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Title: CYTOPATHIC EFFECT OF ENTAMOEBA HISTOLYTICA

ON HEPATOCYTES OF THE SYRIAN HAMSTER.

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The cytopathic effects of intact Entamoeba histolytica (NIH:200) and cell-free extracts of this protozoan on hamster hepatocytes were studied using a trypan blue dye exclusion assay and a lactate dehydrogenase (LDH) assay. Contact lysis was observed in synxenic culture experiments in agreement with the work of several investigators. However, the effects of the sonicate of Entamoeba histolytica on the hepatocytes were ambiguous; though some damage was indicated by the assays, especially the trypan blue assay, lysis was not total even after 4.5 hours. The LDH assay failed to reveal sublethal effects prior to cell death indicated by the trypan blue dye exclusion assay. In one experiment using parabiosis chambers partial damage of the hepatocytes resulted from the products of living Entamoeba histolytica. Before carrying out the toxicity experiments, assays for the selection of the medium were done using the same number of hepatocytes (10^6) in TPS-I medium and in Fisher's medium, with cell-free controls. The results showed that the hepatocytes did better (i.e. leaked less LDH) in TPS-I than in Fisher's. Virulence of E. histolytica (NIH:200) was evaluated in hamster liver following

subcapsular inoculation of 0.2 ml at $4.13 \times 10^6 \text{ ml}^{-1}$ trophozoites.

Hepatic lesions were noticed after 7-9 days.

Cytopathic Effect of Entamoeba histolytica
on Hepatocytes of the Syrian Hamster

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CYTOPATHIC EFFECT OF ENTAMOEBA HISTOLYTICA
ON HEPATOCYTES OF THE SYRIAN HAMSTER

INTRODUCTION

Taxonomy

Entamoeba histolytica is a protozoan parasite inhabiting the colon of man and other vertebrates. It has a worldwide distribution (5% incidence in the U.S. and 40% in the tropical countries (Albach and Booden (1978))). Losch (1875) was the first to describe E. histolytica and indicate its infection in humans. According to Honigberg et al. (1964) the taxonomic position of E. histolytica is as follows:

Superclass	-----	Sarcodina
Class	-----	Rhizopodea
Subclass	-----	Lobosia
Order	-----	Amoebida
Family	-----	Endamoebidae

Morphology and life cycle

The biology of this protozoan has been recently reviewed by Albach and Booden (1978). Two stages are found during the life cycle of E. histolytica. First, the trophozoite is the actively feeding stage found in the intestine and in the freshly passed unformed stool of a patient with amebiasis. Trophozoites vary from approximately 10 μ m to 60 μ m in diameter (averaging 20-30 μ m), and crawl along the intestine with blunt pseudopods. They possess a spherical nucleus with a centrally placed endosome; the chromatin granules are arranged on the inner surface of the nuclear membrane. Food vacuoles in the cytoplasm contain ingested host erythrocytes and glycogen granules.

Mitochondria are absent as this species is anaerobic. Ribosomes are found either in fibrous rods or free (Kress et al., 1971). Thick fibrils (Michel and Schupp 1975), microtubules and surface active lysosomes are also present (Eaton et al., 1969). Dehydration of the fecal matter in the posterior part of the large intestine stimulates the trophozoites to form the next stage, which is the cyst; this is the infective stage and is usually passed in formed stools. Cysts are generally not found in unformed stools. Furthermore, trophozoites which are passed in unformed stools are unable to encyst, and encystment does not take place in invaded tissue. The first stage of encystment when the trophozoite rounds up and eliminates all undigested food material is termed the pre-cyst. A glycogen vacuole is present. The cyst is formed when the precyst secretes a hyaline cyst wall around itself. Precysts and cysts are usually passed in semiformed stool. The single nucleus of the cyst divides to form a 4-nucleated metacyst, which is passed in the formed stool of a carrier and is the infective stage. Depending on the environmental conditions, metacysts are viable for 12-30 days. Following ingestion, excystation takes place in the alkaline conditions of the ileum and the 4-nucleated amoeba divides to form 4 uninucleated amoebae, which feed and grow into trophozoites and multiply within the mucosal crypts of the colon. They feed on starches and mucosal secretions and interact metabolically in poorly defined ways with the enteric bacteria. Occasionally E. histolytica leaves the lumen of the intestine and becomes invasive. Tissue invasion is possible by lysis of mucosal cells (Schmidt and Roberts 1977). Studies have shown that under

optimum conditions of pH and ionic concentration, nonvirulent E. histolytica become virulent (reviewed by Schmidt and Roberts, 1977). Erosion of the intestinal wall by the parasite causes ulcers; the organism erodes through the submucosa into the blood vessels thereby gaining access to the liver, lungs or skin via the blood stream. It is not known what exactly stimulates the trophozoites to become invasive. E. histolytica has an essential requirement for specific carbohydrates, and it has been suggested (Getteridge and Coombs, 1977) that a deficiency of available substrate in the lumen of the intestine brings about the migration of the trophozoites to the carbohydrate-rich tissues of the wall. Other studies imply that amoebae may invade intestinal mucosa which was previously damaged by other intestinal disorders, such as bacterial colitis (Shaffer et al. 1965).

Pathogenesis at the tissue level

The symptoms of intestinal amebiasis are colitis, fever, and amebic diarrhea (with resultant dehydration) with flecks of blood-tinged mucus in the feces (Albach and Booden, 1978). E. histolytica is the only intestinal amoeba known to be capable of host tissue lysis (Schmidt and Roberts 1977). Intestinal lesions are the result of the breakdown of the mucosal lining. On reaching the submucosa, the trophozoites spread laterally (because of the muscular layer below) forming the characteristic "flask-shaped" ulcers. More invasive forms cause intestinal lesions which are sometimes followed by intestinal obstruction by amoeboma, hepatic amebiasis and pulmonary amebiasis. E. histolytica can also be found in the brain, skin, penis, kidneys,

adrenal glands and spleen. Encystment does not take place in these infected tissues. Because of its varied pathogenesis there are still many unanswered questions relating to host-parasite relationships and mechanisms of lysis and invasion of tissues by E. histolytica.

Pathogenesis at the cellular level

The mechanisms of cellular destruction are still not fully understood. Both pathogenic and nonpathogenic strains have proteolytic enzymes. Maegraith (1963) showed that the strains of E. histolytica he used were capable of digesting epithelial proteins in vitro; this was presumed to be due to the active trypsin. Eaton et al. (1969) described enzyme-containing organelles or lysosomes as a feature of the surface of E. histolytica on cultured cell monolayers (RK₁₃, Chang's liver cells and HeLa cells). Eaton et al. (1970) further confirmed their observations. Furthermore, lysosomes were reported to possess a "trigger mechanism" which may be involved in tissue invasion. The lytic effect of lysosomes on tissue cells was also suggested by Proctor and Gregory (1972) on the basis of observations of surface active lysosomes in the trophozoites of E. histolytica from the human colon. Studies on the toxic effect of E. histolytica on leukocytes (human, sheep, rabbit and chicken) by Jarumilinta and Kradolfer (1964) showed that several strains of E. histolytica produced identical changes in live leukocytes of all vertebrate species. After one or two minutes of contact with E. histolytica there was rapid and extensive lysis of cytoplasmic

granules of the leukocytes. It was assumed that "the noxa of E. histolytica may disrupt the lysosomes in the leukocytes, thereby releasing degradative enzymes which cause digestion of other structural elements in the leukocytes and result in cell death and damage." However, there was no definite evidence to confirm that this is the mechanism by which E. histolytica exert toxic influence on leukocytes.

Electron microscope studies of E. histolytica infection in the guinea pig by Takeuchi and Phillips (1975) presented evidence of the mode of penetration of the cecal epithelium by the parasite. It was suggested that the toxic substance produced by the parasite could be responsible for the early focal changes observed in the cecal epithelia. As the amoebae moved towards the epithelial cells, electron dense material was noticed to develop and localize between the advancing portion of the organism and the microvilli. It was not proved whether this glycocalyx was secreted by the amoebae, was the product of both amoebae and epithelial sources, or if it was involved in the observed lysis of epithelial cells.

Studies by the same authors on the epithelial penetration by E. histolytica showed a series of "degenerative changes in trans-epithelial migrating polymorphonuclear leukocytes (PMN). This occurred in the PMN in the immediate vicinity of the amoebae with and without direct contact." Although the precise nature of PMN lysis was not clear, it was suggested that lysates of PMN would probably contribute to changes in host tissue, including separation of cells.

Work on human erythrocyte phagocytosis by different strains of Entamoeba grown in culture (Trissl et al. 1978) showed that the rate of erythrocytic ingestion was higher by the invasive E. histolytica strains than by the nonpathogenic strains. Lopez-Revilla and Said-Fernandez (1980) studied the hemolytic activity of trophozoite homogenates of seven strains of amoebae against the erythrocytes of eight mammalian species; the hemolytic activity observed in vitro correlated with invasive tendencies in vivo, and was dose-dependent and strain-specific. Many subplasmalemmal vacuoles were noted on the sections of trophozoites in human liver and intestine by Deas and Miller (1977). Some vacuoles were adjacent to plasmalemmal extensions and some were not, and in some sections the extensions were seen to contact the host tissue. Their hypothesis was that "the membrane of the plasmalemmal vacuole moves into the plasma dendritic extension via membrane flow and may in certain areas fuse with the plasmalemmal membrane thereby translocating the cytotoxic enzyme in the process. Membrane to membrane contact of these areas of the dendritic extensions with host cell membranes would then provide sites for action for membrane-bound enzymes." Pittman et al. (1973) have postulated that the release of lysosomal-like vesicles from the parasite's surface, or the rupture of the whole parasite could be a way by which toxic products are released. (Lushbaugh and Miller (1974) have suggested that the filamentous projections of the above mentioned glycocalyx play a role in resistance to host defense.)

Visvesvara and Balamuth (1975) described a clear zone, i.e. free

of cells, surrounding Acanthamoeba in synxenic culture. This 'halo' has also been described by Culbertson et al. (1966) for Hartmannella-brain infection. These observations suggested the secretions of lytic substances by the amoebae to form a 'halo'. A similar zone of liquefaction has been observed around the trophozoites of E. histolytica while in the tissues (Siddiqui, 1970) suggesting extensive proteolytic action. The proteolytic enzymes, however, may not be solely responsible for invasiveness, since both pathogenic and nonpathogenic strains have the same enzymatic pattern (Jarumilinta and Maeagraith 1969). Bos et al. (1980) have also suggested that E. histolytica is capable of secreting toxin into its surroundings.

Difference between pathogenic and nonpathogenic strains

The differences between pathogenic and nonpathogenic forms of E. histolytica are not fully understood. Trissl et al. (1978) found differences in certain surface properties such as the agglutinating ability of the plant lectin concanavalin A (Con A), the lack of surface charge of the pathogenic strain of E. histolytica, and the ability to phagocytose different particulate materials including human erythrocytes. A prolonged period of E. histolytica cultivation in axenic conditions usually results in loss of virulence and workers have described various methods of virulence restoration either by Crithidia-association (Bos and Hage, 1975), by temporary reassociation with bacteria (Bos and Hage, 1975; Das et al. 1979), by addition of cholesterol (Meerovitch and Ghadirian, 1978) to the medium, or by serial passaging through rat or hamster liver. Ghadirian and

Meerovitch (1979) tested the pathogenicity of different amebic cultures by intracecal inoculations. Their results showed that the pathogenicity appeared to be inversely proportional to the age of the hamster inoculated.

Lysis of target cells by *Entamoeba histolytica*

Bos (1979) investigated the cytopathogenic effect (CPE) of *E. histolytica* using both intact amoebae and cell-free extracts. In the first case Bos tested 7 strains and several sub-strains of *E. histolytica* on monolayers of baby hamster kidney (BHK) cells. For the second test, he used extracts of *E. histolytica* on monolayers of BHK cells; lysis by "endocellular material" was observed only in the absence of fetal calf serum (FCS). Amoeba cell-free extract at $10 \mu\text{g ml}^{-1}$ was noticed to cause total target cell destruction after 20 hours. However, addition of 5% FCS was found to completely inhibit toxicity. On the basis of his results Bos suggested that there were two ways by which *E. histolytica* may kill their host cells. The first is dependent on cell-to-cell contact and is a rapid process (2-3 hr). This is supported by the results of many workers (Jarumilinta and Kradolfer, 1964; Mattern et al., 1978; Lushbaugh et al. 1979), and is further confirmed by Bos et al. (1980) and Ravdin et al. (1980). The second type of lysis occurs through an endocellular toxin, effective only in the absence of serum (Lushbaugh et al. 1979). Ravdin et al. (1980) used CHO (Chinese hamster ovary) cells to study the CPE of *E. histolytica* (HMI strain) and, as mentioned above, using intact amoebae Ravdin

found that there has to be cell-to-cell contact with the target cells for "cytolethal effect." Using amoebae culture filtrate and sonicates on CHO monolayers, they found, unlike Bos, that the E. histolytica sonicate has a "nonlethal effect."

The Entamoeba histolytica toxin

The toxin secreted by E. histolytica has received much attention recently. Bos (1979) and Lushbaugh et al. (1979), both using ammonium sulphate precipitation and gel filtration, isolated toxic substances from E. histolytica extracts after sonication. The isolated fraction was a heat labile protein with a molecular weight between 35,000-45,000 (Bos 1979) or between 25,000-35,000 daltons (Lushbaugh et al. 1979). They assayed the CPE of this fraction on BHK and HeLa cell monolayers respectively. Both workers demonstrated the inhibition of cytotoxicity by immune IgG and an unknown factor in nonimmune serum. As mentioned earlier, Bos et al. (1980) have reported the binding capacity of both serum and Con A to the E. histolytica toxin. They have further suggested the presence of toxins on the cell membrane of E. histolytica, which act slowly because they are masked by other proteins. The plasma membrane of E. histolytica has a strong affinity for serum proteins. At the site of cell contact between E. histolytica and target cells, it is possible for the exposed toxin to have a lytic effect on the target cells because there is no space for the serum to enter and inhibit the cytotoxic process. Bos found that contact lysis increased in the absence of glucose, suggesting that glucose may also competitively

inhibit toxin binding. This cytotoxin of E. histolytica could be responsible for the invasive property of the pathogenic amoebae.

As we have seen, work on the CPE of E. histolytica so far has shown two possible ways of cell killing. However, much remains to be answered about the role of the toxins in tissue invasion, and the mechanisms of contact-dependent lysis and toxin-induced lysis. Questions remain whether lytic toxins are secreted by living trophozoites and under what conditions cell-free extracts of E. histolytica have a lethal, sublethal or nonlethal effect on target cells. The present study was done to determine (i) whether intact E. histolytica damaged hamster hepatocytes in synxenic culture, (ii) if the sonicate of E. histolytica had a toxic effect on hamster hepatocytes, and, (iii) if any such effect could be detected sublethally by cell leakage of lactate dehydrogenase (LDH) prior to death indicated by the inability of target cells to exclude trypan blue. Experiments were also designed to determine the effect of E. histolytica culture products on hepatocytes in parabiosis chambers. Prior to answering these questions, it was necessary to select the most suitable media, and to determine the contribution of E. histolytica to LDH activity. The pathogenic strain of Entamoeba used was NIH:200. Proficiency in culturing techniques was acquired using the nonpathogenic Laredo strain. The pathogenicity of the strain NIH:200 was determined by intrahepatic injections of approximately 10^6 trophozoites, and looking for liver lesions after a week. The hamster was selected as the source of hepatocytes because it is known to be a suitable laboratory host

for E. histolytica (Bos and Hage, 1975). Hamster liver cells were chosen as target cells for in vitro studies of amoeba-induced damage, because the liver is the primary site of ectopic (secondary) invasion by the E. histolytica trophozoites. Further, it is easier to get a large number of relatively uncontaminated cells from the liver than from the intestinal mucosa.

MATERIALS AND METHODS

Routine cultivation of *Entamoeba histolytica*

The nonpathogenic (Laredo) strain of *Entamoeba histolytica* was purchased from the American Type Culture Collection (ATCC) and a pathogenic strain (NIH:200) was provided by Dr. Lionel G. Warren, Louisiana State University. The strains were cultivated axenically in TPS-I medium (Diamond, 1968). Diamond's TPS-I broth base powder, Diamond's TPS-I (40X) vitamin solution and bovine serum were obtained from North American Biologicals, Miami, Florida. Penicillin-Streptomycin (Difco Laboratories, Detroit, Michigan) were used as antibiotics (200 units ml⁻¹ media). *E. histolytica* was cultured in Leighton tubes; cultures were split and transferred into tubes with fresh medium twice or three times every week depending on the rate of growth. The NIH:200 strain was incubated at 35°C and the Laredo strain was kept at 25°C. The culture tubes for both strains were kept horizontally. Before every transfer the tubes were chilled in ice for five minutes and the sides gently tapped to dislodge the amoebae from the glass; the tube was then tilted up and down to suspend the amoebae, followed by keeping the tubes in a vertical position for ten minutes. Fresh TPS-I was prepared weekly and care was taken to see that the autoclaving of the broth did not exceed 10 minutes as this reduces the cytotoxic activity of the amoebae. Bos et al. (1980) suggested that during excess autoclaving either an inhibitor is produced or an activator destroyed which could result

in the decrease of contact lysis and lysis by the cell-free extract.

Harvesting *Entamoeba histolytica* for experiments

E. histolytica were harvested from TPS-I culture after 72-90 hr (log phase) by chilling in ice for 5 min and gently tapping to dislodge amoebae from the sides. The tubes were then centrifuged at 200 g for 5 min at room temperature (IEC Table-top centrifuge). The supernatant was removed and the pellet was resuspended in 5 ml of serum-free TPS-I broth (+ vitamin + antibiotics). This was centrifuged at 200 g for 5 min at room temperature. The supernatant was discarded and the pellet was washed in serum-free broth again. The E. histolytica were resuspended in 1 ml TPS-I broth and counted in a hemacytometer; viability was determined by a trypan blue dye exclusion method (see below). The final volume was adjusted to the concentration required for the particular experiment in progress.

Preparation of the target cells

The liver cells used were from 4-8 weeks old Syrian hamsters, supplied by Simonsen Laboratories, Gillroy, California.

Liver perfusion. The hepatocytes were obtained by liver perfusion with collagenase. (Procedure obtained from Dr. Donald Reed's laboratory, Biochemistry Dept. OSU.)

Two buffers (25 ml Hanks solution (Ingredients given in Appendix 1) and 225 ml 27.8 mM sodium bicarbonate ($2.333 \text{ g liter}^{-1}$)) were combined, 0.75 g Hepes (12.59 mM) added and the pH was adjusted to 7.4 with 1M NaOH. This was Solution A.

To prepare preperfusion buffer, 150 ml of Solution A was poured into a beaker into which 34.2 mg (.599 mM) EGTA was added. This was warmed to 37°C and oxygenated. To prepare perfusion buffer, 0.3 ml of 1M CaCl_2 was added to the remaining 100 ml of the original Solution A, then this was warmed to 37°C and oxygenated. Collagenase (Sigma Chemical Co.) equivalent to 21,600 units was weighed out and kept aside. This was added immediately before the liver was moved from the preperfusion bath into the perfusion bath.

Media. For the first experiment Fisher's depleted medium was used. To 45 ml Fisher's depleted medium (Ingredients given in Appendix 1), 5 ml heat inactivated fetal calf serum, and 0.15 g Hepes (12.59 mM) were combined, using sterile techniques for the first two stock solutions; the mixture was oxygenated for 10 minutes, and the pH was adjusted to 7.4 using 1M NaOH.

Trypan blue exclusion and lactate dehydrogenase leakage were used to assay viability. The LDH kit with the substrate and coenzyme was obtained as Liquid-STATTM liquid enzyme Reagent from Beckman Instruments, Inc., Fullerton, California.

Perfusion methods

The buffers (pH 7.4) were oxygenated and the pump (Tubing pump Model 375, Sage Instruments) was primed to remove the bubbles. A hamster was anesthetized, the abdomen opened, and 0.2 ml of 1000 U ml^{-1}

heparin was injected into the posterior vena cava. The liver was cannulated via the hepatic portal vein (flow rate $< 5 \text{ ml min}^{-1}$), and the cannula was ligated with surgical thread (00). The flow rate was increased to $< 16 \text{ ml min}^{-1}$: the liver blanched quickly. The liver was removed surgically and suspended in preperfusion buffer (at 37°C) for 3 minutes at 25 ml min^{-1} . The pre-weighed collagenase was added to the perfusion buffer. After 3 min the pump line was switched to the perfusion buffer, the liver was suspended in the perfusion buffer and perfused for 10 minutes at $< 35 \text{ ml min}^{-1}$. After 10 min the flow was turned off, the liver was removed from the buffer and placed in a sterile petri dish. Unless otherwise stated, hepatocytes were obtained as described below. With a blunt pair of forceps the outer membrane of the liver was removed. The hepatocytes were gently swirled out into the petri dish containing 10 - 15 ml TPS-I broth (serum free). Ten or more ml of the hepatocytes in the TPS-I broth were pipetted into a centrifuge tube and pelleted by centrifugation for 3 min at 400 rpm ($\approx 30 \text{ g}$) in a Model HN IEC centrifuge. The supernatant was aspirated off, and the cells were resuspended in 20 ml serum free medium (sometimes the centrifugation had to be repeated depending on the concentration of the cells). The cells were then transferred to a 50 ml Erlenmeyer flask and kept under 5% CO_2 and 95% air at 37°C and rotated at 50 rpm. The cells were suspended in trypan blue solution for 4 min and counts were taken with hemacytometers. It was possible to count total and blue (dead) cells; from these numbers the total number of viable cells was calculated, and the concentration adjusted as required. Viability of hepatocyte preparations exceeded 90% for all experiments.

Generally less than 15 min elapsed between harvesting of hepatocytes and their use in the assays.

Assay methods

Trypan Blue dye exclusion assay. Total cell counts and percent viability were determined by this assay. For the hepatocyte count the trypan blue was a 0.14% solution in 0.9% saline with 2% bovine serum albumin (BSA). Later a 0.28% solution was used in 0.85% saline with 1% BSA. The dilution of cells for the counts was 1:10 in the 0.14% and 1:1 in the 0.28% solution. For the Entamoeba count, a 0.1% trypan blue solution in 0.85% NaCl was used and the dilution was 1:10 cells in trypan blue. Cell suspensions were held in trypan blue for 4 min before being transferred to a hemacytometer, the total number of cells was first counted and then the cells which had taken up the dye (i.e. dead cells) were counted and the average number of live cells were calculated per ml.

$$\frac{\text{The average live}}{\text{The average total}} \times 100 = \% \text{ viability.}$$

Lactate Dehydrogenase assay. To measure the amount of LDH leaked from target cells the LD-L-UV kit from Beckman Instruments Inc., (Fullerton, California) was used. The basic principle of this assay is that LDH acts to catalyze the oxidation of lactate to pyruvate, with the reduction of coenzyme NAD^+ to NADH. The rate of NADH formation, measured by the rate of absorbance increase at 340 nm is directly proportional to the conversion by LDH of lactate to pyruvate. Therefore the amount of LDH in the medium is proportional to the rate of NADH production. The change in absorbance

at 340 nm was followed at 37°C in a Beckman, Model 24 spectrophotometer. For the assay the cell suspension was gently vortexed to suspend cells. One half ml was taken for -t and 0.5 ml for +t in snap cap tubes, where 't' is a 10% solution of Triton X in distilled water. Triton X, a detergent, breaks up the cell membrane; this allows the comparison of the percent of LDH leakage against the total amount in the cell population. The supernatant from the -t was separated from the pellet after 4 min centrifugation at 190 g in an IEC table-top centrifuge. The supernatant was saved for the assay. Twenty five µl of 10% Triton X was added to the +t which was then sonicated in the cold for 30 seconds using a Kontes micro-ultrasonic cell disrupter (Kontes, Vineland, N.J.) with microtip at power 4 (4.6 watts) and 25 KHZ. After 4 min centrifugation at 350 g the supernatant from the +t was retained for the assay. For the first few experiments only, the supernatant from +t was diluted with 0.85% NaCl. For the assay, 0.05 ml of sample was added to 1 ml of the LDH reagent in the sample cuvette. The readings were taken at 30 second intervals for 2.5-3 min.

Preparation of *Entamoeba histolytica* sonicate

The amoebae were harvested (4.7×10^6 in 700 µl of TPS-I) from log phase cultures following the procedures given earlier; this included two washes in TPS-I serum free medium (2-3 ml tube⁻¹), then 5 ml in a single tube. The supernate was removed and the cells were suspended in 2 ml fresh medium. The cells were disrupted by sonication for 3 x 30 seconds using a Kontes sonicator as above.

The tubes containing the E. histolytica were immersed in ice during sonication. After sonication the medium was inspected microscopically for the absence or presence of intact cells, then centrifuged at 4°C at 18000 g for 30 minutes. The supernatant was collected (>800 µl), and placed in ice cold snap-cap tubes.

Parabiotic system

The two chambers of the parabiotic system (Bellco Glass Inc., Vineland, N.J.) were separated by a 0.5 µm Nucleopore membrane filter (General Electric, Pleasanton, California). The small pore size was chosen so as to prevent the amoebae from passing through the filter. Before autoclaving the chambers were assembled with the filters, and the opening of each chamber was fitted with a rubber stopper. The hepatocytes ($2 \cdot 10^6 \text{ ml}^{-1}$) and amoebae (10^6 ml^{-1}) were pipetted into the appropriate sides of three chambers as given in the results section. The chambers were held at 37°C in a desiccator and gassed with 5% CO₂ and 95% air, on a rotary shaker at 50 rpm. Readings were taken at time intervals indicated in result section.

RESULTS

Selection of medium (Experiment no. 1)

Do hepatocytes leak LDH at similar rates in Fisher's and in TPS-I medium? Do these two media contribute significantly to the LDH activity?

Experimental protocol.

TPS-I (Diamond, 1968) and Fisher's media were prepared using sterile techniques. Liver perfusion was carried out following procedures described previously. The hepatocytes obtained were counted and the concentration adjusted to 10^6 ml^{-1} . Eight screw cap culture tubes (Van Water and Rogers, Pyrex 100 mm) were set up as follows:

Tubes 1,2,3 ----- 10^6 hepatocytes in TPS-I with 2.2 ml in each.

Tubes 4,5,6 ----- 10^6 hepatocytes in Fisher with 2.2 ml in each.

Tube 7 ----- 1 ml TPS-I only.

Tube 8 ----- 1 ml Fisher only.

LDH assays were done on this schedule:

Tubes 1 and 4 at T + 45 min. (i.e. 45' after beginning the experiments).

Tubes 2,5,7, and 8 at T + 150 min.

Tubes 3 and 6 at T + 215 min.

The results of this experiment are given in Tables 1 and 2, and illustrated in Figs. 1 and 2.

TABLE I. LDH activity in two test media. Activity is indicated by the absorbance at 340 nm. Samples of media were taken at T + 150'. (Experiment No. 1)

TPS-I medium only.

<u>Time</u>	<u>Absorbance</u>	<u>Change in absorbance (ΔAbs)</u>
0	—	—
1'	1.288	0
1'30"	1.313	0.03
2'	1.338	0.05
2'30"	1.372	0.08
Δ in Abs. $\text{min}^{-1} = .05$ (TPS-I)		

Fisher medium only.

<u>Time</u>	<u>Absorbance</u>	<u>ΔAbs.</u>
0	0.497	0
1'	0.495	-0.002
1'30"	0.494	-0.003
2'	0.494	-0.003
2'30"	0.494	-0.003

Calculation: One International Unit (IU) is defined as the amount of enzyme which will catalyze the conversion of one micromole of substrate per minute under specified conditions. The suppliers of the LD-L-UV kit give the derivation of a value (3376) by which change in absorbance per minute is multiplied to obtain the correct value for IU l^{-1} .

TPS-I medium. $\Delta\text{Abs. min}^{-1} = 0.05 \times 3376 = 168 \text{ IU}$. Therefore, 168 IU l^{-1} has been used as the correction factor in this set of readings, i.e. subtracted from the values obtained for cells in TPS-I.

Fisher medium. The data obtained for Fisher imply that Fisher medium contributed no LDH activity.

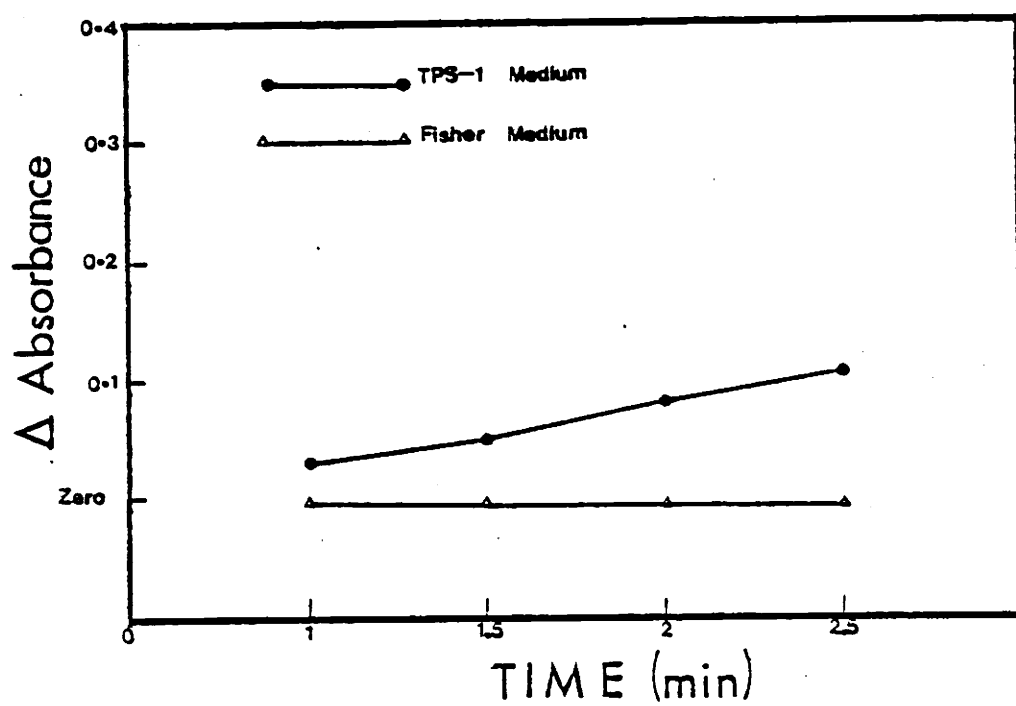


Figure 1. LDH activity in TPS-I and Fisher media, indicated by Δ in O.D. at 340 nm.

TABLE II. LDH leaked from hepatocytes in TPS-I (Tubes 1,2,3) and in Fisher media (4,5,6). Values are given IU l⁻¹. (Appendix 2)

	Tube #	-t ^a	+t ^b	Percent leakage ^c i.e. $\frac{-t}{+t} \times 100$
T+45'	1	270.88	844.8	32%
	4	439.68	844.8	52%
T+150'	2	422.80	1182.4	36%
	5	895.44	1182.4	76%
T+215'	3	507.20	929.2	55%
	6	878.56	1013.6	87%

^aThe -t values (no Triton X) have been calculated as $\Delta\text{Abs. min}^{-1} \times 3376$ from the product of which the correcting factor for TPS-I medium (calculated as 168 IU) has been deducted.

^bThe +t values (Triton X added) have been calculated as in $\Delta\text{Abs. min}^{-1} \times 3376$, the product of which has been multiplied by the dilution factor for the +t sample (in this case the dilution was 1:5). The correcting factor of TPS-I (168 IU) has then been subtracted from the product.

^cThe percent leakage has been calculated as:

$$\frac{\text{Mean } \Delta\text{Abs. min}^{-1} \text{ of the -t sample} \times 3376}{\text{Mean } \Delta\text{Abs. min}^{-1} \text{ of the +t sample} \times 3376} \times 100.$$

These results (Fig. 2) indicate that hepatocytes leak more LDH in Fisher medium than in TPS-I, and that about 50% of the cellular LDH is retained for up to approximately 3 hours when held axenically in TPS-I.

Assay to determine the contribution of *E. histolytica* to LDH activity.
(Experiment 2).

Do *E. histolytica* NIH:200 contain an LDH which can be solubilized and remain active in TPS-I? Does any such LDH leak into TPS-I?
Experimental protocol.

E. histolytica was harvested from 5 culture tubes (72 hr, log

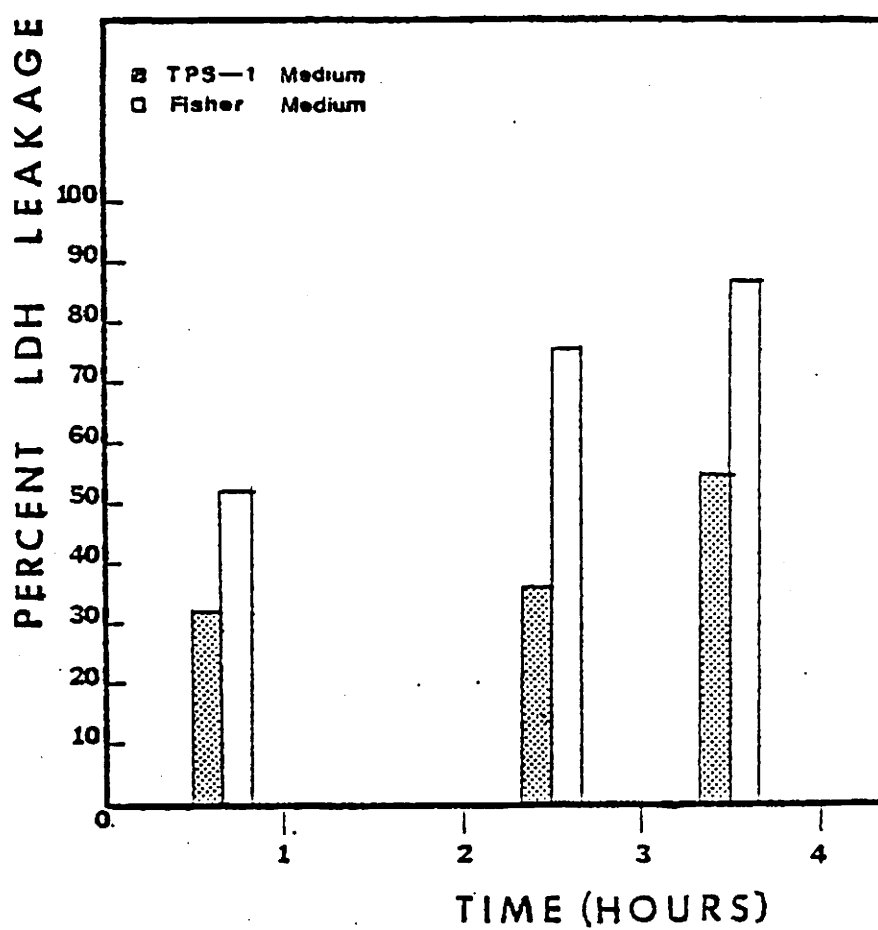


Figure 2. Percent of total LDH leaked by 10^6 hepatocytes in TPS-I and Fisher media.

phase); 0.1% trypan blue solution (in 0.85% NaCl) was used to assess viability of the E. histolytica with a hemacytometer. From the stock suspension ($6.5 \times 10^6 \text{ ml}^{-1}$) cultures were set up as shown below, in screw cap culture tubes (the same type as used in the previous experiment).

Tubes # 1,3 ----- 2 ml TPS-I + 10^6 amoebae.

Tubes # 2,4 ----- " " + $2 \cdot 10^5$ amoebae.

Volumes of tubes 1-4 were made up to 2.2 ml and incubated at 33°C.

Tube #5 ----- 1 ml of fresh TPS-I.

The time of inoculation was noted; readings were taken at T+30' and T+3 hr by LDH assay. At T+30' tubes 1 and 2 were removed from the incubator, chilled for 2 min in ice, and the cells were suspended by shaking the tubes. Aliquots of 0.5 ml were taken and -t and +t samples were prepared (Materials and Methods). At T+3 hr tubes 3,4 and 5 were assayed as above. The results are given in Table III and IV.

TABLE III. Change in Absorbance at 340 nm., due to the TPS-I medium used in this experiment.

Tube #5		
<u>Time</u>	<u>Absorbance</u>	<u>ΔAbs.</u>
1'	1.060	0
2'	1.043	-0.02
3'	1.070	0.01
4'	1.082	0.02
Δ Abs. $\text{min}^{-1} = 0.01$.		

Calculation: The LDH activity = $\Delta\text{Abs. min}^{-1} \times 3376$ (as in Expt. 1)
 i.e. $0.01 \times 3376 = 33.76$ IU.

Therefore 33.76 IU has been used as the correction factor for TPS-I in this set of readings.

TABLE IV. Potential LDH leakage from E. histolytica determined at T+30' and T+3 hr. (Appendix 3)

<u>Time</u>	<u>Tube #</u>	<u>$\Delta\text{Abs. min}^{-1}$</u>
T+30'	1 (with 10^6 amoebae)	-t = 0 +t = -0.02
	2 (with 2×10^5 amoebae)	-t = .013 +t = .04
T+3 hours	3 (10^6 amoebae)	-t = .02 +t = .002*
	4 (2×10^5 amoebae)	-t = .037* +t = .003*

*The dilution for the +t samples for 3 and 4 was 1:5.

The above results show that the maximum change in absorbance is 0.04, which is considered insignificant. Therefore, the readings provide no evidence for LDH leakage by E. histolytica.

Effect of E. histolytica on hamster hepatocytes in a synxenic culture (LDH assay only). (Experiment 3)

Is hepatocyte LDH released by synxenic cultivation with E. histolytica? Do 2.68×10^6 hepatocytes release twice the LDH as 1.34×10^6 hepatocytes? (There might be mechanically caused damage to the hepatocytes due to the 1.34×10^6 E. histolytica in the synxenic culture.)

Experimental protocol.

TPS-I (14 ml) was prepared as before. E. histolytica was harvested from 4 culture tubes (72 hrs log phase) and the count adjusted to $1.34 \times 10^6 \text{ ml}^{-1}$. Hepatocytes were prepared from 6 week old hamsters and the count was adjusted to 1.34×10^6 hepatocytes per ml.

Erlenmeyer flasks (10 ml Kimax, VWR) with serum bottle caps (for gassing) were as follows:

#1 ----- 4 ml TPS-I with 2.68×10^6 hepatocytes.

#2 ----- " " " 1.34×10^6 " + 1.34×10^6 amoebae.

#3 ----- " " " " "

#1,2 and 3 were kept at 37°C, rotated (50 rpm) and gassed (5% CO₂ and 95% air).

#4 ----- 2 ml TPS-I (no gas).

At approximately T+30 min, T+1.5 hr and T+4.5 hr aliquots of 1 ml were removed for LDH determinations. For the last two readings the +ts were undiluted. The results are given in Tables V and VI and illustrated in Fig. 3.

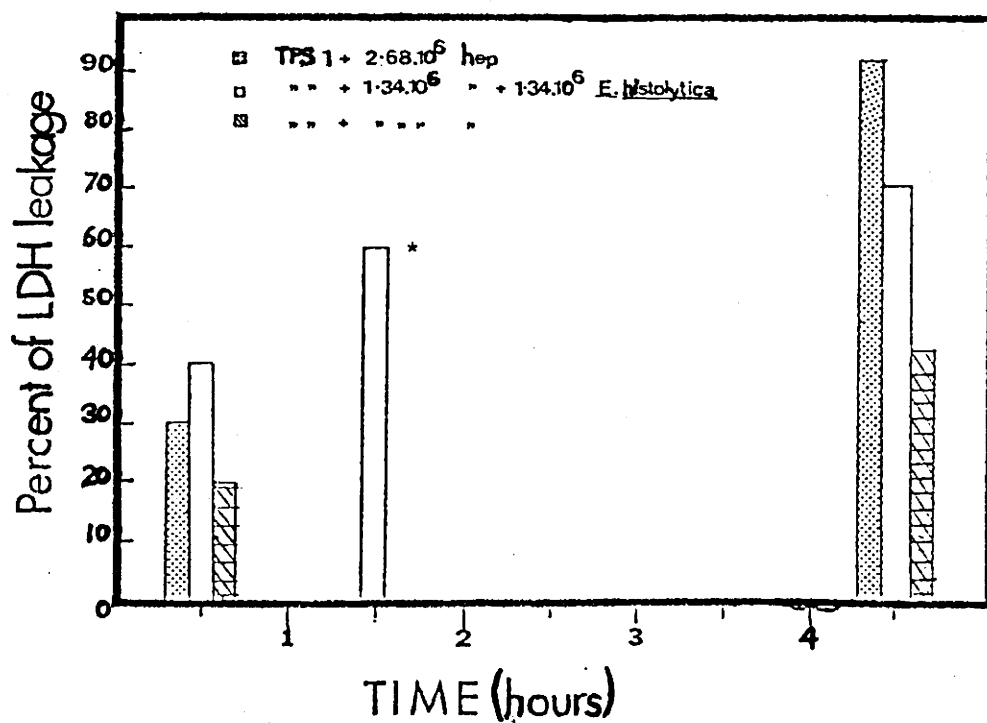


Figure 3. Percent of total LDH leaked by different numbers of hepatocytes when cultured axenically or with *E. histolytica* NIH:200.

* Anomalous values for flasks 1 and 3 at T + 1.5 hr are omitted.

TABLE V. LDH leakage from hepatocytes cultured axenically at two densities, and synxenically with equal numbers of E. histolytica. Data are presented as percent leakage ($\frac{-t}{+t} \times 100$). (Appendix 4)

Time	Flask #	Percent leakage
T+30'	1(2.68 x 10 ⁶ hepatocytes)	30%
	2(1.34 x 10 ⁶ hep. + 1.34 x 10 ⁶ <u>E. histolytica</u>)	40%
	3(1.34 x 10 ⁶ hep.)	20%
T+1.5 hr	1	50%*
	2	60%
	3	55.5%*
T+4.5 hrs	1	92%
	2	71%
	3	43%

*These anomalous values remain unexplained.

TABLE VI. Data to show LDH leakage at T+30', T+1.5 hr and T+4.5 hr. These values have been derived from samples not treated with Triton X (-t).

Time	LDH activity (IU l ⁻¹)		
	#1 (2.68x10 ⁶ hep)	#2 (1.34x10 ⁶ hep + 1.34x10 ⁶ amoebae)	#3 (1.34x10 ⁶ hep)
T+30'	101.28	67.52	33.76
T+1.5 hr	151.92	121.536	168.8
T+4.5 hr	202.56	202.56	101.28

These data imply that LDH leakage occurred in the synxenic culture containing E. histolytica and 1.34 x 10⁶ hepatocytes. Further, the 2.68 x 10⁶ hepatocytes released twice as much LDH as did the axenic culture with 1.34 x 10⁶ hepatocytes after T + 4.5 hr.

To determine the effect of E. histolytica cell-free extract on hamster hepatocytes. (Experiment 4)

Does E. histolytica (NIH:200) sonicate have a toxic effect on hamster hepatocytes? Is any such effect detectable sublethally as LDH leakage prior to death indicated by trypan blue staining? In this and subsequent experiments, serum-free TPS-I was used, since serum inhibits the toxic effect of E. histolytica cell-free toxin on target cells (Bos et al. 1980; Lushbaugh et al. 1979).

Experimental protocol.

Several log phase cultures of E. histolytica were pooled to yield 4.7×10^6 cells in 700 μ l of medium. (These cells were washed twice with serum-free TPS-I.) The cells were then disrupted by sonication (Materials and Methods). Hepatocytes were prepared according to previous procedures, the only differences being the addition of a blunt-ended syringe needle to the end of the usual cannulation tube and that of swirling the hepatocytes immediately after perfusion into serum-free TPS-I instead of into Fisher medium, which was used in the first two experiments. The hepatocytes were suspended at $1.33 \times 10^6 \text{ ml}^{-1}$ in 12 ml i.e. $\geq 1.5 \times 10^7$ hepatocytes. The cell suspension and E. histolytica sonicate were set up in 10 ml Erlenmeyer flasks, as follows:

Flask	Hepatocytes	TPS-I (serum-free)	Sonicate
A	-	3.6 ml + 400 μ l	-
B	4×10^6	" "	-
C	4×10^6	" + 300 μ l	100 μ l
D	4×10^6	"	400 μ l
E	-	made to approximately 500 μ l	

Samples (+t and -t) were assayed from B,C and D at T+30 min, T+1.5 hrs and T+4.5 hrs. Samples from A and E were assayed at T+1.5 hrs.

Trypan blue assays were carried out at approximately the same times.

The results of this experiment are given in Tables VII and VIII, and in Figs. 4, and 6.

TABLE VII. The effect of E. histolytica sonicate on the viability of hamster hepatocytes, as determined by use of trypan blue assay.

Time	Flask #	Percent Viability
T+30'	B(hepatocytes only)	100%
	C(" + 100 μ l sonicate)	100%
	D(" + 400 μ l sonicate)	69%
T+1.5 hrs	B	74%
	C	89%
	D	62%
T+4.5 hrs	B	73%
	C	*
	D	47%

* Anomalous value not presented.

TABLE VIII. The effect of E. histolytica sonicate on LDH leakage from hamster hepatocytes. (Appendix 5)

Time	Flask #	Percent Leakage
T+30'	B(4×10^6 hepatocytes, no sonicate)	-
	C(4×10^6 hepatocytes + 100 μ l sonicate)	3%
	D(4×10^6 " + 400 μ l sonicate)	1%
T+1.5 hrs	B	0
	C	10%
	D	40%
	A and E	0
T+4.5 hrs	B	45%
	C	73%
	D	+t value was too high for spectrophotometer to monitor.

The -t and +t values and the change in absorbance has been calculated as in Experiment 1, except that the TPS-I medium did not contribute any LDH activity so there was no correcting factor due to medium to be considered; further the +t samples were used in full strength so there was no dilution factor either. Percent leakage has been calculated as before.

These results (Tables VII and VIII) imply that the sonicate caused a dose dependent leakage of LDH, and, the results of trypan blue assay showed an enhanced mortality of the hepatocytes.

A repeat of the previous experiment was done using the same number of hepatocytes and the same amount of sonicate. (Experiment no. 5)

The following results were obtained:

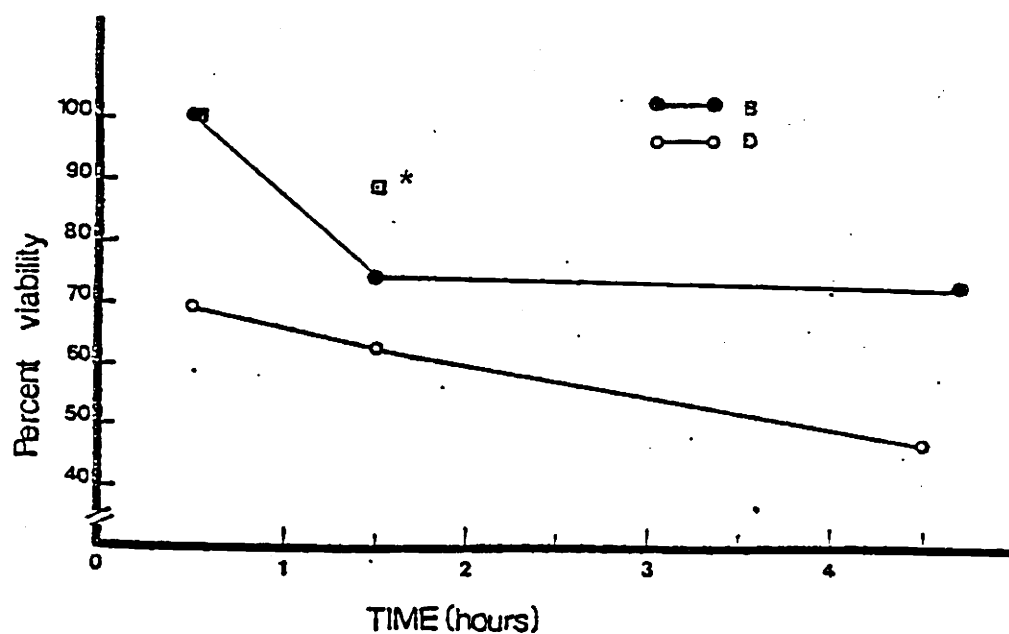


Figure 4.

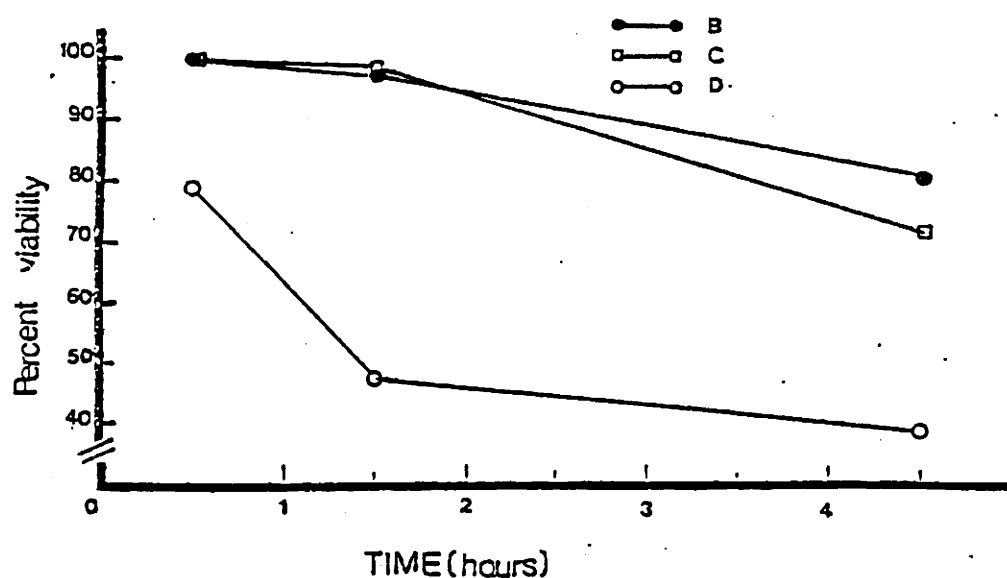


Figure 5.

Figure 4 and 5. Effect of *E. histolytica* cell-free extract on hamster hepatocytes; viability determined by the trypan blue dye exclusion assay. Two separate experiments using the same number of hepatocytes and same amount of sonicate.

B = 4×10^6 hepatocytes
 C = " " + 100 μ l extract
 D = " " + 400 μ l "

* Anomalous value of 'C' in Figure 4 not presented.

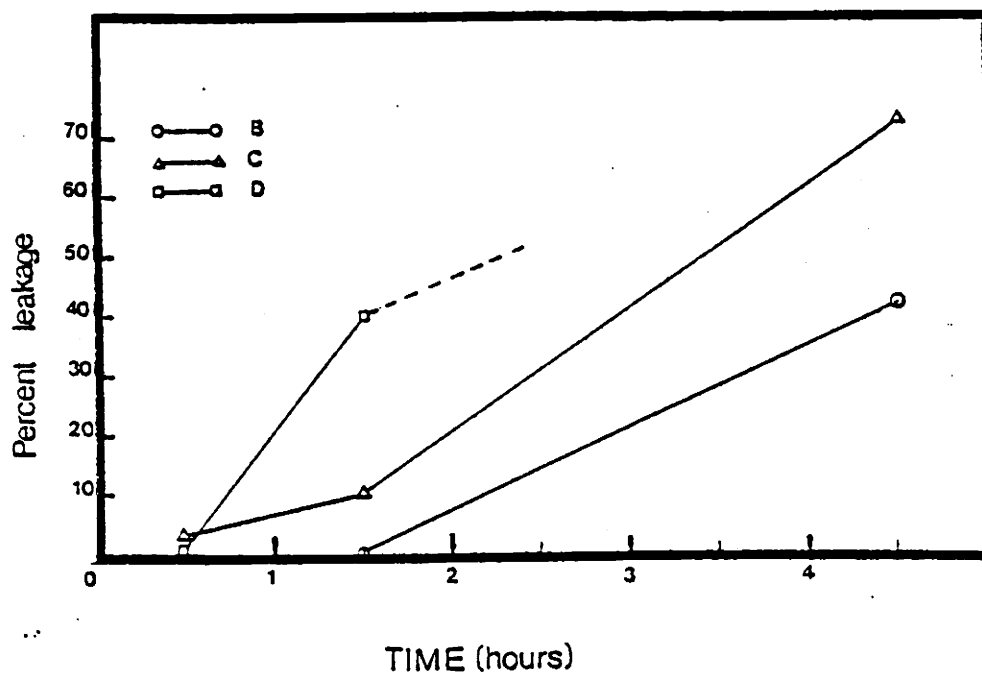


Figure 6. Effect of *E. histolytica* cell-free extract on leakage of LDH from hamster hepatocytes.

B = 4×10^6 hepatocytes
 C = " " + 100 μ l extract
 D = " " + 400 μ l "

* The percent leakage value from sample D at 4.5 hr could not be monitored by the spectrophotometer used.

TABLE IX. The effect of E. histolytica sonicate on the viability of hamster hepatocytes. (Experiment No. 5)

Time	Flask #	Percent Viability
T+30'	B(4x10 ⁶ hepatocytes no sonicate)	100%
	C(4x10 ⁶ hep. + 100 μ l sonicate)	100%
	D(4x10 ⁶ hep. + 400 μ l sonicate)	79%
T+1.5 hrs	B	98%
	C	99%
	D	48%
T+4.5 hrs	B	82%
	C	73%
	D	40%

LDH readings were ambiguous and are not reported here. However, the viability data are given in Fig. 5.

Assay of damage by intact E. histolytica on hamster hepatocytes in synxenic culture. (Experiment No. 6)

Do intact E. histolytica (NIH:200) damage hepatocytes in a synxenic culture, by trypan blue and LDH leakage criteria?

Experimental protocol.

Serum-free TPS-I, prepared as before, was used as the medium. Hepatocytes were prepared using routine protocol, and the viable count adjusted to 10^6 ml^{-1} in 20 ml. E. histolytica organisms were prepared as previously, and the count adjusted to 2.6×10^6 in 2 ml.

Synxenic culture. Three 24 well Falcon tissue culture plates were set up with the following number of cells, and were assayed (trypan blue and LDH) at given intervals.

	<u>Wells 1,4,7</u>	<u>Wells 2,5,8</u>	<u>Wells 3,6,9</u>
Hepatocytes	1.5×10^6	1.5×10^6	1.5×10^6
<u>E. histolytica</u>	-	1.3×10^5	6.5×10^5
TPS-I	0.5 ml	0.4 ml	-

Samples from wells #1,2 and 3 were assayed for LDH activity at T+30'. A 0.28% trypan blue solution was used for the trypan blue assay. Wells 4,5 and 6 were assayed at T+1.5 hrs and wells 7,8 and 9 were assayed at T+4.5 hrs. Using identical protocols, the experiment was repeated (see Expt. 7) and the results of both are reported in Tables X, XI, XII and XIII, and Figures 7,8 and 9. (Appendix 7)

TABLE X. The effect of intact E. histolytica on hamster hepatocytes in a synxenic culture, by trypan blue dye exclusion assay. (Experiment 6)

Time	Well #	Percent Viability
T+30'	1(1.5×10^6 hep.)	96%
	2(" " + 1.3×10^5 amoebae)	84%
	3(" " + 6.5×10^5 ")	84%
T+1.5 hr	4(same as 1)	88%
	5(same as 2)	70%
	6(same as 3)	50%
T+4.5 hr	7(same as 1)	73%
	8(same as 2)	41%
	9(same as 3)	2%

By the end of 1.5 hours the hepatocytes not only looked blue but also showed definite signs of disintegration, whereas the amoebae looked active and 'healthy'.

TABLE XI. LDH leakage from the same concentration of heptaocytes cultured axenically and synxenically with different number of *E. histolytica*. Data presented as percent leakage.

Time of Assay	Well #	Percent leakage
T+30'	1	0
	2	4%
	3	3%
T+1.5 hr	4	5%
	5	*
	6	62%
T+4.5 hr	7	43%
	8	82%
	9	*

* Ambiguous values not reported.

The above experiment was repeated (same number of cells were used) with the following results. (Experiment No. 7)

TABLE XII. The effect of intact *E. histolytica* on hamster hepatocytes in a synxenic culture, by trypan blue dye exclusion assay.

Time	Well #	Viability	Comments
T+30'	1(1.5×10^6 hep.)	92%	The hepatocytes were well spread out.
	2(1.5×10^6 hep. + 1.3×10^5 amoebae)	86%	Hepatocytes starting to clump with amoebae around them.
	3(1.5×10^6 hep. + 6.5×10^5 ")	87%	None.
T+1.5 hr	4(same as 1)	87%	Hepatocytes still dense.
	5(same as 2)	77%	Definite hep. clumping with <i>E. histolytica</i> in between spaces.
	6(same as 3)	25%	Clear areas with amoebae around the hep.
T+4.5 hrs	7(same as 1)	64%	Clumping + presence of debris.
	8(same as 2)	0	
	9(same as 3)	0	Amoebae looked 'active'

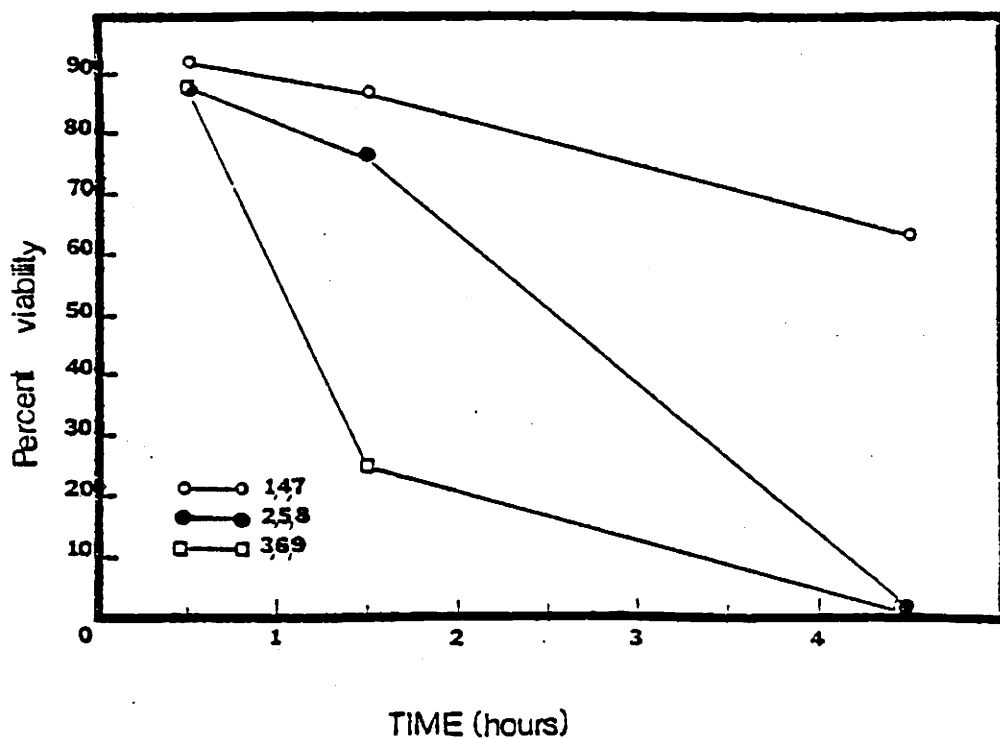


Figure 7. Trypan blue assay to determine the damage of hamster hepatocytes by intact E. histolytica in a synxenic culture using the same number of hepatocytes and different numbers of E. histolytica.

1,4,7	=	1.5×10^6	hepatocytes	
2,5,8	=	"	"	+ 1.3×10^5 <u>E. histolytica</u>
3,6,9	=	"	"	+ 6.5×10^6 "

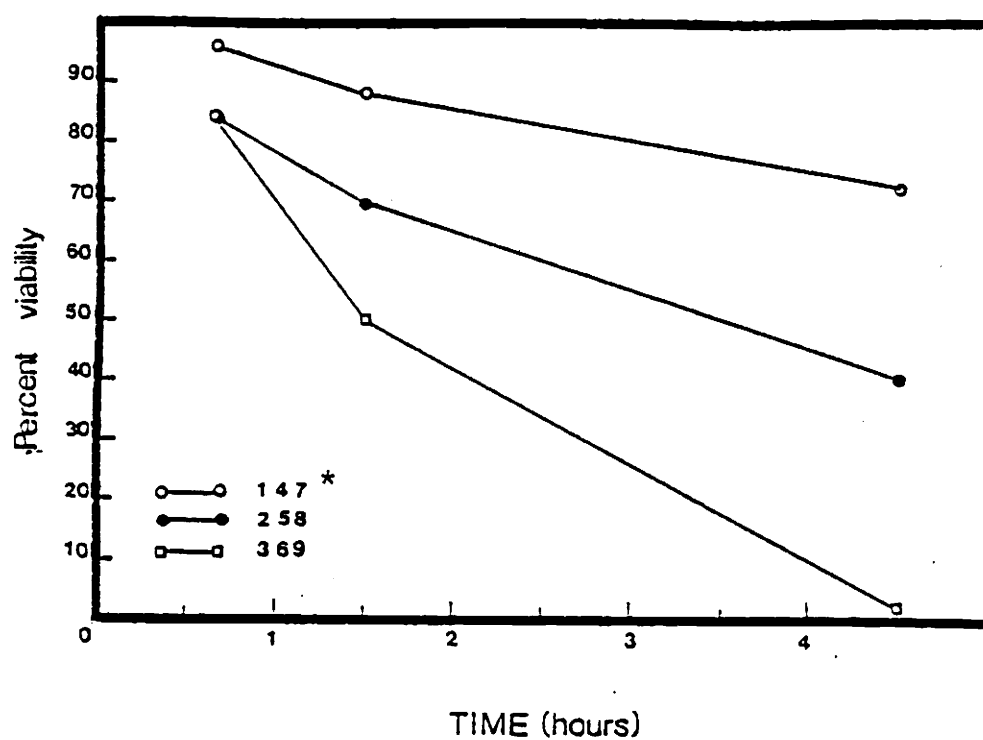


Figure 8. Trypan blue assay to determine the damage of hamster hepatocytes by intact E. histolytica in a synxenic culture using the same number of hepatocytes and different numbers of E. histolytica.

1,4,7	=	1.5×10^6	hepatocytes	
2,5,8	=	"	"	+ 1.3×10^5 <u>E. histolytica</u>
3,6,9	=	"	"	+ 6.5×10^6 <u>E. histolytica</u>

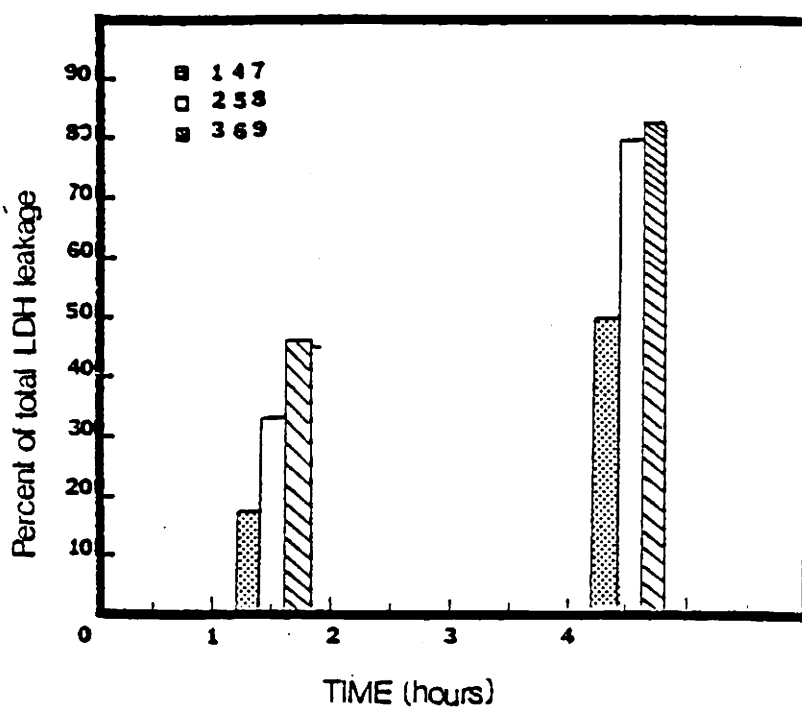


Figure 9. Percent of total LDH leaked by hepatocytes when cultured synxenically with E. histolytica.

1,4,7 = 1.5×10^6 hepatocytes
 2,5,8 = " " + 1.3×10^5 E. histolytica
 3,6,9 = " " + 6.5×10^5 " "

Data for T + 30 min are not presented here since the values were ambiguous.

TABLE XIII. LDH leakage from the same number of hepatocytes cultured axenically and synxenically with different number of E. histolytica. Data presented as percent leakage.

Time of assay	Well #	Percent leakage
T+30'	1	11%
	2	0
	3	89%*
T+1.5 hrs	4	17%
	5	33%
	6	46%
T+4.5 hrs	7	50%
	8	80%
	9	83%

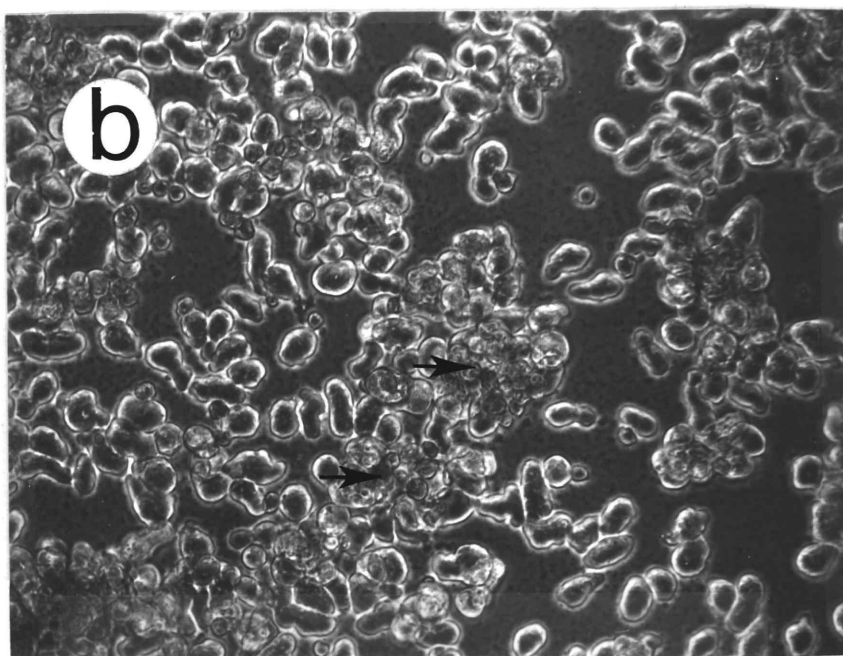
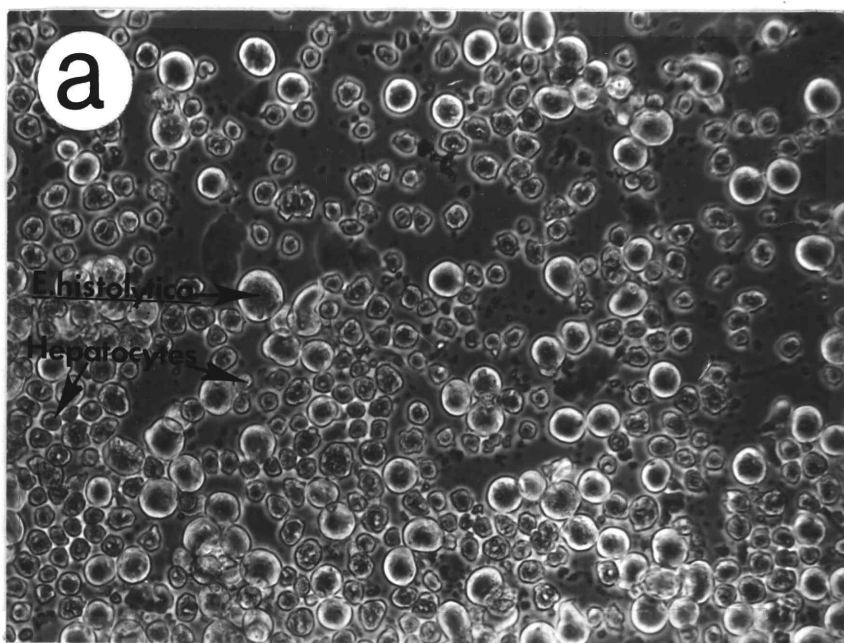
*The erratic value of the T+30' sample from well no. 3 remains unexplained.

The results of both the above experiments (No. 6 and 7) imply that lysis occurs in synxenic cultures, and although the culture with the greater number of amoebae does show more leakage and less viability than the culture with fewer amoebae, still by the end of T+4.7 hr the results apparently are not strictly dose dependent.

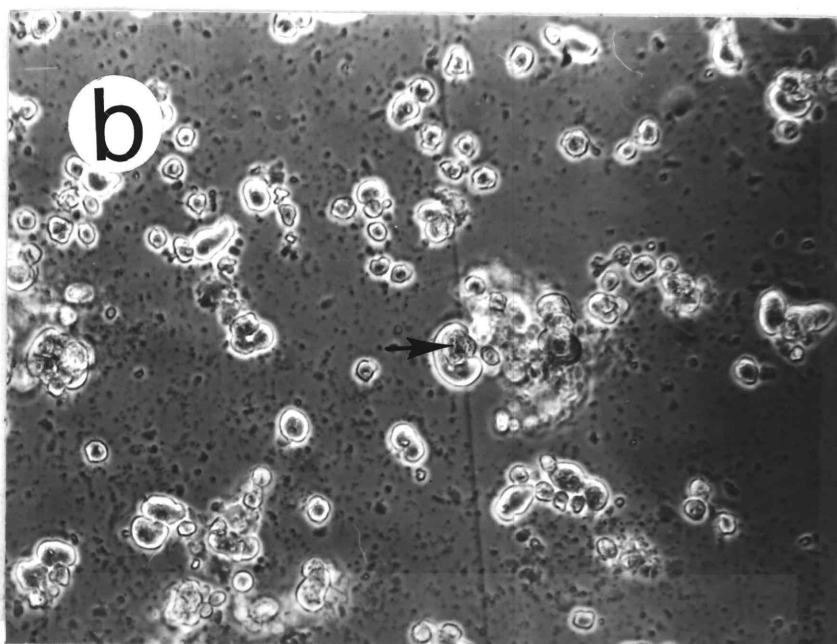
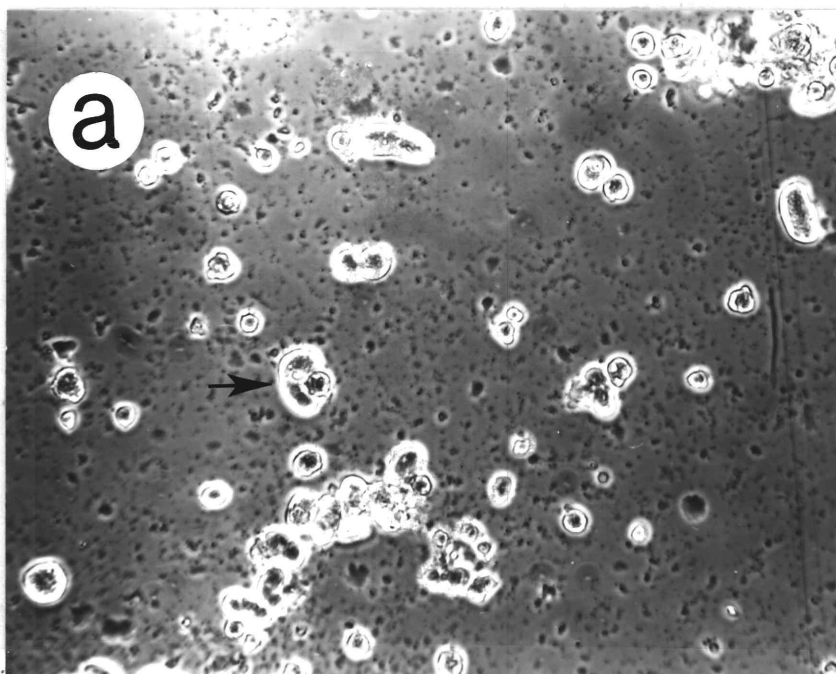
Ring slides preparation for microscopy. Using an experimental system developed in this laboratory (Stibbs et al., 1979), the following amounts were pipetted into ring slides A, B and C.

	<u>A</u>	<u>B</u>	<u>C</u>
Hepatocytes	2×10^5	2×10^5	2×10^5
<u>E. histolytica</u>	-	1.3×10^4	6.5×10^4
TPS-I	The rest was filled with TPS-I medium.		

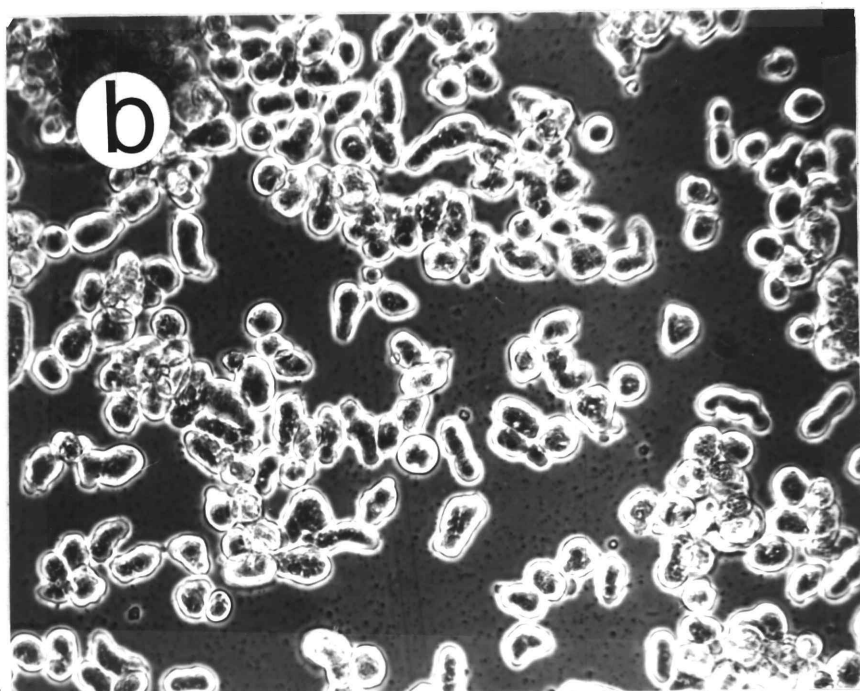
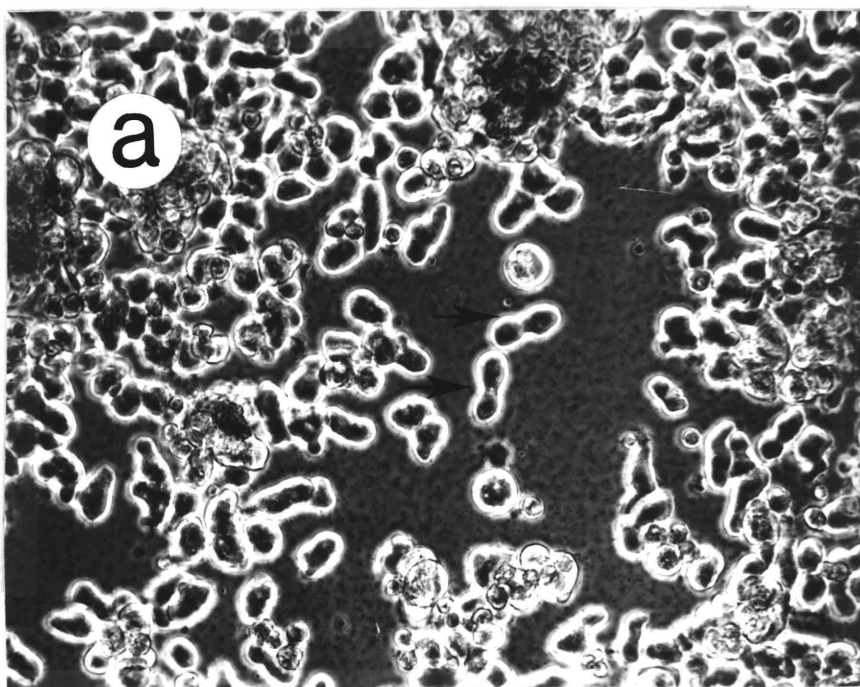
The capacity of each ring slide was 750 μ l approximately. The slides were viewed with an inverted microscope (phase) at selected intervals and the various stages were photographed. Figures 10, 11 and 12 show a sequence of changes that occurred in the synxenic culture con-



- 10a. A synxenic culture of hamster hepatocytes (abundant, small dark cells) and *E. histolytica* (larger, refractile cells). The photograph was taken at T + 18 hr.
- 10b. The same culture as (a) after 22 hr, where the hepatocytes are starting to clump as indicated by arrows.



- 11a. In vitro culture of hepatocytes and E. histolytica. Arrow indicates a hepatocyte being surrounded by an amoeba. Photograph taken at T + 25 hours.
- 11b. This shows a different field of view taken at the same time as above, where one of the remaining hepatocytes is being engulfed by E. histolytica (arrow).



- 12a. This photograph taken at T + 50 hr shows two amoebae dividing (arrow).
- 12b. The synxenic culture after T + 63 hr clearly shows more healthy amoebae and very few hepatocytes.

taining hepatocytes and amoebae.

The effect of *E. histolytica* culture products on hepatocytes in a parabiologic system. (Experiment No. 8)

Are hepatocytes damaged (trypan blue and LDH assay) by *E. histolytica* in a parabiologic system?

Experimental protocol.

The hepatocytes were prepared as before, counted and diluted to 2.10^6 ml^{-1} in serum-free TPS-I. *E. histolytica* was also harvested as before, counted and diluted to 10^6 ml^{-1} in serum-free TPS-I. The parabiologic system was arranged as in Materials and Methods. The hepatocytes and amoebae were pipetted into the appropriate sides of 3 chambers as given below, and illustrated in Fig. 13.

Chamber #	TPS-I	Hep.	<i>E. histolytica</i>
1a	2.4 ml	-	-
b	1.4 ml	$1.0 \text{ ml}(2 \times 10^6)$	-
2a	1.4 ml	-	$1 \text{ ml}(10^6)$
b	1.4 ml	$1.0 \text{ ml}(2 \times 10^6)$	-
3a	2.4 ml	-	-
b	0.4 ml	$1.0 \text{ ml}(2 \times 10^6)$	$1.0 \text{ ml}(10^6)$

Sampling schedule. Chambers were held at 37°C in a desiccator and gassed with 5% CO_2 and 95% air, on a rotary shaker at 50 rpm. At T+4 hr and T+6 hr the chamber was shaken to distribute the cells. Then, 0.5 ml for +t, 0.5 ml for -t, and $2 \times 0.05 \text{ ml}$ for trypan blue were pipetted out from 'b'. An equal volume (1.1 ml) was rejected from 'a'. The results are given in Tables XIV and XV.

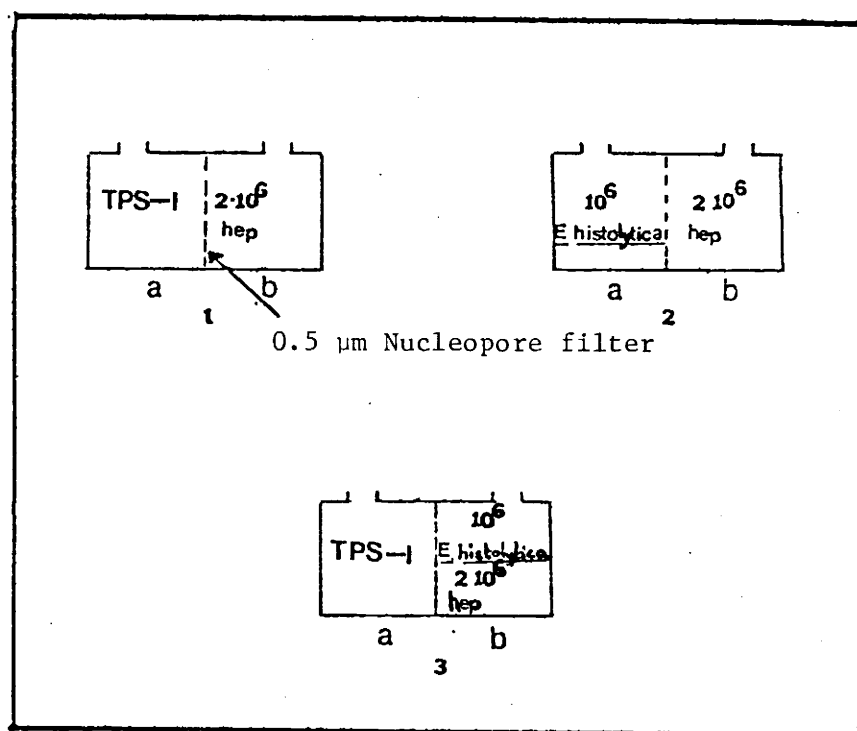


Figure 13. Parabolic system used to study the effect of *E. histolytica* culture products on hepatocytes.

TABLE XIV. Effect of the culture products of E. histolytica on hepatocytes in a parabiatic system, by trypan blue assay. (Experiment No. 8)

Time	#	Viability
T+4 hrs	1	91%
	2	78%
	3	89%
T+6 hrs	1	62%
	2	34%
	3	0

TABLE XV. Effect of culture products of E. histolytica on hepatocytes in a parabiatic system, assayed at T+6 hr by LDH assay.

#	Percent leakage
1	33%
2	50%
3	100%

As was expected from the results of previous experiments, damage did occur by cell-to-cell contact in the chamber with both the hepatocytes and E. histolytica. The results also imply that hepatocytes were damaged by the lytic secretions of E. histolytica. However, more experiments of this sort are necessary before firm conclusions are possible.

DISCUSSION

The research reported in this thesis was undertaken in order to answer questions relating to the specific mechanisms of pathogenicity of E. histolytica. Bos (1979) suggested the existence of two modes of toxicity, one of which was cell-to-cell contact and the other was through an endocellular toxin. The first step of this work therefore was to confirm that when cell-to-cell contact occurred lysis of target cells followed; this was done by using NIH:200 E. histolytica, a pathogenic strain, and hamster hepatocytes as target cells. In contrast to the target cells used by other workers, hepatocytes were used as target cells in this work, because the liver is frequently attacked by invasive trophozoites in extra-intestinal infections of E. histolytica. The results of these experiments confirmed those of other workers i.e. lysis of the target cells did occur when the cells were in contact with the amoebae.

A second group of experiments examined CPE by the lytic secretions of living E. histolytica using parabiosis chambers. Though data were available from only one experiment, there was some evidence that damage of the hepatocytes occurred even in absence of cell-to-cell contact.

During the course of our work, other authors (Ravdin et al., 1980) published results of their research on the sonicate of E. histolytica. Their results were in disagreement with those of Bos (1979) and Lushbaugh et al. (1979) who had reported that the sonicate of E. histolytica damaged the target cells (BHK and HeLa cells).

Ravdin et al. suggested that there is no cytolethal effect of the E. histolytica sonicate on the target cells (CHO). So the second step of this work was to attempt to resolve the differences in the two results using a different mode of assay (LDH backed by the trypan blue dye exclusion assay). The results of these experiments showed a certain amount of killing of hepatocytes by the E. histolytica sonicate, although additional confirming experiments are necessary before firm conclusions can be drawn.

Medium

Fisher's medium is generally used for the preparation of hepatocytes, and TPS-I (Diamond, 1968) is used to culture E. histolytica. However, the results of experiment on the selection of medium showed that hepatocytes leaked LDH at a higher rate in Fisher's depleted medium than in TPS-I. The maximum leakage in both Fisher's and TPS-I media appears to have occurred at T + 215 min. The results led to the choice of TPS-I as the medium to be used for the experiments to follow, especially the synxenic experiments. This choice was further supported by the results of the trypan blue assays which confirmed good viability of hepatocytes in TPS-I medium. On the other hand, the readings of experiment #1 also showed that Fisher's medium did not contribute significantly to the LDH activity, whereas TPS-I did. TPS-I contributed 168 IU l^{-1} (International Units/liter) to the LDH activity. This may have been due to the lot of bovine serum used in the TPS-I medium for that particular experiment. For most of the experiments, therefore, there was a control of only TPS-I to determine its contribution, if any, to the LDH activity. In the

subsequent experiments bovine serum was excluded from the medium not only because of its contribution to the LDH activity, but also due to its ability to bind to the toxic factor of the E. histolytica extract or sonicate thereby inhibiting the cytotoxicity of the E. histolytica (Lushbaugh et al., 1979; Bos et al., 1980). In these experiments, where the medium used was serum-free, there was no contribution to the LDH activity by the medium.

Potential Leakage of LDH from E. histolytica into the TPS-I medium

No evidence was obtained for LDH leakage from the amoebae. The maximum change in absorbance min^{-1} was less than 0.04 O.D. which is considered insignificant, this being within the range of experimental error. The results were consistent with the anaerobic metabolism of this species.

Synxenic experiment

The results of the synxenic experiment showed that the LDH activity of hepatocytes is released by synxenic cultivation with E. histolytica. In the first synxenic experiment there were 3 flasks containing 2.68×10^6 hepatocytes, 1.34×10^6 hepatocytes plus 1.34×10^6 amoebae, and 1.34×10^6 hepatocytes. The flask with the amoebae gave a much higher percent leakage than the control flask with the same number of hepatocytes only. The highest activity was seen to be released at $T + 4.5$ hrs there being an expected increase over time. Besides contact lysis, the death of the hepatocytes may have been due to mechanical damage by cell bumping. However, control

for cell damage by mechanical bumping was provided by the first flask in which the total number of cells was the same as in the synxenic flask, but all cells were hepatocytes. After the first half hour the readings indicated that 2.68×10^6 hepatocytes did not release twice as much LDH as did 1.34×10^6 hepatocytes. However by the end of 4.5 hours the IU l^{-1} values for the flask containing 1.34×10^6 hepatocytes was 101.28, and that for 2.68×10^6 was 202.56. The readings taken for axenic hepatocytes (2.68×10^6 and 1.34×10^6) at T+1.5 hr were anomalous and remain unexplained.

Examination of the cultures set up in the tissue culture plates also indicated the occurrence of contact lysis, as evidenced by both the trypan blue viability count, which showed a steady decrease in percent viability with the increase of time, and by the LDH leakage assay, which in turn showed an increase in percent leakage with time. In both assays, the concentration of amoebae was directly correlated with the extent of contact lysis of target cells.

Photographs of the synxenic cultures in ring slides showed hepatocytes and E. histolytica in close contact. Some of the hepatocytes may have died because of lack of gassing, but the E. histolytica were seen to engulf the hepatocytes. After about 2-3 hours the hepatocytes appeared in several clumps surrounded by amoebae in clean areas. Around the amoebae there were clear areas; these imply either the secretion of a lytic substance or active local phagocytosis.

Effect of *E. histolytica* sonicate on the hepatocytes

The results of this experiment were ambiguous. Although the trypan blue assay indicated hepatocyte death in the presence of the *E. histolytica* sonicate, and the LDH assay showed some leakage, not all the hepatocytes were lysed by the toxin. The LDH assay was not sensitive enough to pick up sublethal effect by the sonicate on the hepatocytes prior to the trypan blue dye exclusion method. Further, some of the absorbance values were so high as to be off scale on the spectrophotometer. Therefore these readings obtained then were not sufficient to support any definite views about toxin-induced lysis. Ravdin et al. (1980) reported that the sonicate of *E. histolytica* did not have any CPE on its target cells (CHO cells). This was contrary to the findings of Bos (1979), Lushbaugh et al. (1979), and Bos et al. (1980), who found toxin-induced lysis when the sonicate and extract of *E. histolytica* were tested on monolayers of BHK and HeLa cells. This difference could be due to the methods employed; Bos (1979) and Lushbaugh et al. (1979) separated the cytotoxin, after sonication, by ammonium sulfate precipitation and gel filtration, thereby using partially purified forms of the toxin for the toxicity assay. The lack of this isolation procedure in the work of Ravdin et al. (1980) and from our experiments could account for the difference in results. There could be some factor in the 'raw' sonicate material which tends to inhibit the toxicity of the antigen. Or, by isolating the antigen, the toxic factor(s) could be more concentrated and thus have a greater effect on the target cells.

Effect of *E. histolytica* culture products on hepatocytes in a parabiotic system

Damage was observed by contact lysis in the chamber containing both the hepatocytes and the *E. histolytica*. In addition, both LDH and trypan blue assays suggest that damage of the hepatocytes did occur by the *E. histolytica* culture products coming through the 0.5 μ m Nucleopore filter. So, if target cell killing occurs by both contact lysis and by the *E. histolytica* secretions as indicated here, the question arises as to what happens to this soluble cytotoxin(s) when the *E. histolytica* are sonicated.

General conclusion

A decrease in the virulence of the pathogenic strain of *E. histolytica* when kept in culture for prolonged periods (2 years) has been reported by previous workers. We maintained our culture no longer than 7-8 months. Furthermore, hepatic inoculation (hamsters) of the trophozoites resulted in lesions at the site of inoculation after one week. Gassing the amoebae suspension along with the hepatocytes did not seem to have any debilitating effect on the amoebae, since active 'healthy' amoebae were seen even after 4-5 hours of gassing with 5% CO₂ and 95% air. This research has shown CPE of target cells by contact lysis. There was also a slight indication of toxin-induced lysis. However, more experiments are required before firm conclusions could be drawn with respect to the presence of toxin induced lysis.

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* Not seen in the original. Referenced by Albach and Booden (1978).

APPENDICES

Appendix 1. Material for Liver (Hamster) perfusion.

Ingredients

A. Hank's Solution.

NaCl	80 g	
KCl	4 g	
MgSO ₄ ·17H ₂ O	2 g	
Na ₂ HPO ₄	.45 g	(or Na ₂ HPO ₄ ·2H ₂ O 0.6 g)
KH ₂ PO ₄	.60 g	

Bring up to one liter with distilled water.

B. Depleted media. (Fisher)

L-amino acids:

L-Arginine HCL	.045 g	
L-Histidine	.180 g	
L-Isoleucine	.225 g	
L-Leucine	.090 g	
L-Lycine	.150 g	
L-Phenylalanine	.180 g	
L-Threonine	.120 g	
L-Tryptophan	.030 g	
L-Valine	.210 g	
L-Tyrosine	.180 g	
Folic acid	.030 g	
L-Asparagine x H ₂ O	.034 g	or .030 if no H ₂ O
L-Serine	.045 g	
NaHCO ₃	3.375 g	
L-Glutamine	.612 g	

Basal Salts and Glucose:

CaCl ₂ ·H ₂ O	.273 g
Na ₂ HPO ₄ (dibasic)	.339 g
Na H ₂ PO ₄ ·H ₂ O (monobasic)	.207 g
KCL	1.2 g
MgCl ₂ ·H ₂ O	.300 g
NaCl	24.0 g
Glucose	3.0 g

Vitamins:

Thiamine - HCl	.003 g
Nicotinamide	.0015 g
Ca Pantothenate	.0015 g
Pyridoxal - HCl	.0015 g
Riboflavin	.0015 g
Choline Cl	.0045 g
i - Inositol	.0045 g
Biotin	.00003 g

Appendix 1 cont.

To dry ingredients add 2670 ml of autoclaved H_2O , 2 mls vitamins (from the x10 stock), and 3 mls phenol red. Dissolve. Filter. (Flame bottle before and after filling with the filtered media).

Appendix 2. Expt. No. 1. Selection of medium. LDH assay. (Table II)

T+45 min. 1-t			1+t		4-t		4+t	
Time	OD	Δ OD	OD	Δ OD	OD	Δ OD	OD	Δ OD
0	1.076	0	0.595	0	0.540	0	0.496	0
30"	1.352	.28	0.624	0.03	0.647	0.11	0.530	0.03
1'	1.363	.29	0.657	0.06	0.740	0.20	0.563	0.07
1-30	1.441	.37	0.686	0.09	0.839	0.30	0.592	0.10
2	1.494	.42	0.716	0.12	0.925	0.39	0.622	0.13
2-30	1.570	.49	0.743	0.15	0.012	0.47	0.652	0.16

T+150 min. 2-t			2+t		5-t		5+t	
0	1.189	0	0.645	0	0.625	0	0.537	0
30'	1.381	0.19	0.715	0.07	0.978	0.35	0.620	0.08
1'	1.469	0.28	0.785	0.11	1.159	0.53	0.659	0.12
1-30	1.560	0.37	0.799	0.15	1.376	0.75	0.698	0.16
2'	1.635	0.45	0.833	0.19	1.473	0.85	0.736	0.20

T+215 min. 3-t			3+t		6-t		6+t	
0	1.385	0	0.691	0	0.640	0	0.520	0
30"	1.658	0.27	0.764	0.07	1.006	0.37	0.596	0.08
1'	1.750	0.37	0.796	0.11	1.177	0.54	0.636	0.12
1-30	1.858	0.47	0.833	0.14	1.338	0.70	0.678	0.16
2	1.970	0.59	0.868	0.18	1.492	0.85	0.716	0.20

Appendix 2 cont.

Sample	$\Delta\text{Abs. min}^{-1} \times 3376$	\times Dilution factor (i.e. $\times 5$ for +t)	Minus correction factor (168 IU)	$\frac{-t}{+t} \times 100$
T+45 min				
1-t(.42-.29)	$0.13 \times 3376 = 438.88$		270.88	
1+t(.12-.06)	$0.06 \times 3376 = 202.56$	1012.80	844.8	32%
4-t(.38-.2)	$0.18 \times 3376 = 607.68$		439.68	
4+t(.12-.06)	$0.06 \times 3376 = 202.56$	1012.80	844.8	52%
T+150 min				
2-t(.45-.275)	$0.175 \times 3376 = 590.80$		422.80	
2+t(.15-.07)	$0.08 \times 3376 = 270.08$	1350.40	1182.4	36%
5-t(.69-.375)	$0.315 \times 3376 = 1063.44$		895.44	
5_t(.16-.08)	$0.08 \times 3376 = 270.08$	1350.40	1182.4	76%
T+215 min				
3-t(.47-.27)	$0.20 \times 3376 = 675.20$		507.20	
3+t(.135-.07)	$0.065 \times 3376 = 219.44$	1097.20	929.2	55%
6-t(.85-.54)	$0.31 \times 3376 = 1046.56$		878.56	
6+t(.19-.12)	$0.07 \times 3376 = 236.32$	1181.60	1013.6	87%

-t and +t are samples without and with Triton X respectively, as described in Materials and Methods and in Results section.

Deduction of '3376' -- as in Result section.

Appendix 3.

Expt. No. 2. Contribution of E. histolytica to LDH activity. The table below gives only the raw data and the overall change in absorbance. The rest of the calculations were worked as in Expt. 1 except for the correction factor (TPS-I) which was 33.76 IU l^{-1} , and the dilution of +t samples was 1:5 only in the T + 3 hr assay.

Time	1-t	^a $\Delta\text{Abs. min}^{-1}$	1+t	^a $\Delta\text{Abs. min}^{-1}$	2-t	^a $\Delta\text{Abs. min}^{-1}$	2+t	$\Delta\text{Abs. min}^{-1}$
<u>T+30'</u>								
1'	1.237		1.248		0.817		1.201	
2'	1.246	0	1.237	-0.02	0.812	0.013	1.214	0.04
3'	1.248		1.247		0.820		1.255	
<u>T+3 hours</u>								
	3-t		3+t		4-t		4+t	
1'	1.164		0.646		1.171		-	
2'	1.215	0.02	0.647	0.002	1.173	0.037	0.672	.003
3'	1.229		0.645		1.206		0.674	
4'	1.258						0.677	

#1 and 3 contain 10^6 amoebae
2 and 4 contain 2.10^5 amoebae

The maximum $\Delta\text{Abs. min}^{-1}$ according to the readings above is 0.04 OD, a value too low to provide evidence for LDH leakage by E. histolytica.

^aValues read from a plot of the $\Delta\text{Abs.}$ values vs time.

Appendix 4.

Expt. No. 3. Effect of E. histolytica on hepatocytes in a synxenic culture. LDH assay. Readings taken at T+30 min, T+1.5 hr. and T+4.5 hr. Only the T+30 min +t samples were diluted (1:5); the rest were used full strength. The raw data (OD) and the leakage values of -t are provided below:

Time	Sample	$\Delta\text{Abs. min}^{-1} \times 3376$	Sample	$\Delta\text{Abs. min}^{-1} \times 3376$
T+30 min				
	1-t		1+t	
1'	1.159	101.28	0.653	337.60
2'	1.147		0.678	
3'	1.121		0.705	
	2-t		2+t	
1'	1.077	67.52	0.657	168.8
2'	1.102		0.670	
3'	1.127		0.678	
	3-t		3+t	
1'	1.126	33.76	0.645	168.8
2'	1.151		0.662	
3'	1.168		0.677	
T+1.5 hr				
	1-t		1+t	
1'	1.110	151.92	1.221	303.84
2'	1.162		1.389	
3'	1.178		1.477	
	2-t		2+t	
1'	1.248	121.54	1.260	202.56
2'	1.252		1.320	
3'	1.289		1.382	
	3-t		3+t	
1'	1.204	168.8	1.137	303.84
2'	1.237		1.212	
3'	1.280		1.304	

Appendix 4 cont.

Time	Sample	^a Δ Abs. min ⁻¹ x3376	Sample	^a Δ Abs. min ⁻¹ x3376
T+4.5 hr				
	1-t		1+t	
1'	1.149	202.56	1.282	219.44
2'	1.206		1.338	
3'	1.265		1.410	
	2-t		2+t	
1'	0.977	202.56	1.303	286.96
2'	1.102		1.471	
3'	1.167		1.601	
4'	1.178		1.645	
	3-t		3+t	
1'	1.088	101.28	1.919	236.32
2'	1.198		2.086	
3'	1.260		2.275	
4'	1.290		2.477	

#1 = 2.68×10^6 hepatocytes.

#2 = 1.34×10^6 hepatocytes + 1.34×10^6 amoebae.

#3 = 1.34×10^6 hepatocytes.

^aValues read from a plot of the Δ Abs. values vs time.

Appendix 5.

Expt. No. 4. Effect of E. histolytica cell-free extract on hamster hepatocytes.

$\Delta\text{Abs. min}^{-1}$ for samples B,C,D are provided below where

flask # B = 4.10^6 hepatocytes only

C = 4.10^6 " + 100 μl E. histolytica sonicate

D = 4.10^6 " + 400 μl " " "

Percent leakage has been calculated as in previous experiments. (No contribution to LDH activity from serum-free TPS-I.)

Time	Sample	^a $\Delta\text{Abs. min}^{-1}$		Sample	^a $\Delta\text{Abs. min}^{-1}$		Sample	^a $\Delta\text{Abs. min}^{-1}$	
T+30'	B-t			C-t			D-t		
1'	0.26	0		0.551	0		0.628	-	
1.5	0.350	0.09		0.543	-0.01		0.616	0	
2'	0.448	0.19	0.18	0.560	0.01	.007	0.615	-0.001	0.002
2.5	0.535	0.28		0.548	-0.003		0.617	0.001	
3'	0.627	0.37					0.618	0.003	
	B+t			C+t			D+t		
1'	0.345	0		0.47	0		0.844	0	
1.5	0.356	.10		0.585	0.12		0.997	0.15	
2'	0.356	.10	0	0.697	0.23	0.22	1.152	0.31	0.3
2.5	0.356	.10		0.809	0.34		1.337	0.49	
3'				0.920	0.45		1.454	0.61	

Appendix 5 cont.

Time	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}}$ min^{-1}	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}}$ min^{-1}	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}}$ min^{-1}
T+1.5 hr B-t				C-t			D-t		
1'	1.402	0		1.325	0		1.385	0	
1.5	1.418	0.02		1.328	-0.003		1.440	0.06	
2'	1.411	0.01	0.04	1.331	0.01	0.03	1.453	0.07	0.07
2.5	1.430	0.03		1.340	0.02		1.432	0.05	
3'	1.453	0.05		1.361	0.04		1.523	0.14	
B+t				C+t			D+t		
1'	2.812	0		1.742	0		2.161	0	
1.5	2.839*	0.03		2.086	0.34		2.506	0.35	
2'	2.839	0.03	0	2.286	0.54		2.684	0.52	0.175
2.5	2.840	0.03		2.536	0.79		2.771	0.61	
3'	2.839	0.03		2.641	0.90		2.857	0.696	
T+4.5 hr B-t				C-t			D-t		
	1.242	0		1.169	0		1.467	0	
	1.252	0.01		1.236	0.07		1.507	0.04	
	1.296	0.05		1.327	0.16		1.529	0.06	
	1.348	0.11	0.075	1.416	0.25	0.18	1.557	0.09	
	1.398	0.16		1.448	0.28		1.587	0.12	
	1.438	0.20							

Appendix 5 cont.

Time	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}} \text{ min}^{-1}$	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}} \text{ min}^{-1}$	Sample	$\Delta\text{Abs.}$	$\overset{a}{\Delta\text{Abs.}} \text{ min}^{-1}$
	B+t			C+t			D+t		
	1.680	0		1.601	0		Values too high to be read off spectrophotometer.		
	2.154	0.47		1.901	0.30				
	2.236	0.57	0.165	2.097	0.50	0.245			
	2.264	0.78		2.222	0.62				
	2.729	1.50		2.497	0.90				
	2.729			2.635	1.03				
	2.839	1.16							

* 2.839 was the approximate limit of sensitivity of the spectrophotometer.

^aValues read from a plot of the $\Delta\text{Abs.}$ values vs time.

Appendix 6.

Expt. No. 6. Damage by intact E. histolytica on hepatocytes. Percent leakage has been calculated as $\frac{\Delta \text{Abs. min}^{-1}}{\text{" " " "}} \times \frac{3376}{\text{for } -t} \text{ for } -t$ X 100 as before, for every sample.

Time	-t	Sample	+t	-t	Sample	+t	-t	Sample	+t
T+30'		1			2			3	
1'	1.364		1.492			1.577	1.360		1.489
1.5	1.356		1.594	1.379		1.677	1.362		1.577
2'	1.333		1.676	1.384		1.774	1.365		1.663
2.5	1.330		1.774	1.390		1.868	1.366		1.745
3'	1.311		1.850	1.391		2.031	1.369		1.893
	Percent leakage = 0.			Percent leakage = 4%.			Percent leakage = 3%.		
T+1.5 hr		4			5			6	
1'	1.174		1.480	1.453		1.403	1.112		1.632
1.5	1.179		1.494	1.548		1.520	1.128		1.648
2'	1.183		1.578	1.518		1.581	1.163		1.746
2.5	1.193		1.670	1.502		1.656	1.203		1.813
3'	1.202		1.768	1.515		1.733	1.242		1.871
3.5				1.487		1.802			1.927
	Percent leakage = 5%.			*			Percent leakage = 62%.		

* Ambiguous value not presented.

Appendix 6 cont.

Time	-t	Sample	+t	-t	Sample	+t	-t	Sample	+t
T+4.5 h5		7			8			9	
1'	1.517		1.481			1.286	1.413		
1.5	1.560		1.616	1.358		1.360	1.516		
2'	1.592		1.690	1.518		1.511	1.634		
2.5	1.579		1.801	1.595		1.450	1.659		
3'	1.545		1.890	1.633		1.479	1.754		
Percent leakage = 43%.			Percent leakage = 82%.			Percent leakage			

9+t values too high to be monitored.