3. MATERIAL AND METHODS

3.1. **In vitro** cultivation of *E. histolytica*

(a). **Axenic culture**: Axenic *E. histolytica*, strain NIH:200, (kindly supplied by Dr. L.S. Diamond) was used for all experimental studies on axenic amoebae.

**Maintenance of axenic culture of E. histolytica**

For axenic cultivation of *E. histolytica*, Diamond's (1968b) monophasic medium, as modified by Dutta and Yadava (1972) was employed.

Its composition is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase (BBL)</td>
<td>15.0 gm</td>
</tr>
<tr>
<td>Panmede (Paines &amp; Byrne)</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.6 gm</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Cysteine HCl (monohydrate)</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Distilled water, to make</td>
<td>875 ml.</td>
</tr>
</tbody>
</table>

The complete TP-S-1 medium thus prepared was dispensed in 9-10 ml volumes in 15 cm x 125 mm Corning screw capped tubes. The tubes were incubated at 37°C for 24-48 hours to ascertain sterility of the medium.

For subculturing, the tubes showing the good growth of amoebae under the inverted microscope and macroscopically (by the formation of characteristic
white beads at the bottom of the tube), were chilled in ice cold water for 5 minutes. These were then gently swirled between the hands and then centrifuged at 500 g for 5 minutes. 7–8 ml. of supernatant was then discarded with a 5 ml. sterile pipette and after mixing rest of the medium and the sedimented amoebae, 0.5 ml of culture was inoculated in the fresh media tubes. This procedure always gave a good growth of amoebae in 3–4 days, with an average yield of 1.5–2.0 million amoebae per tube.

Preparation of vitamin mixture 107.

Vitamin mixture 107 was prepared by mixing the five stock solutions as described below:

Stock solution (i), water soluble B vitamins:

(a) 25 mg of niacin (nicotinic acid) and 50 mg of p-aminobenzoic acid were dissolved in boiling distilled water and the volume made to 50 ml.

(b) 25 mg niacinamide, 25 mg pyridoxine hydrochloride, 25 mg pyridoxal hydrochloride, 10 mg thiamine hydrochloride, 10 mg calcium pantothenate, 50 mg D-inositol and 500 mg choline chloride were dissolved in distilled water and the volume brought to 50 ml.

(c) 10 mg of riboflavin was added to 30 ml of distilled water and dissolved with the aid of 0.1 N sodium hydroxide added drop by drop, the total volume was then brought to 40 ml.

Solutions (a), (b), and (c) were combined and the volume was brought to 200 ml with distilled water.
Stock solution (ii), biotin solution:

10 mg of D-biotin were dissolved in 50 ml of distilled water with the aid of 0.1N sodium hydroxide and the total volume brought to 100 ml.

Stock solution (iii), Folic acid solution:

10 mg of folic acid was dissolved in 100 ml of distilled water heated to 80°C.

Stock solution (iv), lipid soluble vitamins A, D and K.

(a) 100 mg calciferol (Vitamin D$_2$) was dissolved in 20 ml of ethanol, and to this 100 mg of Vitamin A, crystalline alcoholic, was added and dissolved.

(b) 20 mg vitamin K (menadione sodium bisulphite) was dissolved in 100 ml of 5 per cent (v/v) aqueous solution of Tween 80.

Solution (b) was then combined with solution (a) and the volume made to 1250 ml with distilled water.

Stock solution (v), Vitamin E solution:

10 mg of vitamin E (alpha tocopherol acetate) was dissolved in 100 ml of distilled water.

The working mixture of vitamins was prepared by combining the five primary stock solutions in the following proportions:

200 ml of stock solution (i)  
100 ml of stock solution (ii)  
100 ml of stock solution (iii)  
1000 ml of stock solution (iv)  
100 ml of stock solution (v)

The complete mixture, which was clear, was sterilized by passage through a Seitz filter and stored at -20°C until used.
(b) *E. histolytica* cultures with bacterial associates

Two bacterial strains of *E. histolytica* (KA and its serial liver passaged sub strains and BH-95), isolated from formed and semi-solid stool samples of persons attending Out Patient Departments of Balrampur Hospital and Gandhi Memorial & Associated Hospitals, were maintained in modified B & D medium, without altering their bacterial associates.

**Preparation of modified Boeck & Drbohlav (B & D) medium**

The medium as described by Dutta and Mohan Rao (1966) consisted of slants of inspissated whole egg with an overlay of diluted buffalo serum containing rice starch, was prepared aseptically as follow.

Fresh hen's eggs, washed thoroughly with a detergent and water, were wiped with 70% ethanol. Contents of six eggs were poured into a clean, sterile wide-mouthed glass stoppered bottle containing an appropriate number of sterile glass beads. To this was added 100 ml of sterile K/40 phosphate buffered saline, pH 7.0, and the contents of the bottle were thoroughly emulsified by shaking the bottle.

**Phosphate buffered saline, K/40, pH 7.0**

Stock solution:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄, anhydrous</td>
<td>13.35 g.</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.40 g.</td>
</tr>
<tr>
<td>NaCl</td>
<td>32.00 g.</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>1000 ml.</td>
</tr>
</tbody>
</table>

To obtain working solution, one part of the above stock was diluted with three parts of distilled water. The buffer was sterilized by autoclaving at 15 lbs. for 15 minutes.
Egg emulsion prepared as above was filtered through a sterile muslin cloth and dispensed in four to five ml volumes in cotton-plugged sterile glass tubes (16x150mm). Egg slants were prepared by heating the tubes in slanting position at 80°C for one hour on three successive days. The egg slants were overlaid with buffered serum prepared by mixing one part of sterile and inactivated buffalo serum with seven parts of M/40 phosphate buffered saline. Sterility of the medium was checked by incubating the tubes at 37°C for 48 hours.

Sterilization of rice starch: Fine quality rice starch (HDH) was sterilized by dry heating at 160°C for one hour.

To obtain culture from the faecal samples, the positive sample about half a gram containing cysts of *E. histolytica* was inoculated in the medium. To this was added about 20 mg of rice starch and a sterile solution of acriflavine and gentian violet, each at a final concentration of 1:10,000 (w/v). The tubes were incubated at 37°C for 48 hours. The cultures were then examined microscopically for the presence of amoebae. Sometimes even the negative cultures were subcultured for next 48 hours so as to confirm the results. The cultures were maintained by subculturing every 48 hours. For the purpose of subculturing, a small amount of sediment present at the bottom of culture tube, was withdrawn with a sterile pasteur pipette and inoculated into fresh medium, containing rice starch.

3.2. Cultivation of axenic *E. histolytica* with live bacteria

Modified TF-S-1 medium was inoculated with appropriate bacteria and approximately 20,000 amoebae per ml. of the medium. The tubes were then incubated at 37°C for 6 hours. For virulence studies in the
hamster liver, these cultures were pooled after the incubation period and inoculated intrahepatically in the hamsters.

For growing axenic E. histolytica with live bacteria in the B & D medium, amoebae pooled from 48-72 hours old axenic culture tubes were washed twice with sterile phosphate buffer, pH 7.0, and then inoculated into the B & D medium previously preconditioned with particular bacterial species. By repeated attempts, it was possible to establish cultures with different strains of Escherichia coli (B, E-600, K-1262), Aerobacter aerogenes (B-1) and with mixed bacterial flora of amoebic strain KA.

**Strains of bacteria and their maintenance**

Strains of E. coli (B, E-600 and K-1262) and A. aerogenes (B-1) were routinely maintained on the nutrient agar slants (20.0 g Peptone; 2.0 g Glucose; 5.0g NaCl, 2.5 g Na₂HPO₄ and 20.0g Agar, distilled water 1000 ml, pH 7.0, autoclaved at 15 lbs. for 15 min).

3.3. **Initiation of axenic cultures from small inocula**

48 hours old cultures were selected for initiating small inocula cultures of E. histolytica. Serial dilutions of the cultures were made in axenic medium so as to get 100, 50 and 25 amoebae per ml. One ml each of the above dilutions were then inoculated in the modified TP-S-1 medium, well below the surface level. This gave 100, 50 and 25 amoebae inoculum per tube.

In a series of other experiments, cultures from an inoculum of 2-95 amoebae per tube, were initiated by using capillary tube method. Dilutions of stock culture were made so as to get approximately 500-5,000
amoebae per ml. of the medium in different tubes. Small corning glass capillaries (apprx. 50 mm x 1.5 mm size) were filled aseptically with the above dilutions. These capillaries usually revealed one, two or more amoebae inside, when observed microscopically under magnification of X100 and X400. These capillaries were subsequently wiped with ethanol, inserted and broken aseptically with glass rods, in the media tubes containing no antibiotics. Any tube found contaminated next day was discarded.

3.4. Development of clonal cultures of E. histolytica:

Since it was not possible to count exactly the number of amoebae by dilution method described above, capillary tube method was devised for inoculating clonal amoebae in the tube. For initiating clonal cultures, a number of capillaries were filled up aseptically from cultures containing approximately 500 amoebae per ml of the medium. Both ends of the capillaries were sealed in the flame. Thorough microscopic observations of these capillaries (magnification X100 and X400) revealed either one, two or no active trophozoites at all. The capillaries containing single amoeba were subsequently processed and inoculated in the modified TP-S-1 media tubes as described in small inocula cultures.

3.5. Cryopreservation of E. histolytica strains:

Axenic strain NIM-200 and two bacteria associated strains viz., KAE-6 and BK-95 of E. histolytica were used for investigating the possibility of their cryopreservation at -20°C and -196°C.

(a) Freezing at -20°C: The axenic and bacterial culture amoebae were suspended in TP-S-1 medium to give about one million trophozoites per ml. 15 percent dimethyl sulfoxide (DMSO) solution was prepared in
TP-S-1 medium containing 40 percent buffalo serum which was then mixed with equal volume of above amoebic suspension so as to give a final concentration of 0.5 million amoebae per ml, 25 percent serum and 7.5 percent DMSO. This suspension was distributed in 1.5 ml aliquots in small plastic storage vials (capacity 2 ml). These vials were kept at 28°C for 30 minutes before commencement of freezing (equilibration time). The vials were then transferred to Leonard freeze, maintained at -20°C. The samples were usually frozen after 1-2 hrs of transfer. The samples were checked for survival every week during a total storage period of one month.

(b) **Freezing at -196°C** : After freezing to -20°C, some of the vials of amoebic strains NIH-200, KAH-6 and BH-95 were transferred to liquid nitrogen container by lowering the vials very slowly, taking at least 30 minutes in the vapours and then immersed in the liquid phase. The samples were checked for survival after a storage period of one month.

(c) **Testing the survival of frozen material** :

After the specified period, the frozen samples were taken out and thawed in a water bath at 37°C. The axenic and bacterial samples were once washed with TP-S-1 medium and buffered serum, respectively. The strain NIH-200 was inoculated in media tubes containing TP-S-1, whereas the strains KAH-6 and BH-95 were inoculated in the B & D media. These cultures were then examined for the growth of amoebae.

(d) **Supravital staining with eosin**:

Directly after thawing, one drop of amoebic suspension was mixed with 1 percent isotonic eosin solution on the microscope slide and covered with a cover glass (18x18 mm). After 5 minutes, percentage
of the unstained cells was determined by counting 200 amoebae.

3.6. Storage of Diamond's medium

In order to study the effect of storage of axenic culture medium on the growth of *E.histolytica*, TP-S-1 medium (Diamond, 1968b), and the modified TP-S-1 medium described by Dutta and Yadava (1972), were stored up to the period of 85 days. After storage period, the tubes were inoculated with 100, 1000 and 10,000 amoebae per ml. of the medium. The yield of amoebae per ml. was determined after an incubation period of 12, 8 and 4 days, respectively. All the cultures were run in duplicate and the average of two tubes was quantitated by haemocytometer.

3.7. Influence of trypanosomatids on the growth of *E. histolytica*

Trophozoites of *E.histolytica* were inoculated in the modified Diamond's medium, in association with *T. cruzi* or *L. donovani* (approx. 30 million per tube). The inoculum consisted of 5,000, 1,000 and 100 amoebae per ml. of the medium. The counting of amoebae in duplicate tubes was done 4-8 days after the initiation of cultures.

3.8. Maintenance of *Trypanosoma cruzi* and *Leishmania donovani*

The stock cultures of *T. cruzi* and *L. donovani* were maintained at 27 ± 1°C in NNN medium, containing 1 percent glucose saline as overlay. Subcultures were performed once in a week.
3.9. **Virulence of axenic and monoxenic *E. histolytica***

*E. histolytica* grown axenically or monoxenically with *T. cruzi* or *L. donovani*, was used for virulence studies. The virulence was estimated by intrahepatic inoculation of 1-2 million amoebae in the golden hamsters (*Mesocricetus auratus*) of either sex, weighing 25-60 gms, involving laparotomy under sodium pentobarbital anaesthesia (6 mg per 100 gm body weight given intraperitoneally). In addition, golden hamsters 24-48 hrs old were also inoculated with above cultures without any laparotomy. The animals were sacrificed 7-10 days postinoculation.

The scoring system of the hepatic lesions was the same as described by Dutta (1970). (Grade 0 = No amoebic lesion; 1 = Tiny superficial amoebic lesions covering upto 5 percent of the liver surface; 2 = 5-15 percent of the liver surface showing lesions or inflammation with superficial necrosis; 3 = Gross single or multiple lesions involving less than 25 percent of the liver surface with extensive necrosis; 4 = Acute single or multiple lesions covering more than 25 percent of the liver surface with necrosis and pus, may be extending to the whole thickness of one or more lobes).

3.10. **Role of bacteria in restoring virulence of axenic *E. histolytica***

*E. histolytica* (NII:200 strain) associated with different strains of *E. coli* (B, E-600, K-1262), *A. aerogenes* (B-1) and mixed bacterial flora of amoebic strain KA, in TP-S-1 medium, was tested for the pathogenicity in the hamster liver. Axenic *E. histolytica* established in the B & D medium with the above bacterial strains was also studied for its pathogenic potential.
The amoebae from bacterial cultures were prepared by pooling the sediments from the bottom of 48 hour old culture tubes, washed once with buffered serum and quantitated with a haemocytometer. Their virulence was estimated by intrahepatic inoculation in the hamster liver as already described.

3.11. Influence of cholesterol on the virulence of axenic E. histolytica strains:

Method described by Bos and Van de Griend (1977) was used to feed cholesterol in the axenic cultures of E. histolytica. The solution of cholesterol (E. Merck) in chloroform (3.5 mg cholesterol per ml) was prepared and 0.1 ml of this was dispensed per screw cap tubes. After evaporating the chloroform at 37°C for 3-4 hours, the tubes were sterilized by autoclaving. These tubes containing modified TP-S-1 medium were used for feeding cholesterol to axenic E. histolytica. The amoebae fed with cholesterol for a period of 6-25 days (2 subcultures to 8 subcultures) were tested for their pathogenicity by intrahepatic inoculation in the hamster liver.

3.12. Serial in vivo passage of E. histolytica in hamster liver:

Serial in vivo passage of the hamster liver infected with E. histolytica (strain KA) was performed by laparotomy. A small piece, usually 3-4 mm in size was placed well between the liver lobes. The animals were stitched taking care that inoculated liver piece remained well in its position. The serial liver passages were continued uninterrupted upto six passages, when associated bacterial flora also acquired high pathogenic potential. Amoebae from the liver were then cultured in the B & D medium by acriflavin and gentian violet treatment upto six subcultures. Liver passage was again initiated with these cultures to get further more serial passages.

(a) Cytochemical demonstration of acid phosphatase enzyme.

The trophozoites of *E.histolytica* with or without cholesterol feeding in axenic culture were stained for acid phosphatase activity by the modified method of Gomori (1950). The amoebae were smeared on a clean microscopic slide, and care was taken not to let the smear dry completely before immersing in the fixative. The amoebae were fixed for 30 minutes in 5 percent neutral formalin. After washing the smears well with distilled water, slides were immersed for 30 minutes in incubating medium prepared as follows:

0.01M, sodium β-glycerophosphate was dissolved in 0.05M-acetate buffer pH 5.0, containing 0.004% lead nitrate.

In the control incubating medium, either sodium β-glycerophosphate was omitted or 0.1M sodium flouride was included in the reaction mixture.

After incubation period, the smears were washed with distilled water and immersed in dilute yellow ammonium sulphide for 1-2 minutes. After washing, the smears were mounted in glycerine jelly.

(b) Biochemical studies on lysosomal enzymes of *E.histolytica* fed with cholesterol

Preparation of enzymes

For assaying lysosomal enzymes, the amoebae were homogenized in the buffered sucrose 0.25M (Tris-HCl 20mM, pH 7.2). The homogenate was centrifuged at 2,000 g for 15 min to remove nuclear debris and the supernatant
was centrifuged at 16,000 g for 40 minutes. The lysosomal pellet was resuspended in the same medium and the lysosomes were ruptured by freezing and thawing six times, and subsequently used to assay acid proteinase, acid phosphatase, acid ribonuclease and acid deoxyribonuclease.

Assay of enzymes activities

Acid Proteinase

Acid proteinase activity was determined according to slightly modified method of Anson (1938). The assay mixture of 2.0 ml contained 100 μ moles of acetate buffer, pH 5.0, 70 μ moles of cysteine hydrochloride, 0.05% (w/v) haemoglobin and 0.3 ml suitably diluted enzyme. Tubes were incubated at 37°C for 60 min with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) prechilled trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. In controls the enzyme was supplemented after the addition of cold TCA. A suitably diluted aliquot of the protein free supernatant was taken for the colour development with Folin and Ciocalteau's reagent (Lowry et al., 1951).

Unit of enzyme was that amount which liberated one μ mole of tyrosine in 60 min.

Acid Phosphatase

Acid phosphatase activity was assayed according to a slightly modified method of Nelson (1966). The reaction mixture in a final volume of 2.0 ml contained 0.5 ml suitably diluted enzyme, 6 μ moles of ethylenediaminetetraacetic acid (EDTA-sodium salt) 75 μ moles of sodium β-glycerophosphate (pH adjusted to 5.0) and 150 μ moles of Walpole buffer, pH 5.0. The tubes in duplicate were
incubated at 37°C for 60 min with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) cold TCA and the precipitate was removed by centrifugation at 1,500 g for 15 min in cold. The inorganic phosphate (Pi) liberated was measured in the clear supernatant by the method of Fiske and Subba Row (1925).

One unit of enzyme activity was that amount of enzyme which liberated one μ mole of Pi at 37°C in 60 min.

**Acid Ribonuclease**

Acid RNase activity was determined according to a slightly modified method of Ishihara et al. (1967). The typical reaction mixture in a final 2.0 ml volume contained, 0.5 ml suitably diluted enzyme, 200 μ moles of Walpole buffer, pH 5.0 and 0.2% (w/v) purified ribonucleic acid. The incubation was carried out at 37°C for 30 min with occasional shaking. The tubes were then cooled in an ice bath and the reaction was terminated by the addition of 1.0 ml of Mac Fadyan reagent (0.75% (w/v) uranyl acetate in 25% (w/v) perchloric acid) followed by immediate shaking. After a period of 30 min in cold (0-4°C), the contents were centrifuged at 1,500 g for 15 min and an aliquot of supernatant suitably diluted and the absorbancy was read at 260 nm in a Beckman model DU spectrophotometer. The assays were always run in duplicate and under optimal conditions of hydrolysis.

One unit of acid ribonuclease enzyme activity was defined as that amount of enzyme capable of bringing about an increase in OD at 260 nm by 0.001 per min under experimental conditions.
Acid Deoxyribonuclease

The assay of acid DNase was performed according to a slightly modified method of Irie et al. (1966). The final 2.0 ml reaction mixture contained 0.2% (w/v) purified deoxyribonucleic acid dissolved in 200 μmoles of acetate buffer, pH 5.0, 5 μ moles of MgCl₂ and 0.5 ml of suitably diluted enzyme. The tubes in duplicate were incubated at 37°C for 30 min, then chilled in ice bath and the reaction was stopped by the addition of 1.0 ml of Mac Fadyan reagent, followed by immediate shaking of the tubes. After a lapse of 30 min in cold, the contents were centrifuged at 1,500 g for 15 min and an aliquot of the clear supernatant was suitably diluted and the absorbancy measured at 260 nm in a Beckman model DU spectrophotometer.

One unit of deoxyribonuclease enzyme activity was the amount of enzyme capable of bringing about an increase in OD at 260 nm by 0.001 per min under experimental conditions.

Specific Activity

Specific activity of the enzyme was expressed as units of enzyme per milligram protein.

Estimation of protein

The protein contents of the enzyme preparations were estimated colorimetrically using a slight modification of Lowry's method (1951).

8.0% (w/v) solution of anhydrous sodium carbonate was mixed with equal volume of a solution containing 0.06% (w/v) of cupric sulphate and 0.12% (w/v) sodium potassium tartarate. Solutions containing 10 to 150 micrograms of protein were mixed with 5.0 ml of the above reagent, incubated for 10 min at 37°C and then
cooled to room temperature. 0.5 ml of the twice dilute Folin phenol reagent was added and the colour developed was read at 660 nm after 30 min against a proper blank in Klett Summerson photoelectric colorimeter using bovine plasma albumin as the standard. Under these conditions one klett reading was found to be equal to one microgram of the standard protein solution.

(c) Preparation of amoebic samples for transmission electron microscopic studies:

The trophozoites of axenic E. histolytica, NIH-200, with or without cholesterol feeding were collected by centrifugation at 1,000 rpm for 1 minute, and washed twice with normal saline. The sediment containing amoebae was fixed in 5% glutaraldehyde in 0.1M cacodylate buffer (pH 6.8) for 5 hours.

The fixed amoebae were spun at 5,000 rpm for 10 minutes to get a heavy pellet. They were washed 6 times in 0.1M cacodylate buffer (pH 6.8) and doubly fixed in 2% osmium tetroxide (made in cacodylate buffer of same molarity and pH) and kept at 4°C for 4 hours. The pellet was then washed once in distilled water and kept in 1% uranyl acetate overnight. It was then dehydrated through graded alcohol and finally embedded in resin Spurr and araldite 6005 mixture (Mollenhauer, 1964). Silver sections were cut by LKB-Ultratome-III using a glass knife. Sections mounted in copper grids were stained in lead citrate (Reynolds, 1963) for 4 minutes. The observations were made with a Hitachi HU-II E electron microscope operated at an accelerating voltage of 75 kv and photographed on Fuji Electron Microscopic Orthochromatic Sheet Film.
(d) Cytotoxicity effect of *E. histolytica* trophozoites on leucocytes

The peritoneal exudates of guinea pig was taken out by injecting 50 ml of normal saline intraperitoneally 16 hrs before the experiment. At the time of experiment, 20 ml more normal saline was injected intraperitoneally with little massage on the abdomen. The peritoneal fluid was then taken out with a 18 gauge needle and the counts determined. Leucocyte count was 3-4 million/ml of the fluid.

*E. histolytica* trophozoites, from 48 hr cultures with and without cholesterol feeding were pooled and washed twice with normal saline. Amoebae and leucocytes were mixed to give a final concentration in the proportion of 1:5, 0.05 ml of this mixture was then placed on a clean slide and then covered and sealed with paraffin wax. The slide was examined at 37°C to determine the cytotoxic effect of amoebae on leucocytes.

(e) Concanavalin A agglutination of *E. histolytica* strains:

Trophozoites from two different strains of *E. histolytica* cultured under axenic, and mixed bacterial conditions were used for this experiment. Actively growing amoebae were washed three times with phosphate buffered saline (PBS, pH 7.2) by centrifugation at 1000 rpm for 5 min. The viability as determined by the exclusion of eosin stain, was higher than 95%. The final concentration of amoebae in PBS was brought to $1.0 \times 10^6$ per ml. Subsequently, 0.05 ml of amoebeic suspension was mixed by slow shaking with 0.05 ml of Con A diluted in PBS (final concentrations of 10, 100 and 200 μg per ml), covered with a coverslip and sealed by Vaseline and incubated at 36°C for 15-30 minutes.
The agglutinability was estimated at the end of the incubation period as a function of number of amoebae forming the aggregates.

3.14. **In vitro testing of amoebicidal drugs:**

Axenic *E. histolytica* NIH-200, was used for testing the amoebicidal action of commercially available known antiamoebic drugs.

(a) **Effect of pH on in vitro activity of drugs:**

Axenic medium adjusted to pH 7.0 and 5.8, was dispensed in 9 ml volumes in screw capped corning tubes (125 mm x 15 mm size). After checking the sterility overnight, 0.5 ml of the sterile drug dilutions (prepared in normal saline) were added to each tube, and in controls 0.5 ml normal saline was added. After about 30 minutes, 200,000 amoebae in 0.5 ml of the medium, were inoculated in each tube, so as to obtain final count of 20,000 amoebae per ml. Seven systemically active amoebicides, viz., chloroquine phosphate, emetine hydrochloride, dehydroemetine dihydrochloride, mepacrine, camoquin, metronidazole and ambilhar were used. In addition, twelve luminal amoebicides, intestopan, enterovioform, camoform, emetine-bismuth-iodide, furamide, entobex, chlorhexidine dihydrochloride, diodoquin, carbarsone, mebinol, stovarsol and yatren were also employed to study effect of pH on the amoebicidal action of these amoebicides.

The tubes were incubated for 3 to 6 days at 36°C ± 1°C. The amoebicidal end point was recorded by direct observation under the inverted microscope and by subculturing in the fresh medium. The drug dilution at which no motile amoebae could be observed microscopically or after subculturing was regarded as the
amoebicidal end point of the drug. At the drug dilutions of systemic amoebicides at which amoebae were surviving, total count of amoebae was made by haemocytometer. All the tests at pH 7.0 and 5.8 were run in duplicate.

(b) **Influence of active phagocytosis on amoebicidal action of systemically active amoebicides**

_**E.histolytica**_ cultures grown in association with promastigotes of _Leishmania donovani/_epimastigote of _Trypanosoma cruzi_, 30-40 million per tube, were used for testing amoebicidal action of seven systemic amoebicides, at pH 7.0. The end point was recorded after incubating the tubes at 36 ± 1°C for 72 hrs.

(c) **Effect of redox potential (L-cysteine HCl, concentration) and inoculum on action of metronidazole.**

The modified TF-S-1 medium was prepared with different cysteine HCl concentrations, viz., 0.05, 0.20 and 0.40 percent, pH 7.0. The action of metronidazole against axenic _E.histolytica_ was determined at these concentrations of cysteine HCl, as described earlier. The action of metronidazole was also determined against different inocula of amoebae.

(d) **Combined action of some amoebicidal drugs against E.histolytica**

Some combinations of drugs viz., metronidazole + emetine hydrochloride; metronidazole + dehydroemetine dihydrochloride, metronidazole + diloxanide furoate, metronidazole + chloroquine, chloroquine + emetine hydrochloride, chloroquine + dehydroemetine dihydrochloride and furazolidone + enterovioform, were tested for their additive or synergistic action against axenic _E.histolytica_, by the usual method described above.
3.15. Effect of amoebicidal drugs on the incorporation of a radioactive precursor.

\(^{14}\)C-L-leucine (Sp.Act. 76 mCi/m mole, Bhabha Atomic Research Centre, Bombay) was used as the radioactive precursor, for studying its incorporation in axenic \textit{E. histolytica}, and the effect of amoebicidal drugs on its uptake. Trophozoites from logarithmic cultures were pooled and washed twice by centrifugation (850 g x 5 min.) with phosphate buffered saline, pH 7.2. They were suspended in Hanks balanced salt solution containing L-cysteine hydrochloride 0.2 percent, pH 7.2, to give concentration of about \(5 \times 10^6\) trophozoites per ml. Hanks balanced salt solution, containing cysteine hydrochloride was dispensed in 0.6 ml volumes in small screw capped tubes (size 15 mm x 50 mm). To this was added 0.1 ml of the \(^{14}\)C-L-leucine (final concentration of 1 uCi per ml). Serial dilutions of the drugs were added in 0.1 ml volumes per tube. In the end 0.2 ml of the above amoebic suspension was inoculated so as to give a final volume of 1 ml per tube containing \(1 \times 10^6\) active trophozoites. All samples were run in duplicate and incubated at 36 ± 1°C, for 6 hrs. Surviving amoebae were observed under the microscope. The kinetics of \(^{14}\)C-leucine incorporation was studied over periods of zero, one, two, four, eight, twelve and twenty hrs.

**Determination of \(^{14}\)C-leucine incorporation**

The incubation mixtures were chilled in ice, centrifuged at 850g x 5 min, and washed twice with 10 ml of Hanks balanced salt solution. The sediment was precipitated by adding 4-5 ml of 10 percent TCA. The precipitate was then washed once each with ethanol and ether. It was then dissolved in warm 0.5 ml, 2N sodium hydroxide solution. Radio activity was determined
by inoculating 0.2 ml of the dissolved precipitate in 10 ml of diatol solution, in Packard liquid scintillation counter.

**Preparation of Diatol solution (Scintillant).**

**Solution I.**

- Toluene = 250 ml.
- 2,5-diphenyloxazole (PPO) = 3.25 gms.
- 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) = 0.065 gms.

PPO and POPOP were dissolved in toluene.

**Solution II.**

- Methanol = 150 ml.
- Dioxan = 250 ml.
- Naphthalein = 52 gm. (scintillation grade)

First naphthalein was added to methanol in a beaker and shaken well. Then dioxan was also added and mixture was stirred well with a glass rod to dissolve naphthalein.

Solution I and II were then mixed to prepare final diatol solution.

The diatol solution was stored in amber coloured tightly stoppered glass bottle, and was not used after a storage period of more than 10 days.

3.16. **In vivo screening of amoebicidal drugs against hepatic amoebiasis in golden hamsters**

Golden hamsters of both sexes, usually weighing between 40-80 gms. were used. The animals were obtained from the breeding stock of Animal House of Central Drug Research Institute and maintained on diet consisting of pellet, fresh lettuce and water, *ad libitum*. 
Animals within an experiment were infected by inoculation between the liver lobes a small piece of amoebic liver lesion from an infected hamster. The animals were then divided randomly into subgroups of six each. One subgroup received no medication while the others were treated with different drugs. The animals which died next day after infection were excluded from the study. Drugs included in this study were emetine, dehydroemetine, metronidazole, tinidazole, chloroquine, mepacrine, amodiaquine and ambilhar. Emetine and dehydroemetine were given intramuscularly, while all other drugs were administered orally. All drugs were given as aqueous solutions or suspensions for 4 days (two doses were administered orally daily). The first dose was given just after infection and second six hours after the infection. The other 6 doses were given in the morning and later afternoon for next 3 days.

The animals were sacrificed on 5th day of infection and the effect of therapy determined by comparison between untreated and treated groups of (i) infectivity of animals (ii) the grade of liver lesion and (iii) demonstration of amoebae in the liver.