.

II. **Asparagine, glucose and yeast extract**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-asparagine</td>
<td>50 mg</td>
</tr>
<tr>
<td>Mg SO₄·7H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>150 mg</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>350 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>50 mg</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>50 mg</td>
</tr>
<tr>
<td>Agar</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

   Glucose was sterilized by Seitz filtration and was added to the medium after autoclaving along with 0.01% indigo carmine. The pH of the medium was 7.0.
III. Lactic acid and ammonium chloride

Lactic acid   ...   ...   9 g  
\( \text{NH}_4\text{Cl} \)   ...   ...   1 g  
\( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \)   ...   ...   0.1 g  
\( \text{KH}_2\text{PO}_4 \)   ...   ...   1.5 g  
\( \text{Na}_2\text{HPO}_4 \)   ...   ...   3.5 g  
Agar   ...   ...   20 g  
Distilled water   ...   ...   1000 ml.

Indigo carmine (0.01%) was added after autoclaving the medium. The medium had a pH of 7.0.

All the media were kept at 4°C in tubes plugged with cotton wool. A tube contained about 20-22 ml of the medium.

Preparation of viable "sterile" cysts for culture

Singh, Das and Saxena (1963a) have developed a simple and reliable method for obtaining viable sterile cysts of \( \text{E.histolytica} \) from human faeces for monobacterial cultures. They treated the faecal matter, containing the cysts, with 2.0% (w/v) HCl for 48 hours. By the use of this method Robinson (1968a) has also isolated \( \text{E.histolytica} \) in monobacterial culture from human stool. This method was used in the present study to obtain cysts of \( \text{E.histolytica} \) from faeces free of any bacterial contaminations. These cysts were inoculated on \text{Eshk.coli} \) patch in anaerobic petri dish for obtaining monoxenic culture in the plates.
Preparation of plates

About 20-22 ml of nutrient agar was poured into the lid of a dish. After the agar had solidified, a few loopfuls of A. aerogenes or Esch. coli were spread throughout the agar surface and rice starch was mixed with the bacteria. The lid was put in the groove of the dish containing liquid paraffine and nitrogen gas was passed. The plates were incubated at 36°C- 37°C for the bacteria to multiply rapidly and consume oxygen from the agar. After 24-36 hours, when the colour of indigo carmine from the agar had nearly disappeared, a small quantity of a human faecal sample infected with intestinal amoebae was smeared in the centre of the bacterial patch, nitrogen gas was passed and the plates were incubated at 37°C. Amoebae growing on agar were subcultured on freshly prepared plates by cutting a piece of agar containing amoebae and placing it face downwards in the centre of the bacterial patch.

Lead acetate was incorporated in petri dishes in the manner described earlier. The growth of the bacteria on the nutrient media decolourised the colour of indigo carmine as was the case with 0.2% cysteine incorporated into non-nutrient agar.

Different quantities of bovine serum and different concentrations of antibiotics and dyes were added per tube containing nutrient agar at 50°C before pouring the plates.