6. SUMMARY

I. Studies on cultivation of *Entamoeba histolytica*

(a) Small inocula cultures: In order to establish small inocula cultures of axenic *E. histolytica*, a stable negative oxidation-reduction (O-R) potential has been found to play an important role. The modified TP-S-1 medium used in the present study provides a strongly negative O-R potential favourable for culturing axenic *E. histolytica* from small inocula.

Two methods have been developed for initiating cultures from small inocula:

1. By serial dilution: By serial dilution of the cultures, it was possible to get regular cultures of *E. histolytica* from an inoculum of 25-100 amoebae per tube. With an inoculum of 25 amoebae nearly 20,000 fold multiplication was observed after 13-15 days.

2. Capillary tube method: In other experiments, successful cultures could be raised from 2-95 amoebae, sealed in sterile capillary tubes and inoculated aseptically into axenic medium without any antibiotics. This method was useful for inoculation of counted numbers of amoebae in the tubes. Amoebae initially colonize in the capillary tubes and can be observed with the help of inverted microscope.

The earlier workers believed that association of *E. histolytica* with *T. cruzi* or *Crithidia* sp., and the incorporation of agar in the medium, was essential for initiating small inocula cultures. However, by the improved methods used in this study, the small inocula cultures of *E. histolytica* could be initiated with ease without trypanosomatid associates or addition of agar in the medium.
(b) **Clone cultures**: A new method of initiating clonal cultures of axenic *E. histolytica*, has been developed. The method does not involve the association of trypanosomatids or addition of agar in TP-S-1 medium. Small sterile glass capillary tubes containing single trophozoite of *E. histolytica* were inoculated in modified TP-S-1 medium. These clonal cultures produced abundant growth of amoebae in 15-20 days.

Stable redox potential and sterility of the medium were the major controlling factors for successful development of clone cultures. The original TP-S-1 medium, which contains ascorbic acid, did not provide a stable O-R potential and was therefore, not suitable for initiation of clone cultures. The addition of even small amounts of antibiotic mixture in axenic culture medium, had an adverse effect on the clonal cultures.

(c) **Cryopreservation.** Axenic and bacterial cultures of *E. histolytica* with the addition of dimethyl sulphoxide (7.5 percent v/v concentration) were cooled slowly to -20°C and -196°C. The amoebae were stored in small plastic vials. The successful recovery of the cryopreserved amoebae was tested by establishing cultures of rapidly thawed amoebic suspensions and also by staining with eosin. In general, all bacterial cultures could be recovered successfully after storage at -20°C as well as -196°C, for one month. The axenic cultures, at -196°C, however, showed variable recovery, though at -20°C they could be recovered successfully, after storing for one month.

(d) **Successful long-term storage of axenic medium**: Modified TP-S-1 medium (prepared without ascorbic acid but containing 0.2 percent L-cysteine HCl) could support better growth of axenic *E. histolytica*, even
after a storage period of 85 days. The original TP-S-1 medium of Diamond, which contained ascorbic acid did not provide favourable conditions after storage. Interaction of ascorbic acid with cysteine provides an unstable redox potential, shifting it to positive side, which is lethal to amoebae. The modified TP-S-1 medium, which provides a stable negative redox potential, could be used after long storage, for initiation of cultures even from inocula as small as 100 amoebae per ml, which was not possible with the original axenic medium.

(e) **Growth of amoebae with trypanosomatid associates:**

The association of axenic *E. histolytica* with *L. donovani* promastigotes or *T. cruzi* epimastigotes in modified TP-S-1 medium provided a slightly increased multiplication of amoebae. The quantitative differences in growth of amoebae in axenic, A-L and A-T cultures were estimated at various intervals of incubation. The growth in monoxenic cultures was more than the axenic and the association with *T. cruzi* epimastigotes was comparatively more useful than *L. donovani* promastigotes.

II. **Virulence of *E. histolytica***

(a) **Virulence of axenic and monoxenic amoebae**:

New born and weanling hamsters were inoculated intrahepatically with axenic (NIH-200) and monoxenic (with *Leishmania donovani* and *Trypanosoma cruzi*) cultures of *E. histolytica*. Several attempts made to produce amoebic infections from these cultures proved unsuccessful. Association of axenic amoebae with *Escherichia coli* (B, E-600, K-1262) and *Aerobacter aerogenes* (B-1) for six hours in axenic culture medium also failed to produce any amoebic lesions in the hamsters.
(b) Role of bacteria in restoration of virulence:

Attempts to revive the virulence of axenic *Entamoeba histolytica* (NIH-200) were initiated by its association with mixed bacterial flora in the B & D medium. Establishment of amoebae with bacteria in the B & D medium resulted in the production of amoebic lesions of 2 grade in hamster liver, on 30th day of association. Duration of bacterial association was correlated with severity of the infection in the hamsters.

(c) Restoration of virulence by cholesterol:

To restore the virulence of axenic culture, the TP-S-1 medium was supplemented with extra cholesterol. The amoebae were cultured in the cholesterol rich axenic medium for 15 days (5 subcultures). The inoculation of cholesterol fed amoebae in the liver of weanling hamsters produced distinct hepatic lesions in these animals. The isolation of amoebae from the liver lesions in cultures, and further feeding with cholesterol resulted in the increased infectivity rate of hamsters. Serial in vivo passage of the infected liver lesions produced 100 percent infection in the hamsters.

(d) Serial in vivo passage of *E. histolytica*:

Initially, the inoculation of a bacterial culture of *E. histolytica* (strain KA) produced very small lesions in the hamster liver. The infectivity being 16.6 percent only. The virulence of strain KA was greatly enhanced by serial infected liver passages in the hamsters, with simultaneous decrease in the survival period from 6 to 3-4 days after 4-6 liver passages. The increased virulence of amoebae by this method resulted in the formation of acute amoebic abscesses containing liquefied material and necrosed liver cells in its interior.
The mechanism of triggering the virulence of *E. histolytica* by cholesterol feeding was investigated by studying the following parameters.

1. **Phagocytosis**: Light microscopy study of normal and cholesterol fed axenic *E. histolytica* revealed an enhanced phagocytosis of the amoebae cultured in TP-S-1 medium supplemented with extra cholesterol.

2. **Cytochemical study of acid phosphatase**:

   The cytochemical studies on acid phosphatase of normal and cholesterol fed axenic amoebae showed an apparent increase in acid phosphatase activity of cholesterol fed amoebae. The enzyme activity in cholesterol fed amoebae was more intense and prominent than in normal axenic amoebae.

3. **Biochemical estimation of lysosomal enzymes**:

   Biochemical studies on the effect of cholesterol feeding on the lysosomal enzymes of axenic *E. histolytica* revealed an enhanced activity of these enzymes as compared to normal axenic amoebae. The activities of acid phosphatase, acid proteinase, acid ribonuclease and acid deoxyribonuclease were markedly increased.

4. **Ultrastructural changes in cholesterol fed amoebae**:

   Transmission electron microscopy studies on the normal and cholesterol fed amoebae revealed some significant alterations in the cytoplasm and nucleus of the latter. The cholesterol fed amoebae revealed more and large sized cytoplasmic vacuoles as compared to normal amoebae. In the nucleus, distinct 'intranuclear bodies' of high electron density were more in the cholesterol fed amoebae, but few in the normal amoebae.
5. Cytotoxicity: A comparative cytotoxic effect of normal and cholesterol fed amoebae on the guinea pig leucocytes was determined. Cholesterol fed amoebae were found more aggressive and caused on an average cytotoxic effect on 40-50 percent of leucocytes. The normal amoebae killed only 5-6 percent of leucocytes.

6. Concanavalin A induced agglutination: Con A induced agglutination of pathogenic and non-pathogenic strains of *E. histolytica* was also determined. Pathogenic strains NIH(B)-200, KAH-8 and axenic NIH-200 fed with cholesterol were strongly agglutinated by low concentration of con A (10 µg./ml.). The non-pathogenic, axenic NIH-200 amoebae failed to agglutinate under these conditions.

III. Chemotherapeutic studies on *E. histolytica*

(a) Influence of pH on amoebicidal activity of drugs:

In vitro testing of amoebicidal activity of known amoebicidal drugs at pH 7.0 and 5.8 was evaluated using modified Diamond's medium. Among the systemically active amoebicides, metronidazole and ambilhar were equally active at both pH. Other drugs, however, showed high amoebicidal activity only at pH 7.0, viz. Chloroquine 500 µg., emetine and dehydroemetine 7.8 µg., mepacrine and amodiaquine 15.6 µg., per ml. At acidic pH 5.8, Chloroquine showed amoebicidal activity at 4000 µg., emetine and dehydroemetine at 125 µg., mepacrine at 125 µg. and amodiaquine at 62.5 µg., per ml. Since the pus in amoebic liver abscess cases is acidic (pH 5.5-5.8), it is quite likely that occasional failure of emetine, dehydroemetine and chloroquine therapy in unaspirated abscess cases, might be possibly due to very low activity of these drugs at acidic pH due to the presence of pus.
In vitro testing of 12 luminal amoebicides (intestopan, enterovioform, camoform, diloxanide furoate, entobex, chlorhexidine dihydrochloride, diiodoquine, carbarsone, mebinol, stovarsol, chiniofon and furazolidone) did not show any significant variation in amoebicidal activity at acidic pH as compared to neutral pH. These studies suggested that activity of these drugs would not be decreased by the acidity which prevails in diarrhoeal stools.

(b) **Effect of phagocytosis on amoebicidal activity of drugs**:

In vitro active phagocytosis of trypanosomatids by trophozoites of *E. histolytica* simulated an in vivo condition of amoebae feeding on luminal contents. The active phagocytosis in vitro did not influence the amoebicidal activity of 7 systemically active drugs.

(c) **Influence of redox-potential and inocula on amoebicidal action of metronidazole**.

Different concentrations of cysteine hydrochloride (0.05-0.40 per cent) in modified TF-S-1 medium produced different levels of negative O-R potentials. This variation in redox potential had no effect on the amoebicidal action of metronidazole. Similarly effect of different amoebic inocula in the test medium did not effect the amoebicidal activity of metronidazole.

(d) **Combined action of drugs on *E. histolytica***:

Several combinations of amoebicidal drugs which are currently used by clinicians for the treatment of amoebic patients, were compared in vitro for their amoebicidal activity against axenic *E. histolytica*. Out of several combinations namely: metronidazole + emetine or dehydroemetine; metronidazole + diloxanide
furoate; metronidazole + chloroquine; chloroquine +
emetine or dehydroemetine and furazolidone + entero-
vioform, distinct enhanced amoebicidal activity was
observed only with the combination of emetine and
dehydroemetine with chloroquine. The activity of
emetine and dehydroemetine was increased 4-8 fold than
the activity of individual drugs.

(e) Effect of amoebicidal drugs on the incorporation of
14-C-leucine :

The effect of known amoebicidal drugs on the
incorporation of 14-C-leucine was studied in a defined
medium. 50 percent inhibition of uptake of labelled
leucine (median effective dose, MED), was measured in
the presence of various drugs. The MED was found to be
16 µg. per ml for ambilhar, 28 µg per ml for metronidazole
14 µg per ml for emetine and dehydroemetine, 175 µg per ml
for mepacrine and 2,000 µg per ml for chloroquine.

These studies revealed a distinct correlation
between the MED and in vitro activity of these drugs
against axenic E. histolytica. This method can be used
for quantitative determination of amoebicidal activity
of test compounds.

(f) A new method of producing hepatic amoebiosis for
in vivo screening of drugs :

By serial in vivo passage of the infected liver,
large number of hamsters could be infected from one
donor hamster. This method has been found valuable for
in vivo screening of amoebicidal agents against hepatic
amoebiasis. The known amoebicidal drugs used in the
present study showed high activity. Emetine or
dehydroemetine administered intramuscularly (4 mg/kg/day)
suppressed the development of amoebic lesions completely.
Metronidazole and tinidazole (100 mg/kg/day) given orally, also completely inhibited the formation of any amoebic abscess. Chloroquine was effective only at 200 mg/kg dose, at 100 mg/kg, 60 percent animals developed amoebic abscess. Kepacrine (200 and 100 mg/kg/day) was also effective for the adequate elimination of amoebae from liver. Amodiaquine was effective at 200 mg/kg dose, at 100 mg/kg dosage 1 out of 5 hamsters developed a small hepatic lesion. Ambilhar at dosages of 100 and 50 mg per kg was not able to produce 100 percent cure in the animals. At 100 mg/kg 1 out of 5, and at 50 mg/kg, 2 out of 5 hamsters developed amoebic lesions.

The present method of infecting the hamsters was simple and reliable, as the animals do not die of bacteremia following inoculation of a small infected liver piece. The untreated animals always showed 100 percent infection by this method.