# PATHOGENIC MECHANISMS IN AMOEBIASIS

by

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# A B S T R A C T

The delicate balance between host and parasite in the pathogenesis of amoebiasis has been studied in vitro and in vivo.

The cytopathic effect of Entamoeba histolytica upon a tissue cell monolayer is described using light and electron microscopy, and the system has been quantitated by labelling the monolayer with $^{51}$Chromium. The findings emphasize the importance of contact between amoeba and cell, and suggest mechanisms by which damage may occur. The quantitative model allows the dynamics of the interaction to be studied in detail. The method has been applied to several of the physiological variables that affect an amoebic inoculum, and also to the comparison of amoebic strains. The general uniformity of a series of personally isolated strains is shown by antigenic analysis and sensitivity to drugs and low temperature. Two new methods of measuring the median lethal dose of amoebicidal drugs have been devised. Some of the possible roles of cell mediated immunity in amoebiasis have been investigated using mouse spleen cell cultures.

A synergistic relationship was found between E.histolytica in mice and concurrent infection with Schistosoma mansoni or Trichuris muris. The relevance of local tissue damage and immunosuppression is discussed. The importance of dietary factors has been studied in rats; protein deficiency increases susceptibility but carbohydrate supplementation appears to have a protective effect. Two methods were used to produce anoxic liver damage in the hamster; both facilitated localized amoebic abscess formation. A moderate degree of alcohol induced liver damage had no effect.

A study of strain competition *in vitro* led to the development of a mathematical model of amoebic infection, which allows valuable interpretations to be made from epidemiological data. Many of the problems of amoebic pathogenesis in man can only be studied in this way.

It is concluded that while amoebic strains do show some intrinsic differences, the outcome of infection in man is determined mainly by host factors.

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## INTRODUCTION AND REVIEW

### 1. JUSTIFICATION FOR FURTHER WORK ON THE PATHOGENESIS OF AMOEBIASIS

Recent years have witnessed great advances in the diagnosis and therapy of patients with amoebic disease. For example, the recognition of invasive disease, especially liver abscess, is greatly helped by the use of serological tests such as gel diffusion, latex agglutination, indirect haemagglutination and indirect immunofluorescence. Similarly, radio-isotope hepatoscans and ultrasound assist in the localisation of hepatic lesions. Therapy has become more rational and less dependent upon polypharmacy; the use of dehydro-emetine, metronidazole and other nitro-imidazole derivatives means that most patients can now be treated safely and successfully. So effective are these advances that it might be surmised that further work on pathogenesis is unnecessary.

Such complacency would be premature. Amoebiasis is predominantly an infection of developing countries where the aforementioned facilities are rarely available, especially in rural areas where most of the population live. The incidence of disease in many populations is unknown and unstudied. Equally disturbing are the well recognised high disease rates in the crowded and rapidly growing urban populations of the tropics. Because of its relapsing nature and protean clinical presentations, amoebic disease can be notoriously difficult to recognise; even in developed countries known cases are often centred about physicians with a special interest - for example, Oxford, UK (Wright, 1966) or Little Rock, Arkansas, USA (Juniper, 1971).

There is an urgent priority to recognise those populations where invasive disease is particularly frequent, so that appropriate diagnosti-

services and preventative measures may be set up. Furthermore, within populations it is vitally important to identify persons who are especially susceptible to tissue invasion. For example, it is known that latent infections may become overt disease in patients receiving immunosuppressants or corticosteroid therapy; the increasing use of such medications in developing countries is bound to result in many more such cases.

In some places amoebiasis is such a common and serious disease that vaccination must be seriously considered. The development of effective vaccines will depend upon a detailed knowledge of immunological rejection mechanisms in man and experimental animals.

Complacency about the effectiveness and safety of metronidazole is now beginning to wane. Treatment failures are now not infrequently reported (Pittman and Pittman, 1974) and lung tumours and lymphomas have been reported in mice given lifelong medication (Rus'ia and Shulik, 1972). The search must therefore continue for new amoebicides.

It is contended in this thesis that pathogenic mechanisms can only be fully understood by studying the amoeba at three levels of host organisation: the host cell, the individual host and the host population. Studies at one level frequently have explanatory value at another and an overall view can only be obtained by a synthesis of all three approaches.

## 2. SOME IMPORTANT DEFINITIONS, AMOEBIASIS AS A BIOLOGICAL SYSTEM

Diseases are dynamic processes whose causes and mechanisms are included within the general term pathogenesis. In the case of parasitic disease it is useful to try and separate parasite and host factors by introducing the terms virulence and resistance. This is an artificial division and the inverse relation between the terms is an *a priori* one, each being definable only in terms of the other. Neither can be measured on an absolute scale, but both may be ranked on an ordinal scale and the $LD_{50}$ values of two strains of parasite or host may be compared as a virulence ratio or resistance ratio respectively. The raw data is the observed outcome of the host parasite interaction. If both hosts and parasites vary no deductions are possible. Comparisons of virulence or resistance can only be made when either the host or the parasite population is standardised. Wilson and Miles (1961) have discussed these semantic problems in some detail.

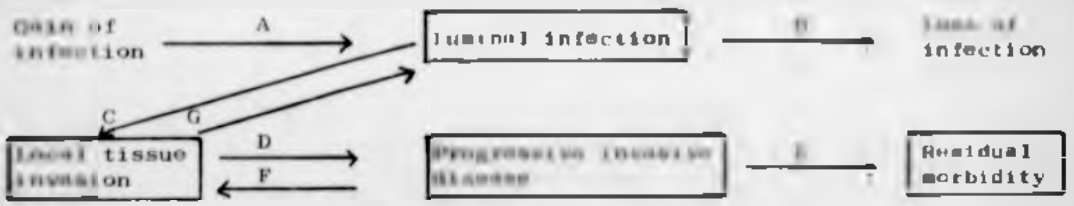
Dubos (1949) has defined virulence as "The ability of a micro-organism to establish a pathological state in a given host is the summation of a number of different and independent attributes such as communicability, invasiveness and toxigenicity ..... Virulence is not a permanent, intrinsic property of a given species. It expresses only the ability of a given strain of the infective agent, in a certain growth phase, to produce a pathological state in a particular host, when introduced into that host under well defined conditions."

Most authors, however, would separate communicability from virulence and define infectivity as the ability of a microbe to establish a primary lodgement on arrival at the body surface, or more simply as the capacity to spread from one host to another under specified conditions.

The term pathogenic is sometimes used as a synonym for virulence but a useful distinction can be made relating to the scope of the two terms. Pathogenicity is best regarded as an attribute of a species, a genus or some other grouping of parasites. Virulence may then be used to refer to the pathogenicity of a stable homogenous strain of the microbe (Miles, 1955).

A microbial strain may be defined as a continuously multiplying population maintained by in vitro or in vivo passage, derived on a unique occasion from a wild population (Lumsden, 1967).

In order to separate the components of virulence and resistance as they relate to amoebiasis it is useful to consider the dynamics of the infective process diagrammatically:




Each of these dynamic processes can be interpreted in terms of both parasite and host factors. In this thesis gain and loss of infection (A and B) are discussed in terms of a deterministic epidemiological model in Part 20, with reference to in vitro studies in Part 8 that are relevant to superinfection. The parasite factors that determine the transition to progressive disease (D) are discussed in Part 10.1, and the host factors that resist this process are discussed in Part 11 (experimental animals) and Part 18 (man). The host factors that determine loss of progressive disease (F) and loss of tissue invasion (G) are discussed in Part 10.2.

The nature of the transition from luminal infection to local tissue invasion (C) is discussed in Part 10.4.

In each of those parts the discussion will refer to the relevant investigational work described in this thesis. The important role of epidemiologic methods in studies of pathogenesis is discussed in Part 20.

## 3. ENTAMOEBA HISTOLYTICA AS A LUMEN DWELLING PROTOZOAN

In order to see the host parasite relationship of E.histolytica in perspective it is pertinent to look briefly at the other pathogenic Mastigophora, Ciliata and Sarcodina that infect the gut and genital tracts of vertebrates. Together with their normal hosts these may be listed as follows (Levine, 1973):

Giardia intestinalis, sensu lato (man, rabbit, rat);
Hexamita meleagridis (quail, pheasant, partridge);
Histomonas meleagridis (wild gallinaceous birds, chickens);
Trichomonas vaginalis (man); T.foetus (cattle); T.gallinae (pigeons);
Balantidium coli (pigs, rats and monkeys);
Entamoeba invadens (lizards).

In each case the parasite normally lives as a lumen commensal in its maintenance host. Prevalence rates are often high, and the infections prolonged and repeated. None have an obligate tissue phase, but pathogenicity may be high in 'abnormal' hosts, e.g. Giardia in dogs and chinchillas, Hexamita and Histomonas in turkeys, T.gallinae in turkeys and chickens, Balantidium in man and rarely the dog, and E.invadens in snakes.

Children are particularly susceptible to symptomatic giardiasis and young animals are susceptible to hexamitiasis, histomoniasis and T.gallinae infections; in the latter 3 infections the 'stress' of husbandry methods is also relevant. Host hormones are implicated in T.vaginalis and T.foetus infections and local mucosal lesions encourage Balantidium and T.vaginalis in humans. High body temperature encourages tissue invasion

by E.invadens in snakes. Metastatic spread to the liver occurs in hexamitiasis (rare), histomoniasis, T.gallinae and E.invadens infections and very rarely in balantidiasis in man (Wenger, 1967).

Differences in strain virulence have been documented in vivo for T.vaginalis and T.gallinae (Frost and Honigberg, 1962), T.foetus and Histomonas. Using chick liver cell cultures, the cytotoxicity of strains of T.vaginalis correlated with pathogenicity in man (Farris and Honigberg, 1970). Virulence usually declines on prolonged in vitro culture. Immunological responses have been studied in histomoniasis (Clarkson, 1963) and in infections with T.vaginalis (Chipperfield and Evans, 1972), T.foetus (Robertson, 1963) and T.gallinae (Stabler, 1954). In each case immunity following local or systemic disease protects against reinvasion, but does not normally eliminate the parasite; local antibody production has been studied in T.foetus and T.vaginalis infections.

# EXPERIMENTAL [illegible] IN VITRO

## 4. INTRODUCTION

### 4.1. The Cytopathic Effect of Amoebae upon Cells

The accidental discovery that hartmanellid amoebae, derived from throat washings, could destroy monkey kidney cells in roller tube cultures (Jahnes et al., 1957; Culbertson et al., 1958) demonstrated in vitro for the first time the cytopathic potential of amoebae upon vertebrate cells. Since that time it has become evident that many strains of soil amoebae belonging to the genera Hartmanella and Naegleria have similar properties (Kingston and Warhurst, 1969).

Earlier Shaffer et al. (1953) had shown that living cellular extracts of chick embryos would support serial transfer of E.histolytica in the absence of bacteria; the mechanisms were not studied. These workers also showed that E.histolytica would enter blocks of liver tissue, but they noted no lytic lesions. Meerovitch (1961) was able to grow E.invadens in chick intestine organ cultures but again no lytic process was noted and damage was attributed to associated bacteria. Maegraith et al. (1959) incubated E.histolytica with suspensions of human gut epithelium for 2 to 18 hours and showed by paper chromatography that the cells had been digested by hydrolytic enzymes.

The next major step forward was the demonstration that E.histolytica exerted a chemotactic effect upon the blood leucocytes of several mammalian species, which were rapidly killed on contact with the amoebae (Jarumilinta and Kradolfer, 1964). Finally, Eaton et al. (1970) showed the cytopathic effect of bacteria-free E.histolytica upon tissue cell monolayers grown in Rose chambers; with the production of lesions very like those noted 12 years before with Hartmanella. The experimental

work described here in Part 6, represents an extension of the work of Eaton and his co-workers. The methodology has been considerably modified, however, so that the process can be replicated more easily and quantified.

4.2. Strain Differences

4.2.1. In vitro. Several approaches have been made towards strain characterisation in vitro and the detection of genetic markers. Antigenic differences have been demonstrated using immuno-electrophoresis (Krupp, 1966) and indirect immunofluorescence (Goldman, 1960; Goldman et al., 1962; Lunde and Diamond, 1969). Amoebal enzymes have been studied by electrophoresis (Reeves and Bischoff, 1969) and by their action upon various substrates (see Part 10.1). Other characters used include ability to grow at room temperature, utilisation of different sugars, and drug sensitivity (Entner et al., 1962; Entner and Most, 1965; Albach et al., 1966). More recently Gelderman et al. (1971) have studied [illegible] and DNA base composition.

The main conclusion from this work has been that diphasic strains such as LAREDO and HUFF are quite distinct from true E.histolytica. Differences have been demonstrated by all the methods employed; of these the most practical are the ability to grow at room temperature and low drug sensitivity (emetine, fumagillin, carbazone, actidione, etc.). The taxonomic position of these strains has been summarized by Goldman (1969); they are now often referred to as E.histolytica-like amoebae. Strains intermediate between these and true E.histolytica have not been found. The situation within the true E.histolytica group appears to be one of general uniformity. Minor differences have been detected by immuno-electrophoresis, immunofluorescence and drug sensitivity but it is not

known how reproducible such characters are, nor their long term stability. Nayebi (1971), using cross absorption of antisera with respective antigens, showed that it was possible to distinguish between 2 strains by immunofluorescence.

4.2.2. Virulence in experimental animals. Many host species have been used to study and compare strain virulence. The earlier work has been fully reviewed by Faust (1960), Neal and Vincent (1955) and Neal (1957). Currently the most widely used method is intracaecal inoculation of trophozoites into weanling rats. This assay method must be carefully standardized; the important host variables being the breed of rat, age of weaning, age at inoculation and diet. Both the infection rate and the caecal score are dose related and for reproducible results at least 50,000 amoebae must be given (Singh et al., 1963).

With this method it has been clearly demonstrated that strains recently isolated from dysentery patients give higher caecal scores than strains from symptomless carriers; furthermore, virulence correlates well with the patient's serological status (Neal et al., 1968). Strains usually but not always lose virulence on prolonged culture, but this may often be restored by hamster liver passage. The virulence of carrier strains cannot normally be enhanced. Encystment does not appear to affect virulence. Although normally examined after 7 days, lesions appear to remain stable in size for many weeks. Repeated isolates from the same patient may differ but few studies have been done. By exchanging the bacterial flora between strains it can be shown that virulence is a characteristic of the amoeba and not the bacterial associate.

While the results obtained by British workers have been relatively clear cut, the findings elsewhere have sometimes been at variance. Minor differences in methodology and real differences in local strain patterns may explain this. Mizgireva (1966) studied 78 strains in Russia and showed that some carrier strains were definitely virulent; however, there was a general downward trend in strain virulence in the following sequence: strains from dysenteric patients, convalescent patients, contacts of patients and carriers with no known patient contact. In India, Gopal Rao and Padma (1971) found several carrier strains to be virulent and some patient strains to be relatively non virulent; however, some of the latter were not recent isolates. These authors commented upon the mild but reproducible ulceration produced by relatively avirulent strains. Both groups, unlike the British, found some strains of intermediate virulence.

Although the rabbit has been little used, Hunninen and Boone (1957) showed a good correlation between caecal ulceration and the source of the strain. Old World monkeys, such as Macacus rhesus and M.sinicus, are easily infected with cysts by mouth, but gut ulceration is exceptional even with dysentery strains (Dobell, 1931). The susceptibility of the guinea pig is similar to that of the rat, except that virulent strains are often lethal.

The findings in kittens are very different. Although variable results may be obtained when cysts are given by mouth, when trophozoites are injected into the ileum all strains of true E.histolytica produce colonic ulceration with only minor strain differences (Meleney and Frye, 1935). Two out of 3 strains repeatedly tested for 5 years maintained their virulence. The virulence of atypical E.histolytica does not appear to

have been tested in the kitten; one might expect them to be non virulent, as in the rat. It would be of great interest to know the virulence in rats of carrier strains passaged through kittens. The dog is another susceptible host and may become naturally infected, many human strains of E.histolytica produce severe ulceration. Some 'small race' strains - possibly E.hartmanni - are apparently virulent in dogs (Tobie, 1940).

To which animal model does the human host most closely correspond? Clearly man is less susceptible than the kitten but more susceptible than the macaque. The strain differences noted in weanling rats must represent true biological differences but it is not known how significant these are relative to host differences. It is quite possible that all strains can cause disease in man when the subject is susceptible enough. What is not known, is the virulence potential of strains in man, before invasion has occurred. To measure it after invasion partly begs the question since some strains may develop an enhanced virulence in vivo. The stability of measurable virulence of strains persisting in a human host has been little studied.

## 5. SOME FACTORS AFFECTING AMOEBIC GROWTH IN VITRO

### 5.1. Introduction. Media Used.

In the present work three methods of amoebic culture have been used. The general characteristics of these media and their main applications in this thesis will now be described; details of composition and methodology are given in Appendix 1.

A. Robinson's medium (Robinson, 1968).

This is bacteria-associated diphasic medium in which amoebae grow in Bijou bottles at the base of a 3 ml liquid overlay on an agar slope. The principal nutrients are horse serum, starch and bacteria. Escherichia coli, strain B, is the main bacterial associate and is replenished at each subculture. Other bacteria are also present, however; these are derived from the primary inoculum at isolation. The culture is thus polyxenic. Bacterial growth is partly suppressed with erythromycin.

This culture method was used for primary isolation of strains from patients and for long term strain maintenance. It was also used to produce gut infection in rats and mice, for the re-isolation of the amoebae from infected animals and for general in vitro studies.

B. Modified Shaffer-Frye technique (MS-F) (Reeves et al., 1957).

Amoebae are grown monoxenically with a penicillin-inhibited inoculum of Bacteroides symbiosus, using 125 x 16 mm tubes, and a monophasic liquid medium. A new inoculum of Bacteroides is added at each subculture. The other main nutrient is horse serum, which also agglutinates the bacteria.

In the present work this medium was used principally for the transfer of strains from Robinson's medium to a crithidia-associated

medium (TTY, see below). Various antibiotics were used in the transfer process. Direct transfer from Robinson's medium to TTY medium was also sometimes possible.

C. Tryptose Trypticase Yeast medium (TTY) (Diamond, 1968a).

This medium is bacteria-free, the amoebae are grown monoxenically in 125 x 16 mm tubes, in a monophasic liquid medium with a Crithidia sp. This flagellate (strain ReF-1: PPR) was originally isolated by Dr. L. S. Diamond in 1958 from a cog-wheel bug, Arilus cristatus, in Maryland, U.S.A. It grows well at 25°C but at 37°C multiplication is largely inhibited, and the organisms round up and eventually die; in this form they become an ideal substrate for amoebae. The other main nutrient is horse serum.

Being bacteria-free, this culture method was used extensively in the in vitro studies with tissue cell monolayers. It was also used in drug sensitivity studies, for the preparation of antigen for the fluorescent antibody studies and for the study of experimental liver abscess in hamsters.

A total of 18 strains of E.histolytica were successfully transferred to this medium from a bacteria-associated culture. Long term maintenance in TTY was not difficult and only strain ZOCKLING had to be retransferred.

The growth characteristics of amoebae in these three media is very relevant to all the subsequent work. Some of the more important features will now be examined. Details of the different strains are given in Appendix 2 and counting methods in Appendix 4.

5.2. Growth Curves in the Different Culture Media

Method. To compare the rates of growth in the three media (Robinson's, MS-F and TTY), 10,000 strain ZOCKLING amoebae were inoculated into new cultures, the inocula coming from the corresponding medium. Two cultures of each medium were counted daily, for 6 days, and then discarded. The total count was calculated as the product of the count per millilitre and the volume.

Result. The growth curves for each medium showed a similar sequence; a lag phase during the first 24 hours, followed by a period of rapid growth until a peak was reached at 48 hours (MS-F and TTY) or 96 hours (Robinson's medium). The counts (Figure 1) then declined quite rapidly, without much evidence of a stationary phase to reach low levels after 5 or 6 days; the precipitate fall in Robinson's medium after day 5, shown here, did not always occur. It is likely that the amoebae are biologically different at different phases of the culture cycle. The precise duration of each phase will be determined by many factors such as inoculum size and the supply of nutrients.

5.3. Inoculum Size

Method. Subcultures were set up using different inoculum counts within the range 500 to 40,000 amoebae; two or three replicates being made of each inoculum. After 48 hours the total count was estimated for each inoculum.

Result. There was a direct linear relationship between the inoculum size and the 48 hour count, the regression line passing through the origin of the graph. This relationship was demonstrated in Robinson's medium with strains EVANS and RUFF, and also with E.invadens; in TTY

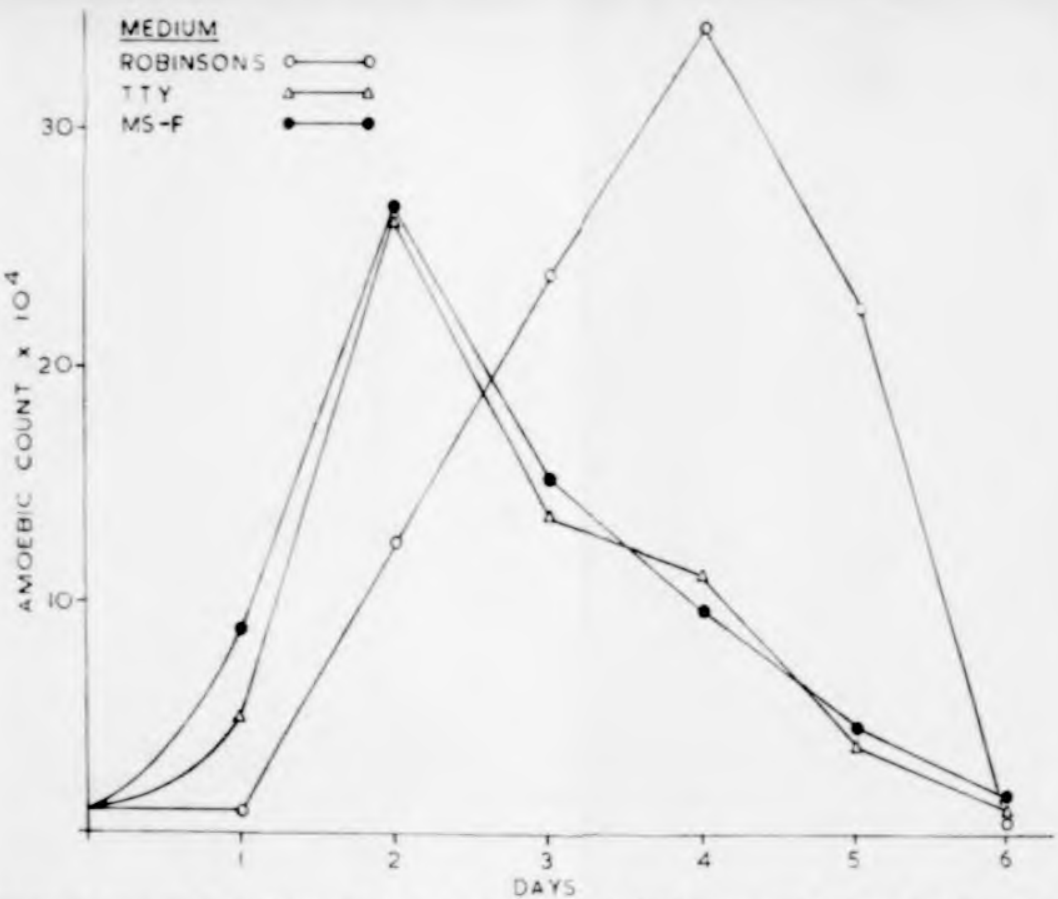



Figure 1. Amoebic growth curves in different culture media. Total daily counts in three media. All inoculated with 10,000 strain ZOCKLING amoebae.

medium with strain DKB (see Figures 6 and 7); and also with E.invadens in axenic culture. With bigger inocula or longer periods of culture there was no linear relationship; see, for example, the experiments in Part 8.2.

5.4. pH

Method. A series of TTY media were made up with their pH adjusted to values between 6.0 and 7.2. These media were inoculated with 10,000 strain EVANS amoebae and counts made daily for 4 days. Two separate cultures at each pH were counted each day and the pH measured; they were then discarded.

Result. At 24 hours the counts (Figure 2) were inversely related to pH, suggesting that pH 6.0 supported maximal initial growth. At 48 hours growth was maximal at pH 6.4 with slightly lower values for 6.0 and 6.8, and a considerably lower value at 7.2. Measurements of culture pH at the time of counting showed constant levels except for the pH 7.2 cultures in which it fell to 6.8. When this experiment was repeated, similar results were obtained.

5.5. Redox Potential

Method. The principal reducing substance in TTY medium is L-cysteine hydrochloride; normally a concentration of 0.8 g.litre is used. To study the effect of different redox potentials, separate lots of media were made up with concentrations of 0, 0.4, 0.8 and 1.6 g.litre. Eight thousand trophozoites (strain EVANS) were inoculated into tubes containing these media and daily counts made for 5 days. Each day 2 cultures were examined at each concentration and then discarded.

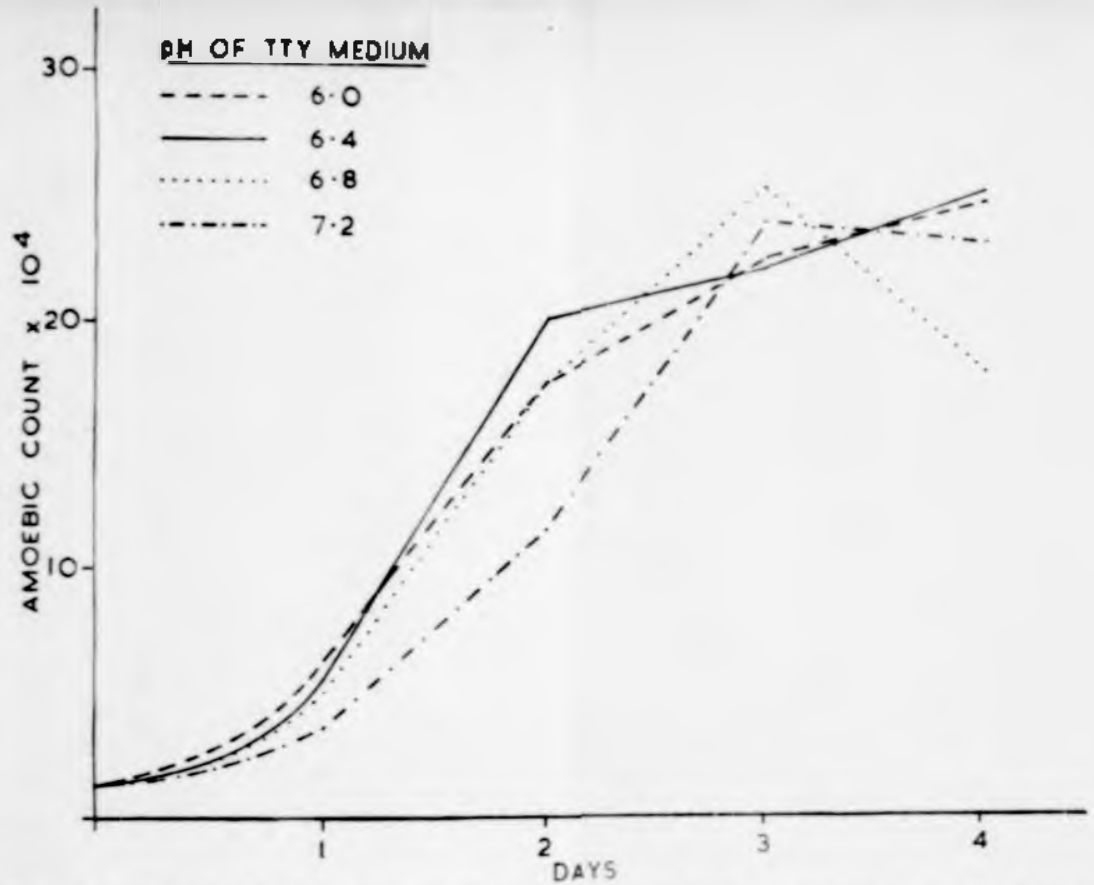



Figure 2. Amoebic growth curves at different culture pH values. Total daily counts at four pH values (TTY medium). All inoculated with 10,000 strain EVANS amoebae.

Results. Growth was reasonably satisfactory in all the media (Figure 3); however, the counts in tubes with no cysteine showed significantly lower counts ($p < 0.05$), at 24 and 48 hours, compared with the other media. At 72 hours, the cultures with no cysteine showed a growth spurt; presumably at this stage cell metabolites have reduced the redox potential to more favourable values. Perhaps the high counts at days 3 and 4 in cultures with 0.4 g.litre cysteine can be explained in a similar manner. The higher counts with 0.8 and 1.6 g litre cysteine, at day 5, may indicate a medium more favourable to prolonged cithidial viability.

5.8. Age of Culture

Method. A series of cultures were set up in TTY medium using 10,000 strain EVANS amoebae. At daily intervals a culture was counted and 6 subcultures made with 10,000 amoebae. Two of those subcultures were counted each day for 3 days and then discarded.

| Donor Culture | | Count of Subculture x $10^4$ | | |
|---|---|---|---|---|
| Age | Count x $10^4$ | 24 hr | 48 hr | 72 hr |
| 24 hrs | 9.7 | 8.3 | 15.9 | 21.7 |
| 48 hrs | 18.8 | 8.5 | 19.7 | 21.4 |
| 72 hrs | 23.7 | 6.5 | 17.3 | 20.6 |
| 96 hrs | 17.5 | 4.1 | 13.3 | 20.1 |
| 120 hrs | 9.5 | 5.4 | 16.1 | 20.2 |

Table 1. Effect of age of amoebic culture upon growth of subculture. Total daily counts, all initiated with 10,000 amoebae.

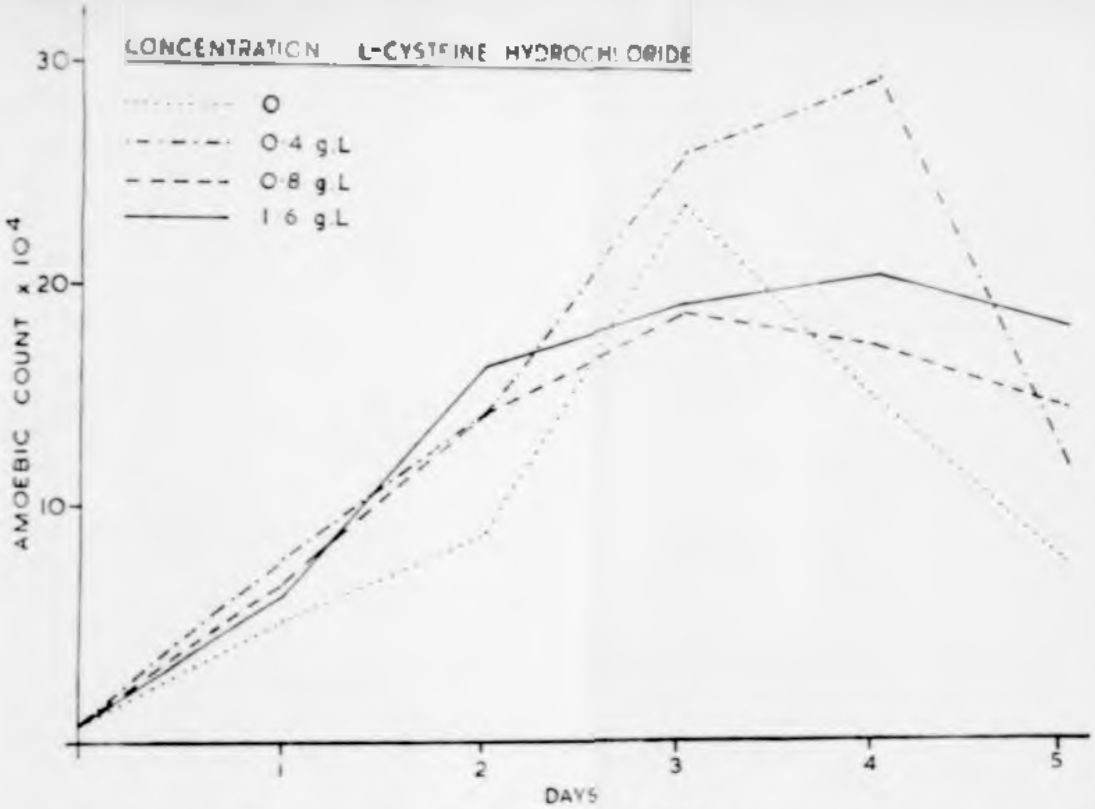



Figure 3. Amoebic growth curves at different culture redox values. Total daily counts at four concentrations of L-cysteine hydrochloride (TTY medium). All inoculated with 8,000 strain EVANS amoebae.

Result. Vigorous growth occurred in all the subcultures, so that very similar counts were obtained after 72 hours (Table 1). At 24 hours, however, subculture counts definitely tended to be lower as the donor culture became older.

5.7. Discussion

The age of an amoebic culture is an important determinant of several of its biological attributes. As shown in experiment 5.6, subcultures grow more rapidly from young cultures. This effect was observed during routine strain cultivation and it is also illustrated in Table 11. Subcultures to be used for experimental purposes should probably always be taken from cultures that are themselves young and growing actively. The effect of culture age upon cytotoxicity to a cell monolayer will be studied in Part 6.3.3.4.

Satisfactory growth was observed at pH 6.8 in TTY medium and this value has been used in this work for routine cultures, rather than pH 7.2 as recommended by Diamond (1968A). Similarly, multiplication occurred in the absence of a reducing agent. These findings suggest that amoebae in TTY cultures, unlike those in polyxenic bacterial cultures (Marinkovic and Marinkovic, 1955), can live under conditions similar to those of mammalian tissues.

The linear relationship observed between inoculum sizes below 40,000 and the total count after 48 hours has been used in the present work to enable estimates to be made of the number of viable amoebae in strain competition experiments (Part 8), or following the exposure of an amoebic population to an amoebicidal drug (Part 7.4.2.A).

## 6. THE CYTOPATHIC EFFECT OF E.HISTOLYTICA UPON A TISSUE CELL MONOLAYER

### 6.1. Introduction and Method

For these studies the rabbit kidney cell line (RK.13) was chosen as the cellular substrate, because it forms an even confluent monolayer that is firmly adherent to the glass surface of a coverslip or Carrel flask bottom. Furthermore, it can withstand for several hours the physiological conditions provided by the amoebic medium TTY when the pH has been adjusted to 6.8 and the osmolarity to 320 milliosmoles. The methods used for maintenance and subculture of this cell line are given in Appendix 5.

Cells to be used for light and phase-contrast microscopic studies were grown upon round coverslips with a diameter of 30 mm. These were placed at the bottom of sterile airtight flat-bottomed plastic containers of 30 ml capacity (Sterilin Ltd.), to which was added a suspension of kidney cells in 10 ml of medium 199. Suitable monolayers were present after 4 to 6 days. The coverslip was then removed and the outer rim wiped free of cells; after inversion it was placed upon a tissue culture chamber (Sterilin Ltd.) and sealed with silicone grease. The chambers were filled with TTY medium together with the amoebic suspension, normally 5,000 trophozoites.

For the quantitative work, kidney cells were grown for 6 or 7 days in 5 ml Carrel flasks using 1.5 ml of medium 199 and silicone rubber bungs (Esco Rubber Ltd.). Just before the experiment the 199 medium was removed and replaced by 4 ml of TTY medium.

The amoebae and other protozoa used in these experiments were grown in bacteria-free cultures, usually TTY. Suspensions of amoebae for inoculation were made by decanting the medium and replacing with fresh

medium at 4 ℃. After 5 minutes, the tubes were inverted a few times and centrifuged at 1,500 rpm for 3 minutes. Most of the supernatant was then removed and the amoebae counted in a haemocytometer. For the Carrel flasks the volume of suspension was adjusted so that the required number of amoebae were present in 0.5 ml.

6.2. Phase-Contrast Observations

Within a few minutes the amoebae became adherent to the cell monolayer. Visible lesions appeared after 20 to 30 minutes, as areas of cell damage that soon enlarged into discrete punched out lesions. These increased progressively in size until at 4 hours a considerable proportion of the monolayer had been destroyed. Within the lesions, the amoebae could be seen close to, and in direct contact with the kidney cells; most appeared to be directly adherent to the glass and they were situated principally at the periphery of the defects (see Plate 2). Kidney cells near to or in contact with amoebae were damaged; the cells appeared to rupture before losing contact with the glass. After several hours the supernatant became finely clouded; after centrifugation cellular debris and amorphous material could be seen but no intact cells. Cells between the lesions appeared quite normal and could not be dislodged even by vigorous agitation of the medium.

These findings suggested that cell damage only occurred at the sites of contact with amoebae. Further evidence for this hypothesis is provided by the following observations:-

(i) No visible damage to the monolayer occurred when any of the following were added to the monolayers:

(a) the supernatant from 48 hour amoebic cultures (see Plate 1);

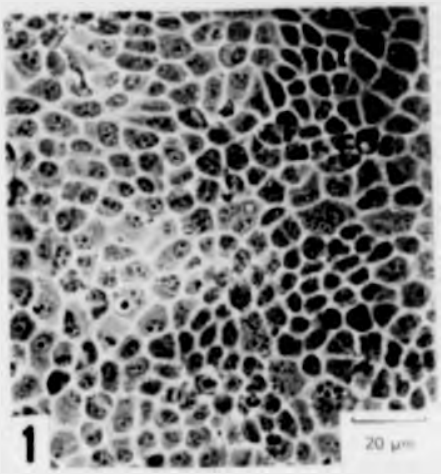



Plate 1. Normal kidney cell monolayer. Phase contrast. Undamaged monolayer after 2 hours exposure to amoebic culture supernatant. (x 637)

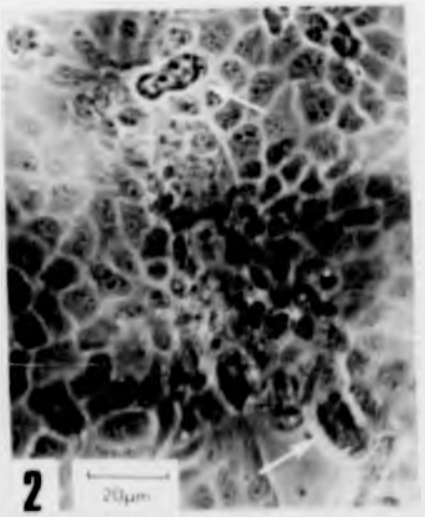



Plate 2. E.histolytica destroying kidney cell monolayer. Phase contrast. Areas of cell damage 2 hours after addition of amoebae (↗). (x 637)

(b) living or ultrasonicated Crithidia, or the medium from an established culture of this organism;

(c) ultrasonicated or freeze-thawed preparations of E.histolytica.

(ii) When a Carrel flask was tilted at 30° soon after addition of the amoebae to the flask, the monolayer was only damaged near the lowest point where all the amoebae had collected.

(iii) A drop of cooling liquid agar (0.7% in TTY) at 37°C was allowed to solidify as a thin disc upon the surface of the central part of the monolayer. After adding a large amoebic inoculum and incubating for 4 hours, all of the monolayer around the disc was destroyed, but that part beneath the agar remained intact despite the persistence of trophozoites upon the surface of the agar.

6.3. A System for Quantitating the Cytopathic Effect

In order to measure the ammount of monolayer damage caused by different amoebic inocula under various conditions, amoebae were allowed to attack $^{51}$Chromium-labelled cells growing in 5 ml Carrel flasks.

6.3.1. Method. On the sixth day of culture the confluent kidney cell monolayer was labelled with $^{51}$Chromium as sodium chromate by adding 1.75 microcuries of this isotope, contained in 0.2 ml of medium 199. Next day, excess isotope was removed by washing with 3 ml of medium 199, followed by elution for 1 hour in a further 3 ml of medium 199; finally, the monolayer was washed twice with 3 ml of TTY amoebic medium.

The amoebae were then added to the flasks, which already contained 4 ml of TTY medium. After careful mixing, the flasks were incubated undisturbed and lying horizontally at 37°C. When the experiment was over,

normally at 4 hours, the flasks were gently shaken and the supernatant poured into the first radioactivity counting tube (88 mm long and 20 mm in diameter with plastic stopper); this was followed by 2 washes with TTY to make a final volume for counting of 10 ml. The cell monolayer was then examined microscopically (x 80) for the presence of defects and the percentage loss estimated visually. To remove the remaining monolayer, 3 ml of distilled water was added to each flask; after 12 hours at 4 °C, all cells could easily be shaken free from the flask bottom and the contents poured into a second counting tube, followed by 2 washes with water to make a total of 10 ml. The radioactivity of the 2 specimens, from each flask, was measured in a well type gamma scintillation counter. The counting time was 400 seconds, the voltage being 25 and the discrimination bias 5 volts. A 10 ml standard was also counted, this was made up from 0.1 ml of the 0.2 ml isotope inoculum put into each flask the day before. The results were calculated in the following manner:-

Let Cn = the isotope count of the supernatant

Cm = the isotope count of the monolayer

Cs = the isotope count of the standard

Then the total percentage loss of isotope from the monolayer (T) is given by:

$$T = \frac{Cn \times 100}{Cm + Cn}$$

and the percentage uptake of isotope by the monolayer (U) is given by:

$$U = \frac{(Cs + Cn)\ 100}{2\ Cs}$$

As some isotope elutes from the undamaged monolayer during the experiment, this must be accounted for. It is assumed that isotope elutes from the undamaged monolayer at the same rate as it does in the control flask. At the end of any experiment let L be the true percentage of the monolayer that is destroyed and K be the percentage isotope loss in the control flask. Then the elution in an experimental flask

$$= \frac{K\ (100 - L)}{100}$$

Now total loss (T) = True loss (L) + Elution loss

Therefore T $$= L + \frac{K\ (100 - L)}{100}$$

And L $$\frac{100\ (T - K)}{100 - K}$$

6.3.2. Basic features of the system. The uptake of $^{51}$Chromium by the monolayer after overnight labelling varied between 12 and 25%; most of the uptake occurred within 2 hours, and by 4 hours a plateau was reached. Measurement of spontaneous loss of isotope from the monolayer into TTY medium showed a linear loss with time reaching about 10% in 8 hours. Control flasks always showed an intact healthy-looking monolayer and a clear supernatant which showed no cells or debris when centrifuged; indicating that the loss of isotope into TTY medium was due to elution and not cell loss from the glass.

In the presence of amoebae there was good agreement between the percentage loss measured isotopically and that estimated by inspection. Even after complete destruction of the monolayer, the amoebae themselves contained less than 3% of the total radioactivity. In all the experiments described in Part 6.3, between 2 and 4 replicates were made of each inoculum.

Incubation time. In most experiments, inocula of between 5,000–40,000 amoebae were used. Following an initial delay of 15–20 minutes, there was a linear increase in percentage isotope loss with time. Figure 4 shows how the percentage loss (corrected for elution) increased with time, using an inoculum of 20,000 strain EVANS amoebae. With most inocula of 20,000 amoebae, the loss was between 30% and 60% after 4 hours, at 8 hours, the monolayer was often completely destroyed so that only amoebae remained attached to the glass.

Inoculum size. Using inocula containing different numbers of amoebae, the percentage loss was linearly related to the number of amoebae added. Thus, for example, in one experiment using different inocula of between 2400 and 81,000 EVANS amoebae, the percentage losses after 4 hours were linear, 40,000 amoebae giving a 25% loss (Figure 5).

Physical damage. To test the sensitivity of the system to minor changes in physical conditions, several factors were considered. Normally, a volume of 1.5 ml of medium was used; when the same number of amoebae were added in different volumes of medium the following percentage losses were obtained: 72% with 1.5 ml, 72% with 3.5 ml, 82% with 2.5 ml and 53% with 1.5 ml. When replicates of the same 4.5 ml of suspension were, before inoculation, subjected to vortex stirring for 2 seconds, the loss was 80% and after 7 or 17 seconds it was 55%. When further replicates of the same suspension were forcibly syringed 2 and 10 times through a gauge 23 needle to produce frothing the respective monolayer losses were 53% and 29%. Further studies showed that when an amoebic suspension was maintained at room temperature for up to 1 hour, the cytopathic activity was reduced by 10–15%.

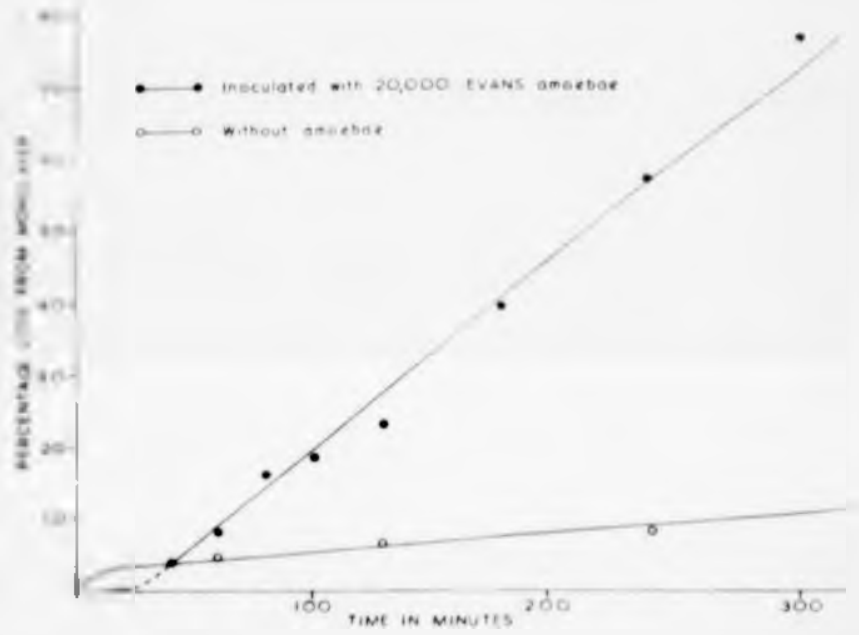



Figure 4. Isotope loss from monolayers in presence and absence of amoebae. Effect of incubation time upon 51-Chromium loss from labelled kidney cell monolayers. The values with amoebae have been corrected for elution.

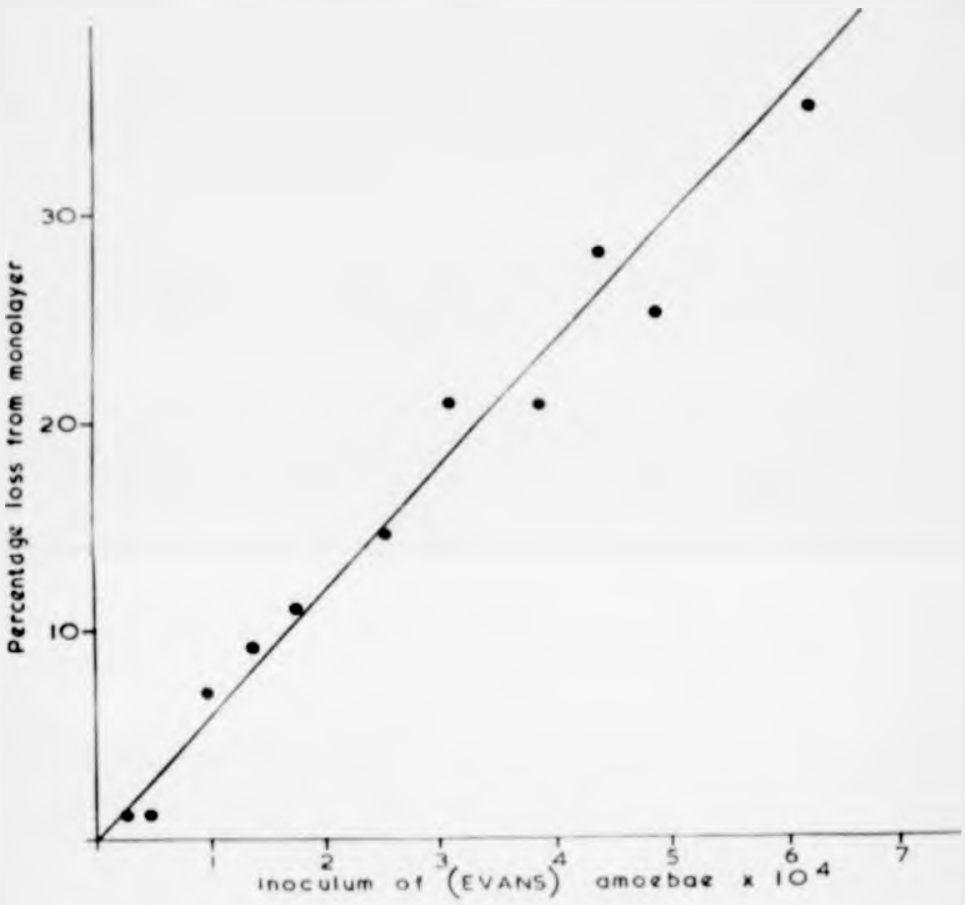



Figure 5. Effect of amoebic inoculum size upon isotope loss from monolayers. 51-Chromium loss, at 4 hours, from labelled kidney cell monolayers. The values have been corrected for elution.

Conclusion. These findings suggested that a suitable standard system would be to use 20,000 amoebae and 4 hours incubation. This gave an adequate cytopathic effect over a time interval short enough to minimise amoebic multiplication and also elution of isotope from healthy cells. The generation time of E.histolytica growing continuously on kidney cell monolayers was 8-10 hours so that a 30% increase might be expected during 4 hours. However, direct counting after 4 hours gave increments of only 10-15%; possibly handling of the amoebae before inoculation temporarily inhibited division.

In view of the susceptibility of the amoebae to physical damage it was important that exactly the same experimental procedure was used for each inoculum being studied.

6.3.3. Some applications of the quantitative cytopathic system

(a) Age of the culture. A series of strain EVANS subcultures was set up on different days. When the amoebae from these cultures were tested together the respective monolayer losses were: 20% from a 7-day culture, 21% from a 5-day culture, 19% from a 4-day culture, 45% from a 3-day culture and 56% from a 48-hour culture.

In another experiment a number of strain EVANS subcultures were set up together from a 48-hour old culture. The amoebae were then tested for their cytopathic effect over the next 48 hours. The percentage monolayer losses were: 56% from the initiating culture, 53% after 4 hours subculture, 50% after 8 hours, 41% after 24 hours, 71% after 32 hours and 57% after 48 hours.

These findings suggest that the age of a culture is a very important variable affecting the cytopathic effect. The fall in activity between

4 and 24 hours may well correspond to the lag phase of amoebic growth. The maximal effect appeared to be produced by cultures between 32 and 48 hours old.

(b) Size of crithidial inoculum used to initiate amoebic cultures

Different amounts of crithidial suspension were added to a number of subcultures containing 20,000 strain HVAH2 amoebae from a 48-hour culture. The amoebae were counted 48 hours later and their cytopathic effect measured (Table 2).

| Volume of Crithidia Suspension Added | Amoebic Count = $10^4$ at 48 hours | Percentage loss from Monolayer |
|---|---|---|
| 0 | 1.4 | 7 |
| 0.05 ml | 2.4 | 34 |
| 0.1 ml | 6.7 | 61 |
| 0.2 ml | 7.5 | 63 |
| 0.4 ml | 7.3 | 68 |

Table 2. Effect of crithidial inoculum upon amoebic count and cytopathic effect

Cultures without crithidia grew very poorly and had little cytopathic effect. However, neither the amoebic count nor the cytopathic effect was significantly increased by volumes of crithidial suspension greater than 0.1 ml. Normally 0.2 ml of suspension was used to initiate cultures.

(c) pH and redox potential of the medium. A series of TPY media was made up with their pH values adjusted to between 5.75 and 7.3. When amoebae were added to these media in Carrel flasks a maximal cytopathic effect occurred between 6.6 and 6.8, but results differed little over

the range 6.4-7.0. Below pH 6.0 the monolayer was damaged directly. Using medium 199 at pH 7.2, instead of TTY, amoebae caused little damage and adhered poorly to the monolayer and glass.

Similar experiments with different TTY media made up with L-cysteine hydrochloride concentrations between 0 and 1.6 g.l showed no significant differences.

(d) Effect of disrupted trophozoites and culture supernatant. Amoebae were broken up by ultrasound or repeated freezing and thawing in water. Inocula of up to 100,000 amoebae, so prepared, produced no cytopathic effect. Culture supernatant similarly caused no isotope loss.

(e) Protozoa other than typical E.histolytica. The crithidial associate normally grown with the amoebae, produced no damage when used alone; even with inocula of 2 million organisms. All the amoebic inocula referred to in this work contained far fewer crithidia than this. A strain of E.hartmanni and 2 atypical E.histolytica strains (HUFF and LAREDO) were grown in TTY with crithidia; none showed any cytopathic effect. A strain of E.invadens, grown axenically, produced no damage at 25°, 34° or 37°C. A strain of Trichomonas hominis grown alone in TTY had no effect. However, a strain of T.vaginalis grown in the same way produced small punched out lesions in the monolayer; the organisms appeared to cluster in great numbers at the edges of the lesions. The supernatant from a T.vaginalis culture, obtained by contrifugation, caused mild diffuse damage and some isotope loss. Unsuccessful attempts were made to establish Dientamoeba fragilis in TTY medium. Studies with this organism on cell monolayers would be of great interest in view of its disputed pathogenicity.

(f) Axenic E.histolytica. Strain NIH: 200 growing axenically produced visible changes apparently identical to these produced by other strains growing with crithidia. 40,000 amoebae from a 24-hour culture gave a monolayer loss of 55% while the same number from a replicate culture gave a loss of 35% a day later.

(g) Alternative cellular substrates and cholesterol. Amoebae were cultured with cellular substrates other than Crithidia.

(1) When grown with human red blood cells for 48 hours, growth was very slow and cytopathic effect negligible.

(2) Amoebae (strain EVANS) were grown upon kidney cell monolayers in Carrel flasks and then removed by chilling, 1 to 24 hours later. The cytopathic effect of 20,000 of these amoebae was then compared with 20,000 crithidia-associated amoebae.

| | Kidney cell associated | | | | | Crithidia associated | |
|---|---|---|---|---|---|---|---|
| Time | 1 hr | 2 hr | 3 hr | 4 hr | 24 hr | 24 hr | 72 hr |
| Cytopathic effect (%) | 21 | 22 | 17 | 14 | 35 | 40 | 21 |
| Donor monolayer damage (%) | 5 | 10 | 35 | 35 | 100 | - | - |

Table 3. Cytopathic effect of amoebae grown with kidney cells compared with those growing with Crithidia.

There was no evidence of an enhanced cytopathic effect in the amoebae taken from the monolayer (Table 3); in this experiment the donor monolayer damage was estimated visually. The low cytopathic effect at 1 and 2 hours

and the even lower activity at 3 and 4 hours, may be due to disturbance or the amoebae entering a lag growth phase. In a similar experiment amoebae associated with kidney cells for 4 and 24 hours produced cytopathic effects of 31% and 14% respectively, compared with 24 and 48-hour crithidia-associated replicate cultures which gave losses of 43% and 20%.

(3) A suspension of normal mouse spleen cells (prepared by the method given in Part 9.1) gave very good growth, with amoeba counts at least as good as crithidia-associated cultures, for up to 8 days.

The growth and cytopathic effect of amoebae grown with spleen cells was studied by culturing 20,000 amoebae (strain EVANS) with spleen cells in the proportion 100 or 25 per amoeba and comparing the results with the same inoculum growing with Crithidia. In this experiment, spleen cells enhanced the cytopathic effect but did not affect growth rate (Table 4). A similar enhancement was obtained when strain DKB was grown with spleen cells.

| | 48 hours | | 72 hours | |
|---|---|---|---|---|
| | Count x $10^4$ | % loss | Count x $10^4$ | % loss |
| Spleen cells 100: 1 | 11.0 | 83 | 21.5 | 77 |
| Spleen cells 25: 1 | 11.2 | 85 | 13.6 | 77 |
| Crithidia alone | 11.4 | 71 | 19.2 | 57 |
| Crithidia + 0.2 mg cholesterol | 7.0 | 50 | 3.2 | 83 |
| Crithidia + 0.1 mg cholesterol | 6.9 | — | 8.6 | — |

Table 4. Growth and cytopathic effect of amoebae cultured with spleen cells, Crithidia or Crithidia plus cholesterol. All cultures set up with 20,000 strain EVANS amoebae.

(4) The effect of cholesterol upon cultures was studied in several experiments by making up a 1% suspension in water, a 1% emulsion in peanut oil (cholesterol dissolved in oil and emulsified with saline) and a 1% solution in ethanol. In each case when 1.0 or 2.0 mg of cholesterol was added to a TYI culture amoebic growth was almost completely suppressed, while 0.5 mg and 0.1 mg nearly always produced some inhibition (see, for example, Table 4). Cytopathic effects were similar to controls using 0.1 mg but at 0.5 mg per tube the effect was often diminished (Table 4).

8.3.4. Discussion

The experiments described here demonstrate the usefulness and wide application of this new *in vitro* system. Many factors relevant to pathogenic mechanisms can be studied in detail under controlled conditions.

The phase contrast observations make it clear that contact between amoeba and cell is necessary for cell damage to occur; furthermore, disrupted trophozoites and culture supernatants cause no isotope loss from labelled cells. The linear increase in cytopathic loss with time suggests that progressive and sequential amoeba cell contacts occur with no toxic accumulation of cell metabolites; a conclusion consistent with the observed linear relation between inoculum size and cytopathic loss, and also with the phase contrast appearances.

Since isotope loss is proportional to inoculum size, this system can be applied to the measurement of amoebic viability after damage. This concept will be used in Part 9.2 to study the effect of immune serum upon amoebae, and in Part 7.4.2B to develop an assay method for measuring emetine sensitivity.

The effect of different cultural conditions upon cytopathic loss was studied in several experiments. The observation that the maximal cytopathic loss was produced by amoebae from cultures 32 to 48 hours old, suggests that biological vigour is greatest during the logarithmic growth phase; a conclusion consistent with the findings in experimental infections in animals. The shape of the growth curve for a particular culture may be of great importance. The situation is clearly complex since the growth curve is affected by the age of the initiating culture, the inoculum size, the medium characteristics and possibly the amoebic strain. These considerations are very important when different strains are being compared. Amoebae growing poorly with a small erithidial inoculum or human red blood cells had a reduced cytopathic effect compared with those growing well on a kidney cell monolayer. Similarly, amoebae grew well with mouse spleen cells and showed a greater cytopathic effect than those growing with erithidia. The finding that amoebae taken from a kidney cell monolayer while actively destroying it, had no enhanced cytopathic effect, suggests that the amoebae were already exerting their maximum effort.

For satisfactory growth in vitro, E.histolytica has a lipid requirement that is normally provided by the cholesterol contained in horse serum (LaTour and Reeves, 1965). An enhancement of virulence in animals has been reported by Sharma (1959), using culture media supplemented with cholesterol; however, the relation between virulence and cholesterol remains controversial. In the present work it has been demonstrated that supplementary cholesterol produces a dose related depression of both amoebic growth and cytopathic effect.

It was further shown that the pH and redox requirements of bacteria-free amoebae were not critical, for cytopathic effects were observed at values not very different from those of mammalian tissues.

This observation suggests that amoebae can destroy cells in relatively healthy tissue.

The destructive capacity of protozoa, other than E.histolytica, can be assayed using the same methodology, and several examples have been given. The pathogenicity of axenic E.histolytica has frequently been doubted; the present studies have demonstrated, for the first time, that these amoebae have a definite cytopathic potential in vitro.

6.4. Fine Structural Changes at E.histolytica Kidney Cell Interface.

6.4.1. Method of preparing specimens. Kidney cell monolayers were grown for 7 days upon circular glass discs (30 mm diameter and 1 mm thick), or Millipore filters (25 mm diameter and 3 micron pore size). These were placed at the bottom of 30 ml flat-bottomed plastic bottles, in 10 ml of medium 199.

Just before adding the amoebic suspension to the monolayer, the [illegible] medium was removed, and replaced by TTY medium. The usual inoculum was 5,000 trophozoites. Preparations were selected for fixation by light microscopic examination of parallel control preparations. The procedure was to pipette off the medium and gently add 3% glutaraldehyde in 0.066 M cacodylate buffer (pH 6.8) warmed to 37°C. Fixation was then completed at room temperature (25°C) for 30 minutes. Specimens for electron microscopy were further fixed in 1% osmium tetroxide in cacodylate buffer at 4°C, stained with uranyl acetate, dehydrated through serial dilutions of ethanol, embedded in araldite and later removed from the glass disc and mounted on the conical end of a 1.5 cm length of transparent plastic for section cutting. Sections were cut on a Reichert OMU2 ultramicrotome, mounted on Smethurst New 200 grids and further stained with lead citrate before examination in an EM[illegible]

6.4.2. Electron microscope findings. These demonstrated the fine structural changes in the kidney cells and amoebae that occurred after or during contact. Although the findings reported here refer particularly to strain EVANS, there was no evidence to suggest that this strain differed in any way from the other 15 cytopathic strains.

The structure of the normal undamaged kidney cells is illustrated in Plates 3 and 4; the latter showing in detail the contact zone between adjacent cells. On initial contact with an amoebae these cells appeared to be still undamaged. However, when contact was more prolonged, as seen in Plate 5, where an amoeba has burrowed between kidney cells and the Millipore filter substrate, the cell membrane remains intact and unaltered, but gross and rapid degeneration takes place in the mitochondria, with obvious vacuolation and loss of cristae. A general view of this early change is seen in a group of kidney cells at the periphery of a defect in the monolayer (Plate 6). At this stage other cytoplasmic organelles appear to be unaltered. But a careful study of other amoeba-cell interfaces revealed that other changes were taking place, although the order in which they are presented here does not necessarily indicate the true sequence of events.

(a) Changes in the kidney cells. Plates 7 and 8 show that in addition to the mitochondrial degeneration there is a concentration of peripheral cell lysosomes and microbodies, while in Plate 9, the rough endoplasmic reticulum is seen to be fragmented, with a tendency to vacuolation; and the cytoplasmic ribosomes and polyribosomes are less numerous than in control cells. In addition, the Golgi membranes appear active and there is an increase in prominence of the peripheral tonofibrils.

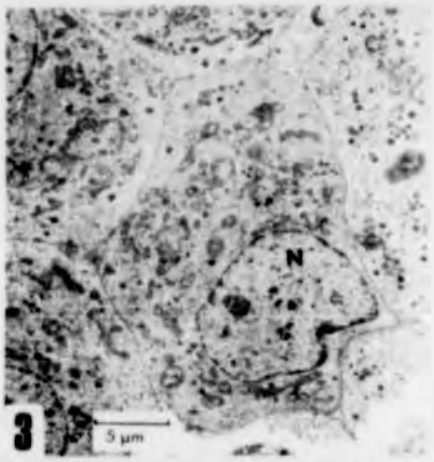



Plate 3. Normal kidney cell monolayer. Electron micrograph (EM) 1. Longitudinal section of healthy monolayer. (x 2,720)

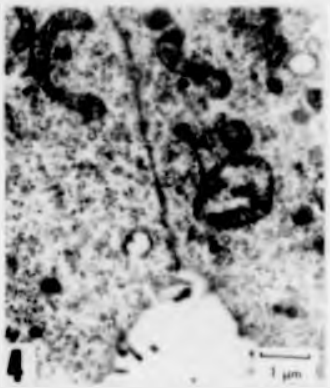



Plate 4. Normal kidney cell monolayer. EM 2. Detail of cell contact zone. Cytoplasmic organelles appear normal. (x 10,710)


Plate 5. Amoeba burrowing between kidney cell monolayer and supporting millipore filter. EM. Note mitochondrial ballooning in contacted kidney cells. (x 1,420).

Abbreviations: E.h. - E.histolytica; M - mitochondrium; MF - millipore filter; N - nucleus

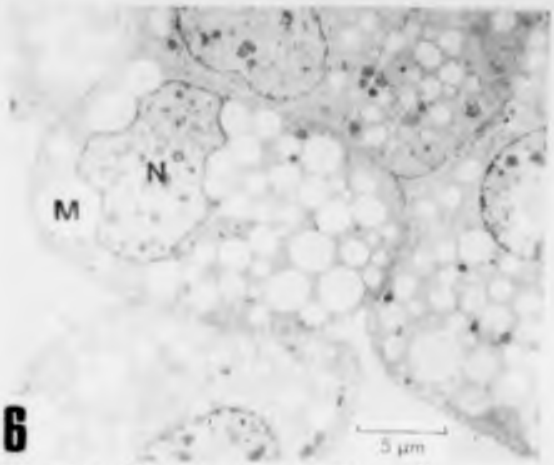



Plate 6. Early kidney cell damage. Low power. EM. Kidney cells bordering an area of amoebic cell-denudation. Note mitochondrial ballooning in contacted kidney cells. c.f.P13. (x 2,272)

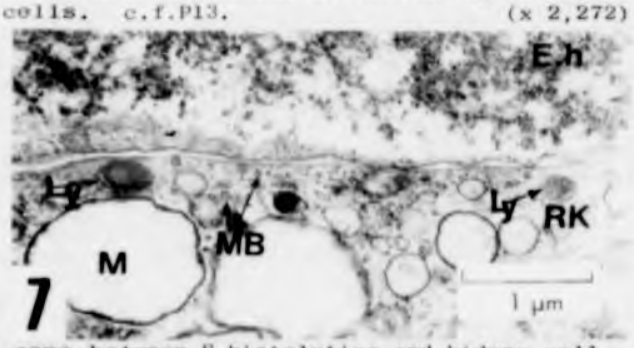



Plate 7. Contact zone between E.histolytica and kidney cell. EM 1. Both cell and parasite membranes are intact and stain densely. Kidney cell mitochondria are vacuolated, and there is crowding of microbodies and lysosomes towards cell margin. (x 14, 200)

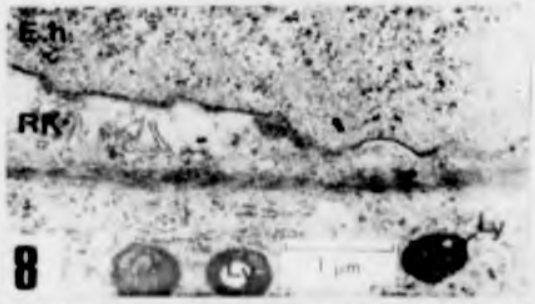



Plate 8. Contact zone between E.histolytica and kidney cell. EM 2. After 1½ hours contact some kidney cells show patchy loss of membrane, condensation of tonofibrils and early disintegration of cytoplasm adjacent to the amoeba. (x 14,200)

Abbreviations: E.h. - E.histolytica; Ly - lysosome; M - mitochondrium; MB - microbody; N - nucleus; RK - kidney cell; T - tonofibril.

Where attachment has been more prolonged, as illustrated in Plate 10, there is patchy localized cell membrane degeneration. The membrane extending beyond the patch appearing normal in both trilaminar structure and osmiophilic properties. Internal to localized patches of membrane destruction, discontinuity of tonofibrils was noted (Plate 11); together with very evident disruption of normal endoplasmic reticular pattern, mitochondrial swelling and vacuolation (see also Plate 14).

As the process of cell destruction progresses (Plate 12), the cytoplasm appears rarified, metabolically inactive and in a state of disintegration. Although the inner membrane of the nuclear envelope is still intact in this Plate, the outer membrane is absent and connections with the endoplasmic reticulum are absent. The nucleoplasm is rarified with islands of chromatin more granular and less compact than in normal cells. The final stage is illustrated in Plate 13 where there is complete disintegration of cell cytoplasm, escape of cell debris into the surrounding medium, and pseudopodial activity by the amoeba prior to the ingestion of some of this debris.

(b) Relevant findings within the parasite. Where patchy degeneration of the kidney cell membrane has occurred there is frequently a discontinuity of the amoebic surface membrane with no barrier between the cytoplasm of the cell and the parasite (Plates 8, 9, 10 and 11). The small membrane-bound vesicles seen in Plates 9 and 15 may indicate transference of cytoplasmic content from cell to amoeba. The digestive food vacuoles within the amoebae sometimes contained intact segments of trilaminar membrane, derived presumably from the mitochondria or surface membranes of the kidney cells.

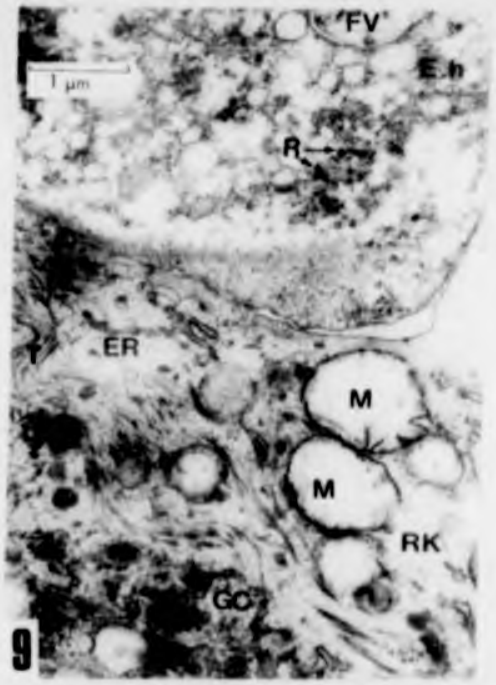



Plate 9. Contact zone between E.histolytica and kidney cell. EM 3. Contact area shows interrupted loss of amoebic pellicle and kidney cell membrane. The kidney cell mitochondria are vacuolated, the tonofibrils prominent and condensed, the Golgi complex seemingly active; but the endoplastic reticulum shows early degeneration. (x 17,000)

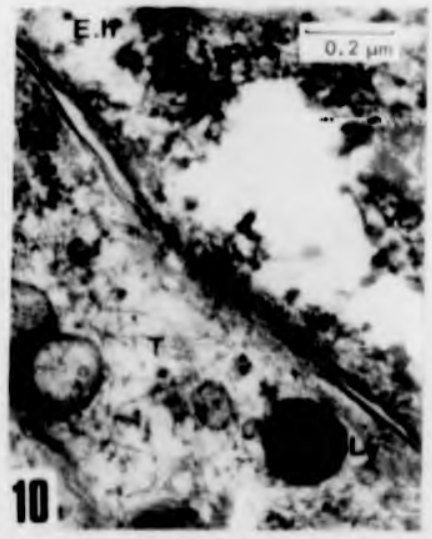



Plate 10. Contact zone between E.histolytica and kidney cell. EM 4. High magnification of an area where both surface membranes are absent with resulting continuity of amoebic and cell cytoplasms. (x 68,000)

Abbreviations: E.h. - E.histolytica; ER - endoplasmic reticulum; FV - food vacuole; GC - Golgi complex; Ly - lysosome; M - mitochondrium; R - rhabdovirus particle; RK - kidney cell; T - tonofibril.

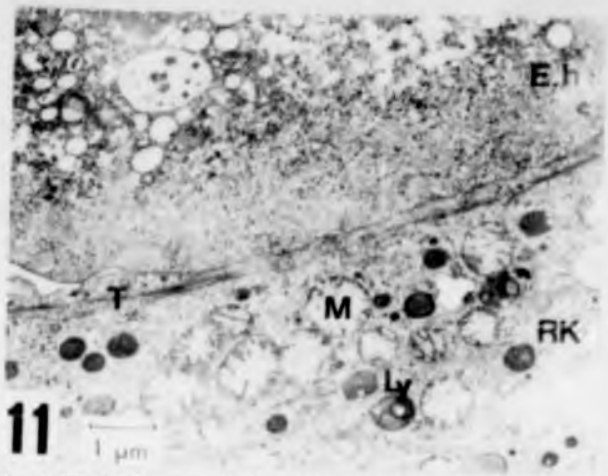



Plate 11. Contact zone between E.histolytica and kidney cell. EM 5. A broad contact area. The condensed tonofibril layer is being resorbed and the cell cytoplasm internal to it is rarified. (x 8,032).

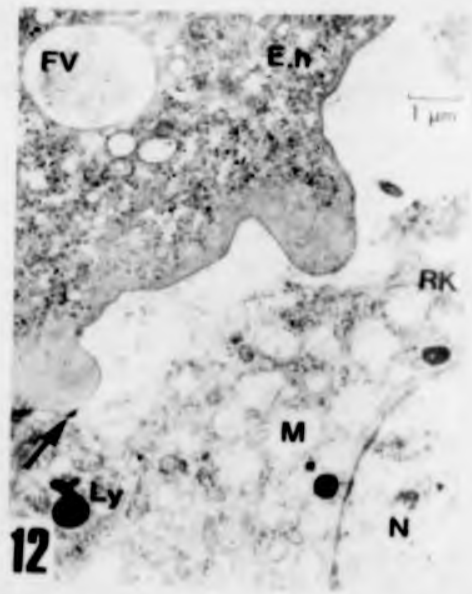



Plate 12. E.histolytica destroying kidney cell. EM 1. Cytoplasmic destruction of kidney cell with nuclear involvement. Note electron dense small liposome (↗). (x 8,032)

Abbreviations: E.h. - E.histolytica; FV - food; Ly - lysosome; M - mitochondrium; N - nucleus; RK - kidney cell; T - tonofibril.

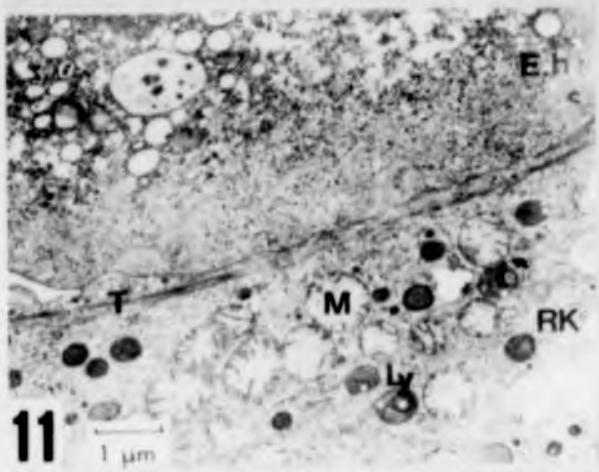



Plate 11. Contact zone between E.histolytica and kidney cell. EM 5. A broad contact area. The condensed tonofibril layer is being resorbed and the cell cytoplasm internal to it is rarified. (x 8,032).

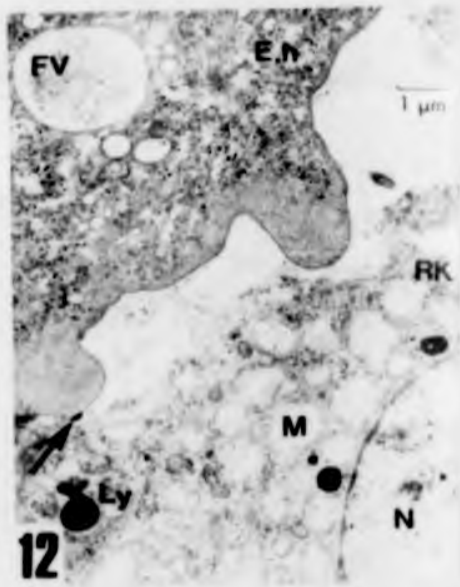



Plate 12. E.histolytica destroying kidney cell. EM 1. Cytoplasmic destruction of kidney cell with nuclear involvement. Note electron dense small liposome (↗). (x 8,032)

Abbreviations: E.h. - E.histolytica; FV - food; Ly - lysosome; M - mitochondrium; N - nucleus; RK - kidney cell; T - tonofibril.

One frequently noted feature was the presence of small (up to 150 nm in diameter) irregularly shaped osmiophilic bodies, seen sometimes in the cytoplasm and at others in contact with the inner lamina of the surface membrane (Plates 15 and 15a).

It is possible that these bodies are cytotoxic as in Plate 15 the outer mitochondrial membrane of the kidney cell adjacent to one of these bodies shows localized disintegration. Other micrographs suggest that they may be discharged by the amoebae into an adjacent kidney cell or the surrounding medium (Plate 13).

Bodies closely resembling rhabdovirus particles (Bird et al., 1974) have been found in all of the 12 strains of typical E.histolytica examined, and also in the non-cytotoxic strain LAREDO. In many trophozoites (Plate 16), these regular membrane-bound bodies (up to 250 nm long and 100 nm diameter) were seen singly or clustered as a rosette close to the cell contact zone.

6.4.3. Discussion. These micrographic studies show that substantial damage takes place within the cell cytoplasm before the surface membrane is visibly affected, and while the cells are still firmly adherent to the glass. When cell membrane damage does occur it is localized initially and cytoplasm of cell and amoebae appear to become continuous. Fusion with cells may be one of the ways that enable the amoebae to discharge their enzymes. The electron-dense osmiophilic particles seen in the amoebic cytoplasm and beneath the surface membrane may well be small liposomes (lipid droplets containing enzymes). Possibly these bodies originate in the nucleus, move across the cytoplasm and so come to lie beneath the internal lamina of the membrane; they may later fuse with it and on cell contact appear to be discharged into the cell or surrounding medium.

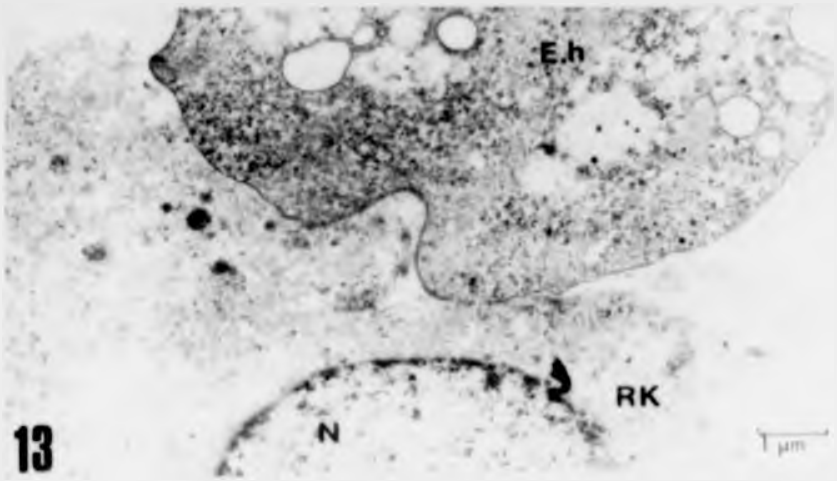



Plate 13. E.histolytica destroying kidney cell. EM 2. Advanced stage. Amoebic pseudopodium engulfing cell debris prior to ingestion. (x 8,820)

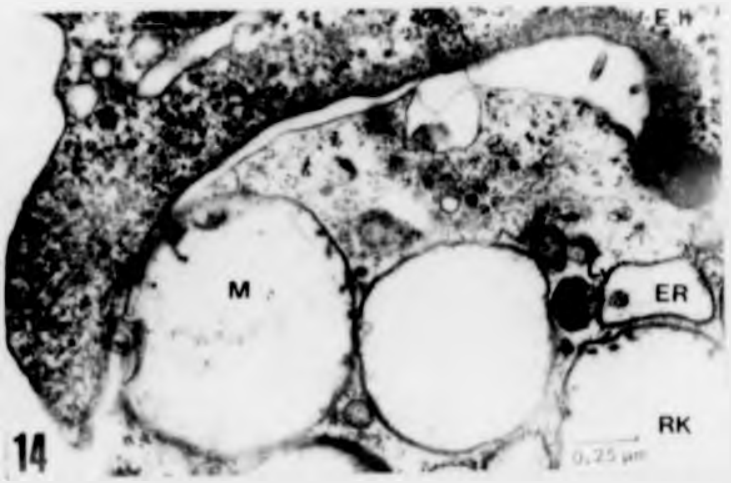



Plate 14. E.histolytica engulfing kidney cell. EM. Amoebic pseudopodium with patchy membrane fusion and disintegration of kidney cell cytoplasm.

(x 35,000)

Abbreviations: E.h. - E.histolytica; ER - endoplasmic reticulum; Ly - lysosome; M - mitochondrium; N - nucleus; RK - kidney cell.

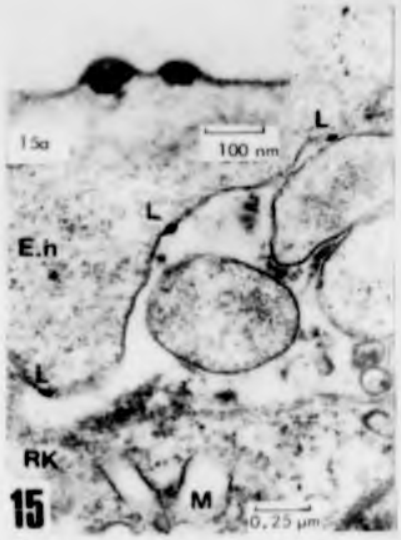



Plate 15. Liposomes on pellicle of E.histolytica. EM. Three small 'liposomes' on the pellicle of amoebae in contact with kidney cell. Nearby mitochondrial membranes are degenerating. (x 35,000)

Inset - 15a. Two such 'liposomes' in greater detail. (x 84,000)

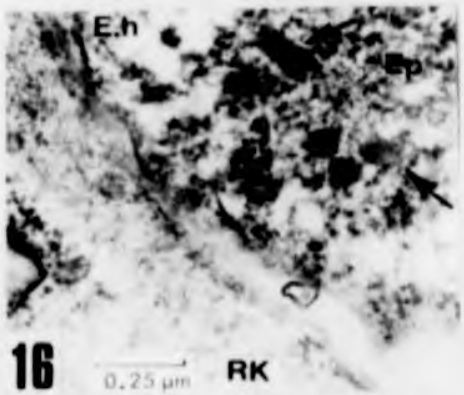



Plate 16. Rhabdo-virus bodies in E.histolytica. EM. Virus bodies (↗) close to the kidney cell contact area and clustered round a possible episome. (x 42,000)

Abbreviations: E.h. - E.histolytica; Ep - episome; L - liposome; M - mitochondrium; RK - kidney cell.

These findings of a contact-related effect is in agreement with Eaton et al. (1970) who used various cell monolayers, including RK.13; however, the fine structural changes which they describe differ considerably. They reported that 'the earliest effect on the cell was a loss of stainability of the plasmalemma affecting the entire periphery of the cell'. Plate 6 shows clearly that quite extensively damaged cells still have an apparently intact cell membrane, a normal shape and a firm attachment to the supporting surface. Their original suggestion (Eaton et al., 1969) that damaged cells are completely depolarized appears untenable. In the preparations described here there was no evidence of the surface lysosomes that they describe and it is likely that such structures are digestive vacuoles or other vacuolar structures sectioned near the amoebal surface. Another source of confusion may be partly digested Crithidia, ingested before the amoebae were added to the monolayer. The stacked membranes seen in Figure 4 of their paper (Eaton et al., 1970) and interpreted as possible Golgi-like bodies, may be so derived. Their scanning micrographs illustrating lysosomes with a vermiform trigger on the surface of bacteria-associated trophozoites could represent fixation artefacts. Proctor and Gregory (1972) have also described these structures from colonic biopsy specimens, but their illustrations are not convincing; they were not found by El-Hashimi and Pittman (1970) or by Griffin and Juniper (1971), who also used colonic material.

The methodology described here is a considerable improvement upon that used by Eaton et al. (1970). They used Rose chambers throughout and to overcome loss of $CO_2$ from the rubber gaskets of the chamber and anaerobiosis in the central part of the monolayer, it was necessary to

introduce an air bubble when the chamber was filled. Their chambers were then incubated in a perspex cabinet with a slow stream of 95% air and 5% $CO_2$, and lying upon a mechanical rocker that was activated for 5 minutes every half-an-hour. Relatively large tissue cell inocula were used and the amoebae usually added 48 hours later. Preparations were fixed for syringing 10 ml of glutaraldehyde solution slowly through the chamber whose volume was 2 ml. The lower coverslip was carbon coated, to a critical thickness, to allow separation of the embedded blocks from the glass.

## 7. STRAIN HETEROGENEITY

Several methods were used to determine whether biological differences could be detected among a series of strains of E.histolytica. Details of the source of the various strains are given in Appendix 2.

### 7.1. Growth at Room Temperature

Subcultures from strains growing in Robinson's medium were placed in incubators at 30.5°C and 25°C. A total of 25 conventional strains were tested; none grew at 25°C and survival at 30.5°C never exceeded 5 days. The strains HUFF and LAREDO, of course, grew well at either of these temperatures.

### 7.2. Antigenic Analysis using Fluorescent Antibody Staining

The antigenic relationships of 14 strains growing in TTY medium were determined by measuring the titration end points against different human antisera obtained from patients with invasive disease. Details of the fluorescent antibody staining method are given in Appendix 3. Antigen slides were made from each strain.

A preliminary titration was performed using fourfold serial dilutions and one antigen (DAWSON) to determine the approximate titre of each serum. For the definitive titration six twofold dilutions were used, with the previously determined end point in the third well. All slides were read as unknowns.

For six of the strains, sera were available from the patients from whom the strains were isolated. Table 5 shows the titres obtained when these sera were titrated against the corresponding antigens. There was no tendency for homologous reactions (underlined in the Table) to differ from heterologous ones.

| SERUM | ANTIGEN | | | | | |
|---|---|---|---|---|---|---|
| | ARNELL | ASANTE | LIGGINS | DAWSON | SWANWICK | RUSSELL |
| Arnell | 120 | 120 | 120 | 120 | 60 | 60 |
| Asante | 80 | 160 | 80 | 40 | 40 | 40 |
| Liggins | 60 | 60 | 60 | 60 | 30 | 60 |
| Dawson | 60 | 60 | 60 | 60 | 30 | 60 |
| Swanwick | 120 | 120 | 120 | 120 | 60 | 120 |
| Russell | 1000 | 500 | 500 | 500 | 500 | 500 |

Amoebic antibody titrations. 1.

Table 5. Reciprocal end point fluorescent antibody titres of six human sera reacting with antigens prepared from the corresponding strains.

The same sera, and also serum TOURMENTIN from a liver abscess patient, were then reacted against a further 8 strains (Table 6), together with strain SWANWICK which had been tested before.

| SERUM | ANTIGEN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SCOTT | BRUNT | LL77 | DKB | 106 | EVANS | SWANWICK | HUFF | LAREDO |
| Tourmentin | 250 | 250 | 250 | 250 | 250 | 250 | 250 | <250 | <250 |
| Arnell | 60 | 60 | 120 | 60 | 60 | 60 | 60 | 30 | 30 |
| Asante | 40 | 40 | 40 | 40 | 40 | 40 | 40 | < 20 | < 20 |
| Liggins | 60 | 60 | 60 | 60 | 60 | 60 | 60 | < 15 | < 15 |
| Dawson | 60 | 60 | 60 | 60 | 60 | 60 | 60 | < 15 | < 15 |
| Swanwick | 120 | 120 | 60 | 120 | 60 | 120 | 60 | N.D. | 30 |
| Russell | 500 | 500 | 500 | 500 | 500 | 500 | 500 | <125 | <125 |

Amoebic antibody titrations. 2.

Table 6. Reciprocal end point fluorescent antibody titres of seven human sera reacting with the antigens prepared from nine strains of E.histolytica.

It is apparent that strains HUFF and LAREDO, the atypical room temperature strains, are reacting at considerably lower titres. The strain SWANWICK gave the same results as before apart from differences of one dilution with sera LIGGINS and DAWSON. The titres for each serum may be summarized as follows, excluding HUFF and LAREDO:

| | | | |
|---|---|---|---|
| ARNELL | 1/60 - 7 strains; | 1/120 - 5 strains; | homologous 1/120 |
| ASANTE | 1/40 - 9 strains; homologous 1/160 | 1/80 - 2 strains; | 1/160 - 1 strain; |
| LIGGINS | 1/60 - 11 strains; | 1/30 - 1 strain; | homologous 1/60 |
| DAWSON | 1/60 - 11 strains; | 1/30 - 1 strain; | homologous 1/60 |
| SWANWICK | 1/120 - 9 strains; | 1/60 - 3 strains; | homologous 1/60 |
| RUSSELL | 1/500 - 11 strains; | 1/1000- 1 strain; | homologous 1/500 |
| TOURMENTIN | 1/250 - 7 strains. | | |

It is clear that using this methodology the strains were antigenically uniform.

In an attempt to quantify the end points more precisely, the value of a fibre-optic system was assessed. Details of this method are given by Taylor et al. (1971). A light sensitive probe is centred over each organism, at a magnification of 400, and the light meter read immediately. Using E.histolytica trophozoites as antigen it was found that the variance of the light meter readings between organisms was considerable. This was at least partly attributable to the unequal size and non-uniform staining of individual organisms. As an example of the results obtained the following is quoted. When LIGGINS serum was reacted against 4 strains the mean readings were [illegible] 20.6 (S.D. 9.3) [illegible] 14.7 (S.D. 6.8);

SCOTT 16.1 (S.D. 7.2); and LAREDO 7.0 (S.D. 4.5). LAREDO is significantly different, but the other strains are not significantly different among themselves.

7.3. Cytotoxicity

Following their establishment in TTY culture, strains were repeatedly tested for their ability to damage a tissue cell monolayer. Strains were grown for 48 hours in TTY medium and then 20,000 trophozoites were added to a $^{51}$Chromium labelled kidney cell monolayer. Percentage loss was measured after 4 hours. Details of methodology are given in Part 8.3.1. Three to 5 replicate flasks were used for each strain. Strain CYANN was included each time as a reference strain. The results of all the experiments where 3 or more strains were compared are shown in Table 7.

Two important conclusions may be drawn by simple inspection. Firstly, all the strains were cytotoxic; this applies also to strains MARGRETA, SCOTT and INGRAM which were tested once or twice and not studied further. Secondly, there was no general tendency for cytotoxicity to rise or fall during the 16-month observation period.

There was considerable variation in the loss of any one strain on different days. Two groups of factors will affect all the strains examined on a particular day:

| DATE | EVANS | SCOTT | RUSSELL | DKB | ASANTE | LIGGINS | SWANWICK | ARNELL | ZOCKLING | 106 | DAWSON | BRUNT | LL77 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25. 1.72. | 73 (1/4) | 46 (2/4) | 32 (4/4) | 33 (3/4) | | | | | | | | | |
| 4. 2.72. | 47 (1/4) | 24 (2/4) | 17 (4/4) | 18 (3/4) | | | | | | | | | |
| 22. 2.72. | 59 (2/4) | | | | 56 (3/4) | 81 (1/4) | 30 (4/4) | | | | | | |
| 25. 2.72. | 26 (2/3) | 48 (1/3) | 20 (3/3) | | | | | | | | | | |
| 3. 3.72. | 40 (1/4) | | | | 15 (4/4) | 25 (3/4) | 30 (2/4) | | | | | | |
| 9. 3.72. | 48 (2/4) | | | | 41 (4/4) | 42 (3/4) | | | 59 (1/4) | | | | |
| 15. 3.72. | 65 (2/4) | 58 (3/4) | | 54 (4/4) | 82 (1/4) | | | | | | | | |
| 13. 4.72. | 55 (1/3) | | | | 35 (2/3) | | | | | 29 (3/3) | | | |
| 6. 7.72. | 74 (1/4) | 41 (2/4) | 16 (4/4) | 27 (3/4) | | | | | | | | | |
| 6.10.72. | 84 (1/4) | | 7 (4/4) | 17 (3/4) | 20 (2/4) | | | | | | | | |
| 18.10.72. | 93 (1/4) | 28 (2/4) | 24 (3/4) | 14 (4/4) | | | | | | | | | |
| 16.11.72. | 68 (2/6) | 10 (6/6) | 50 (4/6) | 19 (5/6) | | | | 51 (3/6) | | | 75 (1/6) | | |
| 23.11.72. | 30 (4/6) | | | | | 24 (5/6) | 38 (2/6) | | 63 (1/6) | 16 (6/6) | | | 33 (3/6) |
| 10. 5.73. | 25 (4/4) | | 40 (2/4) | | 34 (3/4) | 46 (1/4) | | | | | | | |
| 23. 5.73. | 77 (1/12) | 70 (2/12) | 56 (4/12) | 28 (10/12) | 29 (9/12) | 36 (7/12) | 57 (3/12) | 50 (5/12) | | 34 (8/12) | 26 (12/12) | 28 (11/12) | 40 (6/12) |
| 31. 5.73. | 61 (3/12) | 47 (7/12) | 32 (11/12) | 48 (6/12) | 43 (10/12) | 45 (9/12) | 45 (8/12) | 65 (1/12) | | 50 (5/12) | 51 (4/12) | 11 (12/12) | 61 (2/12) |

Table 7. Repeated strain comparisons of cytopathic effect.
Cytopathic effect (% loss from monolayer) and rank (in parentheses) of 13 strains tested on different dates.

1. The quality of the kidney cell monolayer. Although the monolayers used were always confluent and 6 or 7 days old it is likely that there were minor physiological differences between batches. Thus, the $^{51}$Chromium uptake and elution loss in control flasks varied somewhat between experiments. It is likely that the size and quality of inoculum used to set up the cell culture partly determines the subsequent growth and 'maturity' of the monolayer.

2. The amoebic culture itself will be affected by:

   (a) the age of the medium;

   (b) the number, age and 'quality' of the Crithidia used to set up the TTY culture; and

   (c) the number of amoebae used to set up the culture, and their biological condition at that time.

All these factors may alter the amoebic growth curve and affect the physiological status of the amoebae when they are harvested at 48 hours.

Because it is difficult to standardize all these variables completely, the relative activity of the different strains is best studied by ranking methods. In Table 7, the ranks have been given for each experiment (rank as numerator and number of ranks as denominator).

Inspection does suggest some real difference in strain ranking. Thus, although there are exceptions, EVANS usually ranks high as does ZOCKLING. Strains SCOTT and ARNELL give high or moderate values, SWANWICK and 1177 give moderate values, while strains RUSSELL, DKB and BRUNT generally rank low. The rankings of ASANTE, LIGGINS and DAWSON are ver.

variable. To examine this ranking in more detail the 4 strains that have each been examined together on 7 occasions may be further analysed:-

| Date | EVANS | SCOTT | DKB | RUSSELL |
|---|---|---|---|---|
| 25. 1.72. | 1 | 2 | 3 | 4 |
| 4. 2.72. | 1 | 2 | 3 | 4 |
| 6. 7.72. | 1 | 2 | 3 | 4 |
| 18.10.72. | 1 | 2 | 4 | 3 |
| 16.11.72. | 1 | 4 | 3 | 2 |
| 23. 5.73. | 1 | 2 | 4 | 3 |
| 31. 5.73. | 1 | 2 | 3 | 4 |

Table H. Ranking stability of cytopathic effect for four strains

Calculation of the coefficient of concordance from the data in Table H gives a value of 0.75, Snedecor's F = 17.9, DF greater 2,7 and lesser 18,1. The 1% level of F is 3.6, hence the R value is highly significant ($p \ll 0.01$).

The relationship between amoebic growth rate and cytopathic effect was studied in four of the experiments. On these occasions the same inoculum size, 20,000 amoebae, was used to initiate all the strain cultures that were to be assayed for cytotoxicity 18 hours later.

Table 9 shows that growth rates varied between strains and also for the same strain in different experiments. When the rankings were analysed using Kendal's coefficient of correlation, there was found to be no correlation between the growth rates on 23. 5.73. and those on 31. 5.73. However, in each of these experiments growth rank was positively correlated with the rank for cytopathic loss, although the correlation was not strong ($p = 0.25$ and $p = 0.2$). Growth rates were noticeably higher on 23. 5.73. (mean = 17.5) than on 31. 5.73. (mean = 8.6); however, the mean cytotoxicity was not different, being 44.3% and 45.6% respectively.

## 7.4. Drug Sensitivity

### 7.4.1. Acriflavine and emetine in bacteria-associated cultures

Acriflavine. Subcultures from 21 strains were made into a series of 5 culture bottles containing liquid phase acriflavine concentrations of 167, 100, 66, 33 and 15 mcg.ml. At 48 hours the highest concentrations with live amoebae were 66 mcg.ml (5 strains), 33 mcg.ml (11 strains) and 15 mcg.ml (4 strains). One strain (SCOTT) failed, on two occasions, to survive at 15 mcg. ml.

Emetine. Subcultures from 9 strains were made into a series of 6 culture bottles containing liquid phase emetine concentrations of 333, 111, 38, 12.6, 4.2 and 1.4 mcg.ml. At 48 hours the highest concentrations with live amoebae were 38 mcg.ml (2 strains), 12.6 mcg.ml (5 strains) and 4.2 mcg.ml (2 strains).

| | 16.11.72. | | 23.11.72. | | 23. 5.73. | | 31. 5.73. | |
|---|---|---|---|---|---|---|---|---|
| | Count | CP % | Count | CP % | Count | CP % | Count | CP % |
| EVANS | 16.6 (1) | 68 (2) | 8.8 (1) | 30 (4) | 31.9 (2) | 77 (1) | 6.8 (10) | 61 (3) |
| DKB | 15.3 (2) | 19 (5) | | | 12.6 (9) | 28 (10) | 15.0 (1) | 48 (6) |
| RUSSELL | 10.5 (3) | 50 (4) | | | 16.6 (6) | 56 (4) | 8.5 (6) | 32 (11) |
| ARNELL | 7.2 (4) | 51 (3) | | | 7.2 (11) | 50 (5) | 14.2 (2) | 65 (1) |
| SCOTT | 5.0 (5) | 10 (6) | | | 29.2 (3) | 70 (2) | 8.6 (5) | 47 (7) |
| DAWSON | 3.2 (6) | 75 (1) | | | 18.0 (5) | 26 (12) | 6.8 (9) | 51 (4) |
| LIGGINS | | | 6.9 (2) | 24 (5) | 12.9 (8) | 36 (7) | 6.9 (8) | 45 (9) |
| LL77 | | | 6.2 (4) | 33 (3) | 34.6 (1) | 40 (6) | 12.5 (3) | 61 (2) |
| SWANWICK | | | 5.9 (5) | 38 (2) | 8.5 (10) | 57 (3) | 9.8 (4) | 45 (8) |
| 106 | | | 4.8 (6) | 16 (6) | 22.7 (4) | 34 (8) | 3.6 (11) | 50 (5) |
| ZOCKLING | | | 6.7 (3) | 63 (1) | | | | |
| ASANTE | | | | | 14.5 (7) | 29 (9) | 8.5 (7) | 43 (10) |
| BRUNT | | | | | 1.3 (12) | 28 (11) | 2.2 (12) | 11 (12) |

Table 9. Relationship between amoebic growth rate and cytopathic effect. Four experiments comparing the amoebic counts (x $10^4$) and rank (in parenthesis) at 48 hours, and their subsequent cytopathic effect (CP) and rank (in parenthesis). All cultures initiated with 20,000 amoebae.

7.4.2. Emetine sensitivity in Crithidia-associated cultures.

In order to study the dynamics of emetine's amoebicidal properties in more detail, two new methods of sensitivity testing were devised. Both give an estimate of the $LD_{50}$, the dose of emetine that kills 50% of the amoebae under the test conditions, together with a measure of the dispersion of sensitivity within the population of amoebae.

A. Depression of growth after brief drug exposure

Method. This was based upon the observation that with inocula of 40,000 or less, the amoebic counts at 48 hours are directly proportional to the inoculum size. Dilutions of emetine were made up in 3 ml volumes of TTY medium and to these were added a series of amoebic suspensions (3 ml TTY medium containing 20,000 or 40,000 amoebae but no crithidia), to give final drug concentrations of 500, 250, 100, 33, 10 and 3.3 mcg.ml. The tubes were incubated vertically for 3 hours at 37°, centrifuged and washed once in TTY medium. The tubes were then filled with TTY medium (with crithidia), incubated for 48 hours and the amoebae counted. Control tubes containing 4,000, 8,000, 20,000 and 40,000 amoebae were made up in 6 ml volumes and then treated in the same manner as those with emetine.

Results. Using 40,000 strain DKB amoebae it was found that the 48-hour count after emetine exposure was linearly related to the logarithm of the emetine concentration, except at the lowest concentration used (Figure 6). From the regression line for non-exposed amoebae it can be seen that 50% of the inoculum (i.e. 20,000 amoebae) would give a count of $4.5 \times 10^4$ at 48 hours, this value intercepts the dose response line to give a log $LD_{50}$ of 0.98 (dotted line). The experiment was repeated using

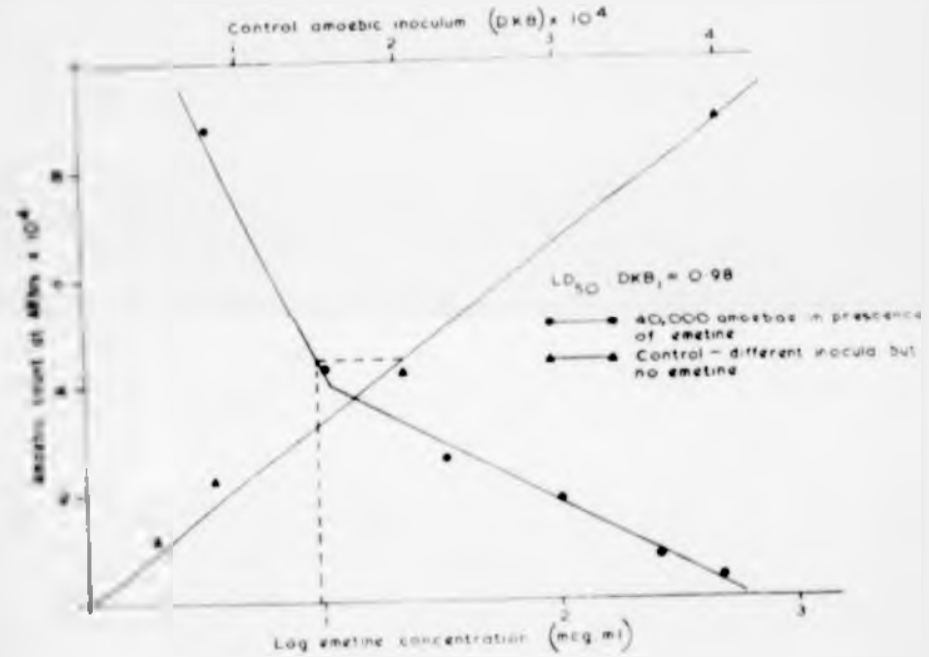



Figure 8. Amoebic growth after emetine exposure; i. Counts at 48 hours following 3 hour exposure of 40,000 strain DKB amoebae to emetine, and the growth of different inocula without emetine. Intercept from 20,000 untreated amoebae gives $LD_{50}$.

40,000 strain DKB amoebae that had been growing for 4 days upon rabbit kidney cell monolayers. In this instance (Figure 7) the dose response line was again linear, but the non-exposed amoebae grew more rapidly so that 20,000 produced 10 x $10^4$ amoebae in 48 hours; this value intercepts the dose response line to give a log $ID_{50}$ of 1.78. The higher value obtained with amoebae growing on kidney cells may be due to a change in physiological status, for when strain EVANS, from crithidia-associated cultures, was tested on two occasions, the values for log $ID_{50}$ were 1.8 and 2.2. The theoretical implications of a linear log dose response line and the meaning of its slope will be discussed in Part 10.3.

Since both log emetine concentration and non-exposed inoculum size were linearly related to subsequent growth, it was decided that the system could be simplified to a two point assay. Thus, 20,000 amoebae were exposed to emetine at 25 and 5 mcg.ml and the non-exposed inocula were 20,000 and 6,000. The log $ID_{50}$ value was estimated from the intercept of the interpolated 48-hour count from 10,000 non-exposed amoebae, upon the dose response line. When 4 strains were tested in this way, the estimated log $ID_{50}$ values were: SWANWICK 1.06, DAWSON 0.9; DKB 0.22 and ARNELL 0.08 (see Figure 8). The last two values are probably spuriously low since the intercept does not fall near the chosen assay concentrations. Another four strains were then tested using 20 mcg.ml and 3 mcg.ml as the two emetine concentrations. In this instance the intercepts indicated log $ID_{50}$ values as follows: RUSSELL 0.93, EVANS 0.8 and ASANTE 1.87 (see Figure 9). The fourth strain LIGGINS gave a very steep dose response line so that no meaningful intercept could be drawn.

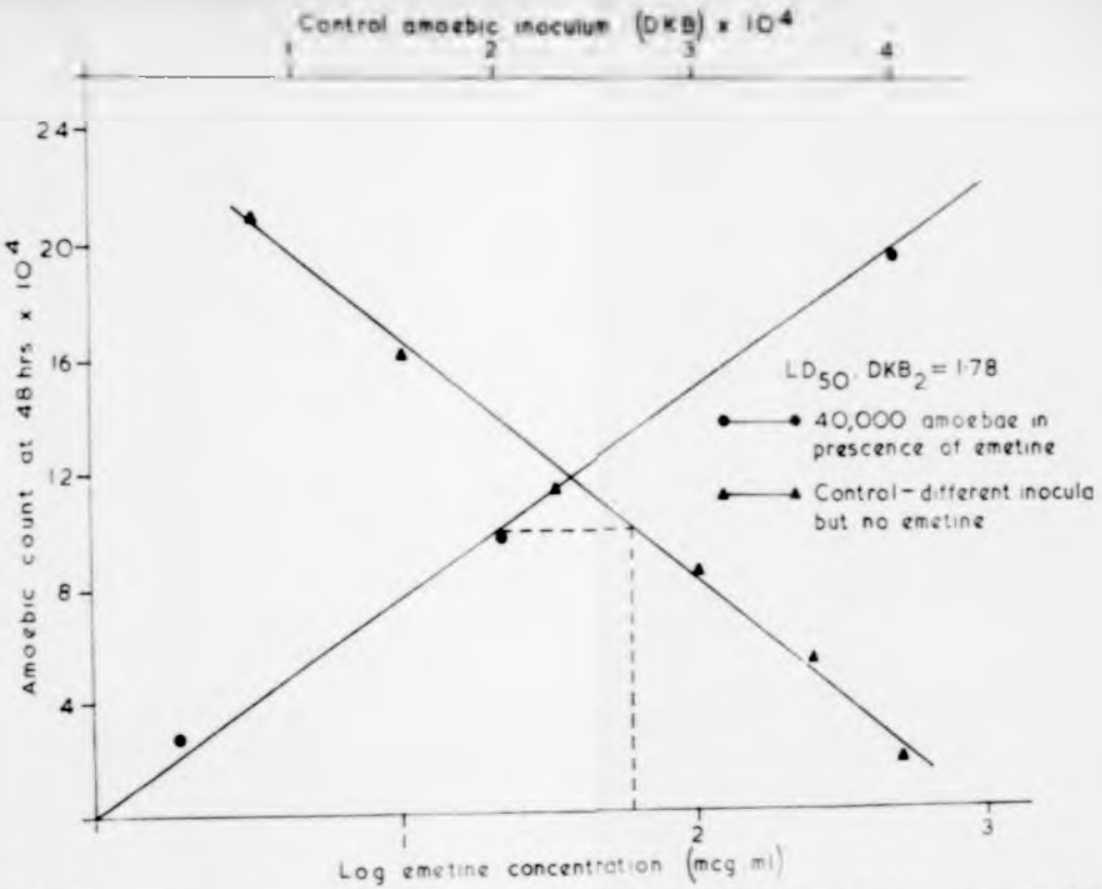



Figure 7. Amoebic growth after emetine exposure. 2. Counts at 48 hours following 3 hour exposure of 40,000 DKB amoebae (previously grown on kidney cell monolayers), and the growth of different inocula without emetine. Intercept from 20,000 untreated amoebae gives $LD_{50}$.

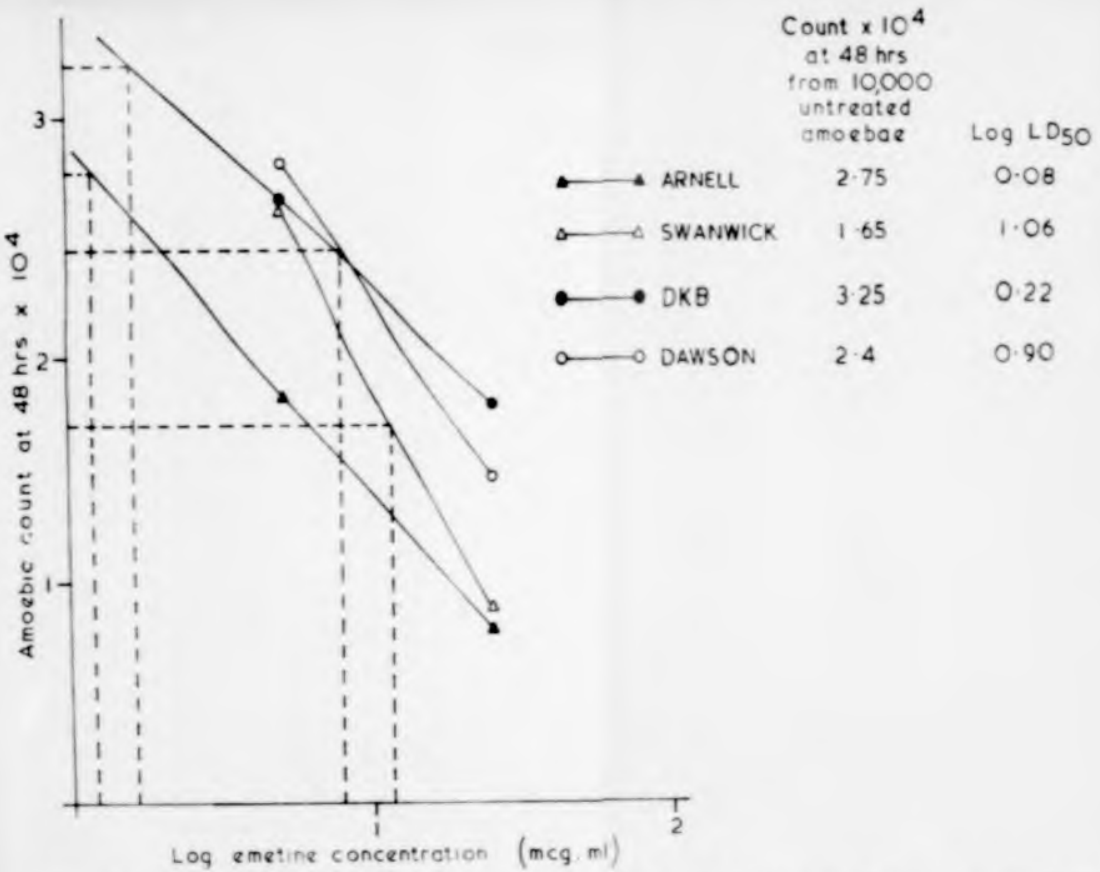



Figure 8. Amoebic growth after emetine exposure. 3. Counts at 48 hours following 3 hour exposure of 20,000 amoebae (4 strains) to 5 or 25 mcg emetine per ml. Intercepts from 10,000 untreated amoebae gives $LD_{50}$ values.

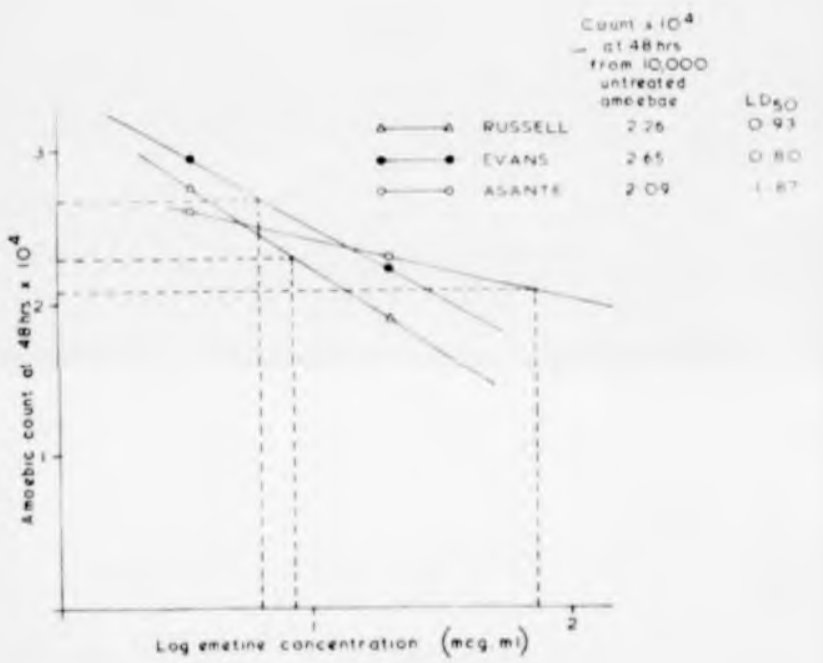



Figure 9. Amoebic growth after emetine exposure. 4. Counts at 48 hours following 3 hour exposure of 20,000 amoebae (3 strains) to 3 or 20 mcg emetine per ml. Intercepts from 10,000 untreated amoebae gives $LD_{50}$ values.

B. Cytopathic effect in the presence of drug

Method. Amoebic inocula were added to $^{51}$Chromium labelled kidney cell monolayers in Carrel flasks in the presence of various concentrations of emetine and the cytopathic effect measured by isotope loss from the monolayer. Details of this labelled tissue cell methodology have already been described in Part 6.3.1. Emetine alone did not cause isotope loss.

In a preliminary experiment the effect of the duration of emetine exposure was studied. 20,000 strain EVANS amoebae in 4 ml TTY medium (without crithidia) were added to 5 ml Carrel flasks containing the $^{51}$Chromium labelled monolayer. Two solutions of emetine (1,000 and 100 mcg.ml) were made up in TTY medium and at hourly intervals 0.45 ml was added to separate flasks. The experiment was concluded after 5 hours. A series of control flasks received 0.45 of plain TTY at hourly intervals.

| Duration of exposure (hrs) | Isotope Loss (%) | | |
|---|---|---|---|
| | Emetine | | Plain TTY |
| | 100 mcg.ml | 10 mcg.ml | |
| 5 | 18 | - | 41 |
| 4 | 19 | 27 | 43 |
| 3 | 28 | 31 | 14 |
| 2 | 33 | 30 | 47 |
| 1 | 48 | 50 | 47 |

Table 10. Effect of duration of emetine exposure upon cytopathic effect.
Percentage isotope loss from kidney cell monolayer caused by E.histolytica in the presence of emetine for 1 to 5 hours.

It was found that when emetine was added 1 hour before the end of the experiment it had no retarding effect upon monolayer damage (Table 10). Exposure for 2 to 5 hours produced a progressive retardation in monolayer damage. In the absence of emetine it has already been shown that damage increases linearly with time (see Part 6.3.2). Four hours was chosen as a suitable duration for further sensitivity studies employing cytopathic effects. By this time the damage was about half that of untreated amoebae at emetine concentrations of 10 mcg.ml. When exposure was further prolonged, for example to 48 hours, it was found that no amoebae (strains EVANS or DKB) survived at 25 mcg.ml but some would survive at 12.5 mcg.ml.

The effect of emetine concentration was then studied in more detail. 2.25 ml of amoebic suspension, containing 20,000 amoebae in TTY medium, was added to a series of Carrel flasks containing $^{51}$Chromium labelled monolayers. 2.25 ml of emetine diluted in TTY medium was added to give final drug concentrations of 500, 200, 50, 10 and 2 mcg.ml. Further flasks without emetine were inoculated with 20,000, 10,000 and 4,000 amoebae in 4.5 ml of medium, to measure the effect of inoculum size upon isotope loss. Two replicates were made throughout.

Results. This methodology was applied, in separate experiments, to strains EVANS and DKB. With both strains, the central part of the log dose response line was more or less linear but at low emetine concentrations the line must curve upwards to reach, at zero emetine concentration, a loss value equal to that of 20,000 non-exposed amoebae. This extrapolated part of the dose response line has been indicated on the graphs (Figures 10 and 11) by a dotted line. The

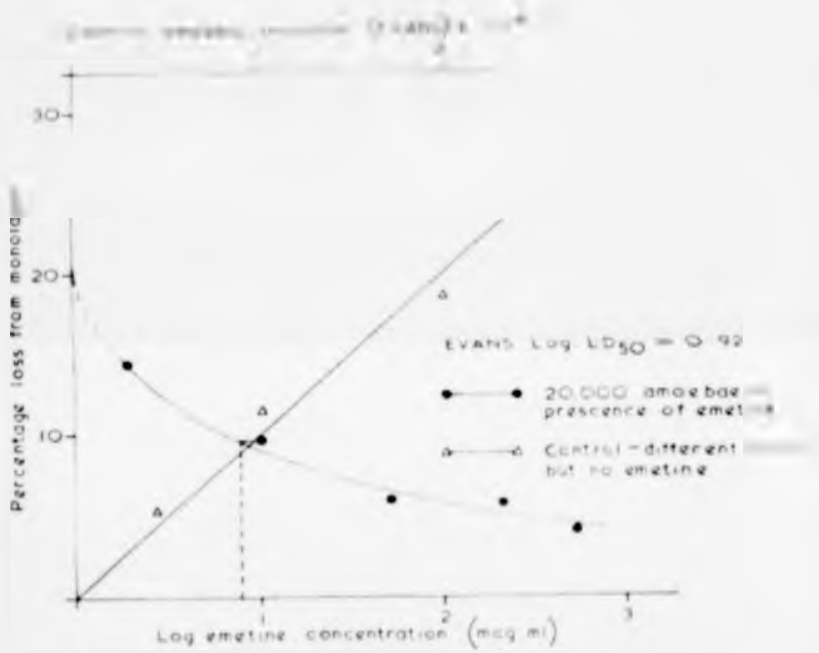



Figure 10. Cytopathic effect of amoebae in the presence of emetine. 1. 51-Chromium loss, at 4 hours, from labelled kidney cell monolayers, using 20,000 strain EVANS amoebae. Intercept from 10,000 untreated amoebae gives $ID_{50}$.

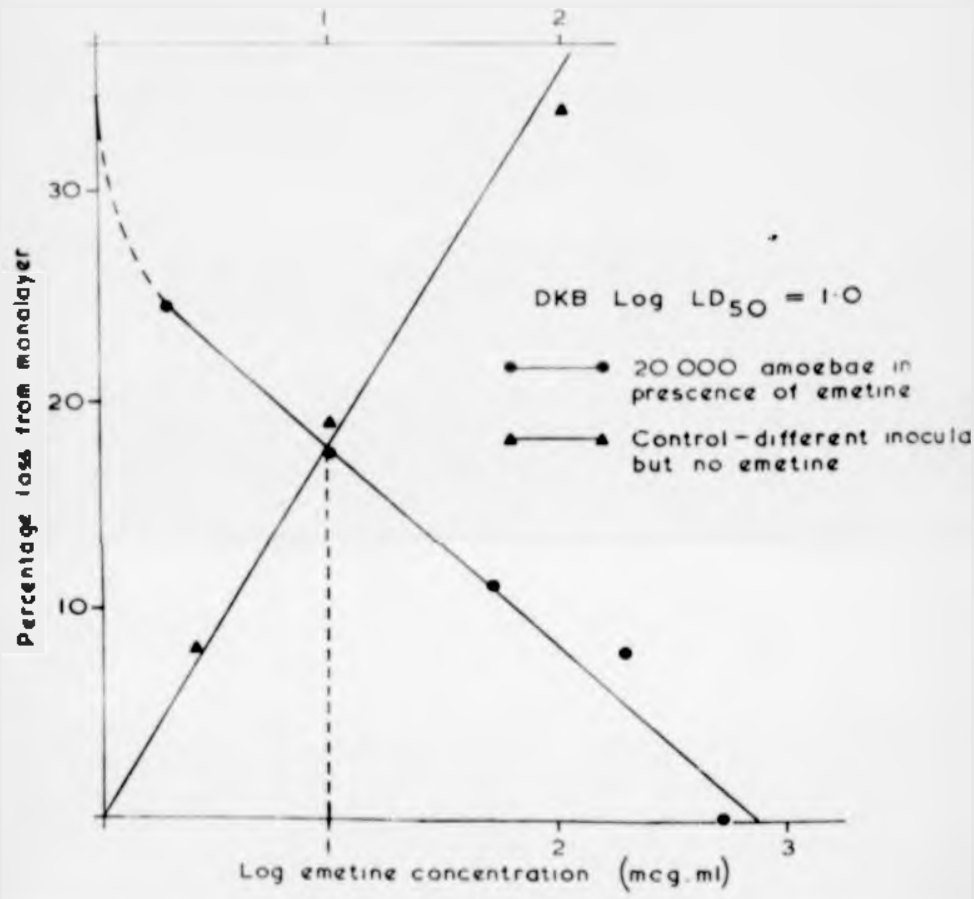



Figure 11. Cytopathic effect of amoebae in the presence of emetine. 2. 51-Chromium loss, at 4 hours, from labelled kidney cell monolayers, using 20,000 strain DKB amoebae. Intercept from 10,000 untreated amoebae gives $LD_{50}$.

central tendency equivalent to the $LD_{50}$ used in Method A may be considered to be the concentration of emetine that reduces the cytopathic effect of the inoculum by 50%, i.e. to that produced by 10,000 normal amoebae. The intercepts have been drawn on Figures 10 and 11 and it can be seen that in these particular experiments the strains EVANS and DKH gave log $LD_{50}$ values of 0.92 and 1.0 respectively. When the experiment was repeated again with strain EVANS on two different dates, log $LD_{50}$ values of 1.28 and 1.2 were obtained.

7.5. Discussion

The main conclusion to be drawn from these strain comparison studies is that, apart from strains HUFF and IARE80, all were very similar in many of their characteristics. Thus no other strains grew at room temperature and the fluorescent antibody titre, using 12 different sera was always within the range of one doubling dilution from the mode. There was no tendency for homologous reactions to give a higher titre. Drug sensitivities were also similar. The differences in the observed sensitivity to acriflavine and emetine in bacteria-associated cultures are likely to be within the range of experimental error for a simple endpoint system.

Two new methods of measuring drug sensitivity have been devised and their theoretical implications will be discussed in detail in Part 10.3. Both methods appear to give reproducible results, but once again there was no definite evidence of strain differences among the small number of strains tested. Very accurate amoebic counts are

necessary for both methods, and if a large number of strains were to be compared, the use of a Coulter counter should be considered.

In contradistinction to the above findings, some reproducible differences in strain cytotoxicity to a kidney cell monolayer were demonstrated by ranking analysis. The difficulties encountered in these studies have already been mentioned; however, it is possible that the true cytopathic potential of some of the strains was so similar that further differentiation would be nearly impossible. There was no apparent correlation between cytotoxicity and the disease state of the patient from whom the strain was isolated. Latter (1972) has reported a correlation between virulence in rats and the culturability and growth rate of different strains. In the present work only a weak correlation was noted between growth rate and cytopathic effect in vitro.

An important implication of the observed antigenic uniformity is that any of the conventional strains could be used as antigen to measure the fluorescent antibody titres of unknown sera.

## 8. STRAIN COMPETITION

### 8.1. Introduction

In many endemic areas man is repeatedly infected with E.histolytica and superinfection must be common. Thus a new inoculum may enter the ecological niche already occupied by a resident strain. The effect of such interaction in vivo is not known.

In order to study the outcome of strain interactions in vitro, a number of experiments were performed using conventional strains, strains HUFF and LAREDO, and E.invadens, in mixed and non-mixed cultures growing in Robinson's medium. The design and interpretation of the experiments are based upon the following observations:-

(1) Strains HUFF and LAREDO grow freely at either 25° or 37°C. A change from one temperature to the other gives growth rates nearly equal to those of the same strain 'adapted' by serial subculture.

(2) E.invadens will not grow at 37°C.

(3) The 48-hour count given by a subculture is normally directly proportional to inoculum size, provided the inoculum is less than about 40,000.

In all these experiments at least two replicates were made of each culture. Cultures were discarded after counting. The counts shown in Tables 11 to 15 are the number of amoebae per millilitre of overlay medium. All the culture bottles contained 3 ml of liquid medium and 2.5 ml of agar slope.

8.2. Results

Experiment 1. Strains EVANS and HUFF were grown alone and as a mixture for 6 days at 37 C. At 24, 48, 72 and 98 hours the cultures were counted. Subcultures were set up, using in each case 5,000 amoebae, and grown for 48 hours at 25 . The subculture count allowed the proportion of HUFF in the mixture to be estimated.

| INOCULUM | AMOEBIC COUNT x $10^4$ | | | | | |
|---|---|---|---|---|---|---|
| | 24 hrs | 48 hrs | 72 hrs | 98 hrs | 120 hrs | 144 hrs |
| EVANS 15,000 | 1.5 | 7.7 | 8.8 | 7.8 | 9.4 | 9.1 |
| HUFF 15,000 | 12.9 (40.9) | 28.9 (20.8) | 13.5 (29.7) | 4.7 (13.4) | 1.8 | 1.2 |
| EVANS 15,000 + HUFF 15,000 | 11.3 (35.9) | 34.8 (22.4) | 32.8 (30.9) | 8.2 (18.1) | 4.0 | 1.3 |

Table 11. Strain competition (Expt. 1). Counts after 1 to 6 days culture at 37 . Figures in parenthesis are counts after 48 hours subculture at 25 .

Table 11 shows the rapid early growth of HUFF, and the initially higher counts of the mixture suggesting that the strains were growing independently. The ratio $\frac{12.9}{40.9}$ for the subcultures made at 24 hours suggests that at this stage the proportion of HUFF in the mixture was 0.83. This ratio thereafter tended towards unity showing that the more rapidly growing strain soon dominated the mixture.

Experiment 2. Strain LAREDO and E.invadens were grown alone and as a mixture at 25°C. Counts were made at 48 and 96 hours and 2 sets of subcultures made, each with 5,000 amoebae; these were incubated at 25° and 37° respectively and counted after 48 hours.

| | AMOEBIC COUNT x $10^4$ | | | | | |
|---|---|---|---|---|---|---|
| INOCULUM | 48 hours | | | 96 hours | | |
| | | 25° | 37° | | 25° | 37° |
| LAREDO 15,000 | 18.2 | 19.1 | 5.4 | 57.6 | 21.9 | 17.5 |
| E.invadens 15,000 | 5.3 | 11.3 | 0 | 38.0 | 7.6 | 0 |
| LAREDO 15,000 + E.invadens 15,000 | 22.5 | 32.8 | 3.4 | 72.3 | 16.2 | 2.9 |

Table 12. Strain competition (Expt. 2). Counts after 48 hours and 96 hours culture at 25° and the 48-hour subculture counts at 25° and 37°.

Table 12 shows the more rapid initial growth of LAREDO. The ratios of the 37° subcultures is 3.4/5.4 at 48 hours and 2.9/17.5 at 96 hours, suggesting that the proportion of LAREDO in the mixture was 0.63 at 48 hours and 0.17 at 96 hours. It might be suggested that the low LAREDO counts in the 37° subcultures from the mixtures indicated poor viability, however this is contradicted by the good growth of the same inoculum at 25°.

Experiment 3. Strains HUFF and LAREDO and E.invadens were grown alone and as mixtures at 25 °C for 6 days. Subcultures were made with 5,000 amoebae and counted after 48 hours growth at 37°.

| INOCULUM | | AMOEBIC COUNT x $10^4$ | |
|---|---|---|---|
| | | Six Days | 48-hr Subculture |
| HUFF | 15,000 | 33.1 | 13.4 |
| | 30,000 | 31.8 | 14.8 |
| LAREDO | 15,000 | 33.7 | 15.1 |
| | 30,000 | 31.7 | 14.5 |
| E.invadens | 15,000 | 18.1 | 0 |
| | 30,000 | 17.8 | 0 |
| HUFF 15,000 + LAREDO 15,000 | | 34.2 | 8.9 |
| HUFF 15,000 + E.invadens 15,000 | | 47.4 | 5.5 |
| LAREDO 15,000 + E.invadens 15,000 | | 34.9 | 6.9 |

Table 13. Strain competition (Expt. 3). Counts after 6 days culture at 25° and the counts after 48 hours subculture at 37°.

Table 13 shows that for each strain growing alone the count on the sixth day was independent of inoculum size (15,000 or 30,000). The counts for the mixtures HUFF and LAREDO, and LAREDO and E.invadens are also similar to either HUFF or LAREDO growing alone, indicating that in the mixtures the counts of at least one of the strains was depressed. The count for the HUFF and E.invadens mixture shows much less depression.

The subculture figures suggest that after 6 days the HUFF and E.invadens mixture contain 5.5/13.4 = 0.41 HUFF, and the LAREDO and E.invadens mixture contain 6.9/15.1 = 0.46 LAREDO. Thus, as in Experiment 2 the more slowly growing E.invadens has not been dominated. The low subculture count for the HUFF and LAREDO mixture might be interpreted as indicating poor viability of at least one of the strains; however, when this interaction was studied again in Experiment 5, this finding was not repeated.

Experiment 4. Strains LIGGINS, RUSSELL and EVANS were grown alone and as mixtures for 72 hours at 37°. Subcultures were made, using 5,000 amoebae and counted after 48 hours at 37°.

| INOCULUM | | AMOEBIC COUNT x $10^4$ | |
|---|---|---|---|
| | | 72 hours | 48-hr Subculture |
| LIGGINS | 30,000 | 3.1 | 8.5 |
| RUSSELL | 30,000 | 6.3 | 7.6 |
| EVANS | 30,000 | 27.4 | 6.9 |
| LIGGINS | 15,000 | 23.2 | 7.6 |
| + EVANS | 15,000 | | |
| RUSSELL | 15,000 | 4.7 | 8.0 |
| + LIGGINS | 15,000 | | |
| RUSSELL | 15,000 | 24.4 | 5.4 |
| + EVANS | 15,000 | | |

Table 14. Strain competition (Expt. 4). Counts after 72 hours culture at 37°, and the counts after 48-hour subculture at 37°.

Table 14 shows that EVANS was growing much more rapidly than the other 2 strains. The mixture counts are all lower than the sum of the 2 strains growing alone, indicating depression of at least one of the strains. The subculture counts are all similar suggesting that the viability of the mixtures was not impaired.

Experiment 5. Strains HUFF and LAREDO and E.invadens were grown alone and as mixtures for 7 days at 25 . Two subcultures were made, using 5,000 amoebae, and grown for 48 hours at 25 and 37 C respectively.

| INOCULUM | | AMOEBIC COUNT x $10^4$ | | |
|---|---|---|---|---|
| | | [illegible] Days | 48-hr Subcultures | |
| | | | 25 | 37 |
| HUFF | 15,000 | 72.8 | 19.3 | 26.3 |
| | 30,000 | 42.4 | 21.8 | 23.1 |
| LAREDO | 15,000 | 69.3 | 34.2 | 28.3 |
| | 30,000 | 49.6 | 29.3 | 35.3 |
| E.invadens | 15,000 | 26.0 | 9.0 | 0 |
| | 30,000 | 33.1 | 13.4 | 0 |
| HUFF 15,000 + LAREDO 15,000 | | 48.9 | 26.4 | 23.6 |
| HUFF 15,000 + E.invadens 15,000 | | 39.8 | 24.6 | 26.7 |
| LAREDO 15,000 + E.invadens 15,000 | | 88.8 | 28.7 | 30.9 |
| HUFF 15,000 + LAREDO 15,000 + E.invadens 15,000 | | 38.7 | 23.3 | 23.7 |

Table 15. Strain competition (Expt. 5). Counts after 7 days culture at 25 , and the 48-hour subcultures counts at 25 and 37 .

Table 15 shows that as in Experiment 3 the counts for HUFF and LAREDO growing alone were smaller with a bigger inoculum: possibly the medium becomes more depleted by the higher initial counts produced by a larger inoculum. The mixture counts are all less than the sum of the 2 strains growing alone, the mixture of 3 strains showing this particularly. The 25° subculture counts are unremarkable apart from the rather high value for the triple mixture. The subcultures of the HUFF and LAREDO mixture suggest no loss of viability. The counts of the 37° subculture from all the 3 mixtures containing E.invadens suggest that this organism has been nearly or completely eliminated.

8.3. Discussion

The interpretation of the findings in any one of these experiments must be tentative but the following general conclusions may be made:

(1) During the later stages of a culture the count is not related to inoculum size and may be lower with a bigger inoculum.

(2) After 72 hours the total count of a mixture is usually less than the sum of the counts of each component growing alone. Thus the count for one strain has been reduced by the presence of the other. If inhibition did not occur, this would suggest that strains could occupy different ecological niches.

(3) The proportion of a mixture may change while the culture is in progress. Sometimes the more rapidly growing strain becomes dominant, as in Experiments 1 and 5; but on other occasions this does not occur, as in Experiments 2 and 3.

(4) Subcultures taken from older cultures tend to grow less well; this is also shown in Table 1 in Part 5.6. The viability, however, of the strains growing together as mixtures, was as good as those growing alone.

During the logarithmic phase of growth each component of a mixture probably grows independently so that the proportion of a mixture will be determined by the respective division rates. During the stationary and decline phases of the culture further amoebic growth may be limited by nutrient depletion, toxic metabolites and changes in physiological variables such as pH and redox potential. It is possible that strains differ in their nutrient requirements and their tolerance to other limiting factors. Although the principal bacterial component of the Robinson's culture used in this work was Escherichia coli, strain "B", other bacteria are present, especially in the later stage of a culture. The bacterial 'contaminants' of different amoebic strains are not necessarily the same; and it is possible that strain mixtures are affected by this factor. The dynamics of strain interactions in cultures are very complex and difficult to standardize; for this reason it may be difficult to reproduce experimental results very precisely.

These findings do suggest that, in vivo, different strains may occupy the same ecological niche and that the total population size is determined by a set of unstable host factors that cause the population to fluctuate in size. New incoming strains may have difficulty in establishing themselves and they may be lost during population fluctuation. Thus a non-virulent resident strain could protect the host against a new infection with a virulent strain. Using a human volunteer

Williams Smith (1969) showed that resident strains of Escherichia coli prevented the colonisation of the host gut by small ingested inocula of other strains of the same bacterial species.

Loss of amoebic infection is probably a random process that occurs when the total population happens to reach a critically low level. The presence of a mixed infection, derived from superinfection, is unlikely to affect the duration of infection.

When models of amoebic infection are being devised these considerations are of great importance (see Part 20).

## 4. MECHANISM OF PROTECTIVE IMMUNITY

The following experiments were carried out to study *in vitro* some of the ways in which a sensitized host might destroy invading amoebae.

### 4.1. Method of Preparation and Maintenance of Spleen Cell Cultures.

Adult T0 mice were injected subcutaneously at weekly intervals with 200,000 sonicated trophozoites from TYI cultures. The amoebae were thoroughly mixed with 0.5 ml of complete Freund's adjuvant before the injection. Control groups of mice received either complete Freund's adjuvant in TYI medium or no injections. After a series of at least 5 injections the spleens were removed aseptically, cut up and then forced through a fine stainless steel mesh into culture medium (see below) using a glass rod; the suspension was then taken up into a syringe and pushed through a gauge 22 needle. The cells were washed twice and then set up in 16 x 125 mm culture tubes containing 2 ml of medium (Eagle's minimal essential medium with 10% foetal calf serum, 100 u.ml of penicillin, 100 mcg.ml of streptomycin and 50 mcg.ml of mycostatin). Ten million cells were placed in each tube. Giemsa stained smears showed that 60-80% of the cells were small and medium lymphocytes. Cultures were gassed with 5% $CO_2$ in air and incubated for 48 hours. Sera from antigen treated mice gave a low titre positive antibody test using fluorescein labelled antimouse globulin. The methodology used here is similar to that of Granger and Williams (1968).

9.2. Experiments

(1) Growth of amoebae with spleen cells

Method. Inocula of 10,000 amoebae (strain EVANS) were added to spleen cell cultures. At hourly intervals a drop of the mixed culture deposit was examined microscopically. Amoebic viability was assessed by lack of eosin staining and the ability to grow on kidney cell monolayers with the production of cytopathic lesions. All studies were repeated on at least 3 occasions with several replicates. Results were read qualitatively.

Results.

A. Using normal mouse spleen cells: The amoebae were undamaged and proliferated rapidly, ingesting the spleen cells. Cultures of amoebae could be maintained in this way by serial subculture (see Part 6.3.3.g).

B. Using spleen cells from mice receiving Freund's adjuvant only: Amoebic movement and viability declined rapidly between 3 and 8 hours, by 24 hours all the amoebae were dead. Dying amoebae were frequently surrounded by a cluster of adherent spleen cells, many of them lymphocytes.

C. Using spleen cells from mice receiving amoebic antigen and Freund's adjuvant: The findings were very similar to those with adjuvant only (B), but in most instances amoebic death appeared to be more rapid.

(2) Viability of amoebae with spleen cells in the presence of antiserum or complement

Method. A rabbit anti-amoebic serum was prepared by giving 3 intravenous injections, at weekly intervals, of 1 to 3 million washed

amoebae. 0.1 ml of the serum was then added to the mixed spleen cell and amoebic culture. Results were read qualitatively.

Results. In the presence of normal mouse spleen cells the amoebae were undamaged by antibody, but in the presence of sensitized cells (adjuvant alone or adjuvant with amoebic antigen) all the amoebae were dead within 2 to 4 hours. There was very definite cell clustering about the amoebae when sensitized cells were used. The addition of 0.5 ml of fresh normal human serum (CHEW) or 0.1 ml of guinea pig complement to the spleen cell, amoebic antiserum mixture accelerated amoebic death in the presence of sensitized cells, but not in the presence of unsensitized cells.

In the absence of spleen cells the amoebic antiserum caused immobilisation and eventual death of the amoebae at the concentration used (1 in 20); similarly, the guinea pig complement appeared to be toxic. The apparent protective effect of spleen cells might be due to their adsorption of some of the antibody or complement.

(3) Attempts to demonstrate toxic lymphokines

Method. 30 mcg of phytohaemagglutinin (Wellcome purified, MR68 and 69) was added to the spleen cultures of normal mice and the excess removed at 2½ hours by replacing the supernatant medium. After 48 hours the culture supernatant was separated by centrifugation.

Results.

A. When 1.5 ml of the culture supernatant was added to a monolayer of kidney cells growing in a 5 ml Carrel flask there were definite morphological changes after 24 hours with rounding up of cells and partial detachment from the glass substrate. The supernatant from a spleen cell culture untreated with PHA had no visible effect upon the monolayer.

B. In a similar manner, cultures of spleen cells were set up in the presence of amoebic antigen (the supernatant from 100,000 sonicated trophozoites grown in crithidia-associated cultures). After 48 hours the spleen cell culture supernatant was added to kidney cell monolayers. No visible changes were noted after 24 or 48 hours, irrespective of whether the spleen cells were derived from sensitized mice or controls. However, when the monolayers were labelled with $^{51}$Chromium using the method described previously (see Part 6.3.1) there was at 24 hours greater elution of isotope from the kidney cells exposed to spleen culture supernatants from amoeba sensitized mice (mean loss in 5 flasks = 30.1%, S.D. = 2.5) compared with culture supernatants from normal or adjuvant treated mice (mean loss in 4 flasks = 26.9%, S.D. = 0.59). This difference is significant at the 1% level. A control flask with no amoebae showed an isotope loss of 25.2%. Even with larger amounts of amoebic antigen it was not possible to produce spleen cell culture supernatants that were visibly toxic to kidney cells.

C. In order to observe any direct toxicity of lymphotoxin upon amoebic trophozoites, the supernatants from PHA stimulated and amoebic antigen treated spleen cell cultures were added to amoebae growing as crithidia-associated cultures or upon kidney cell monolayers. No toxic effects were observed, even at a concentration of 50%.

(4) Direct action of antibody upon amoebae

Method. To study the effect of human serum upon the viability of trophozoites, 20,000 amoebae (strain EVANS from a crithidia-associated culture) were added to tissue flasks with $^{51}$Chromium labelled monolayers

to which had been added dilutions of serum. Serum from RUSSELL (amoebic fluorescent antibody titre (FAT) 1/500), TOURMENTIN (FAT 1/250) and GOULD (FAT negative) were used at final concentrations of 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320; one flask being used at each dilution.

Results. After 4 hours the cytopathic losses were not greatly dissimilar and there was no tendency for losses to correlate with serum dilution. The mean values were: control with no serum (5 flasks) 47.3%, S.D. = 1.96; RUSSELL 47.5%, S.D. = 5.2; TOURMENTIN 43.7%, S.D. = 2.8; and GOULD 43%, S.D. = 3.2. The means for both the normal serum (GOULD) and TOURMENTIN were significantly lower, at the 5% level, than that of flasks with no serum.

9.3. Discussion

The first two experiments show that the spleen cells from sensitized mice can destroy amoebae in vitro. However, it was clear that non-specific stimulation with Freund's adjuvant could itself produce a substantial damaging effect. Either lymphocytes or macrophages could be involved in this process, but it was observed that at least a large proportion of the cells adherent to the dying amoebae were lymphocytes. The third experiment demonstrated the production of a toxic substance when spleen cells from amoeba-sensitized mice were incubated with amoebic antigen, it is likely that this is a lymphotoxin similar to that produced by normal lymphocytes in the presence of phytohaemagglutinin. The amoebae themselves appeared to be unaffected by either lymphotoxin.

It is known that the serum of many persons with active or recent amoebic disease will immobilise and destroy trophozoites (Brown and Whitby, 1955),

especially in the presence of complement. The titres are, however, usually low. Several of the high titre (as demonstrated by immunofluorescence with amoebic antigen) human sera used in this work, were tested for direct immobilisation of amoebae, but none gave a titre greater than 1 in 5. Nevertheless, Experiment (2) does suggest that, even at concentrations less than that necessary to immobilise the trophozoites, the presence of antibody can accelerate cell-mediated damage. In Experiment (4) it was shown that even normal human serum may have a mild damaging effect upon amoebae.

These experiments will be discussed further in Part 10.2.

## 10. GENERAL DISCUSSION OF IN VITRO EXPERIMENTS

### 10.1. Mechanisms of Host Damage

The studies of enzymes produced by E.histolytica have been extensive and they are reviewed by Jarumilinta and Mcgrath (1969). Proteolytic activity has been demonstrated using substrates such as gelatin, casein, fibrin, haemoglobin and gut epithelial suspensions. The findings with pathogenic and non-pathogenic strains are in general similar, with the possible exception of carboxypeptidase, which appears to be absent from some pathogenic strains. The free-living Acanthamoeba produces many similar proteolytic enzymes. Many non-proteolytic enzymes have been identified but these may relate more to the internal economy of the parasite rather than to its pathogenic potential. Of greater interest is the finding of hyaluronidase activity in a number of pathogenic strains (Jarumilinta, 1962). If invading amoebae are to move between cell interfaces this enzyme may be of greater relevance.

Work on amoebal enzymes has often involved both intact amoebae and cell-free extracts. When intact amoebae have been used it is not clear to what extent enzymes are set free into the medium, released on contact with the substrates or released by dying amoebae. The work described in Parts 6.2. and 6.4. emphasises the importance of cell contact. Amoebal extracts and culture supernatants produced no visible damage, no changes on electron microscopy and no release of isotope from $^{51}$Chromium labelled cells; furthermore, interposition of an agar disc prevented cytopathic attack. The electron micrographic findings described here show that cells and amoebae come into very close contact before cell damage occurs. It is possible that proteolytic enzymes or

their precursors are transferred across points of temporary contact or cytoplasmic fusion; such a mechanism would certainly be less wasteful. Recently Visvesvara and Callaway (1974) have studied the cytopathic attack of Naegleria fowleri upon monkey kidney cell cultures. Their findings were very similar to those obtained here with E.histolytica; discontinuity of cell sheet, shrinkage of cell cytoplasm, nuclear pyknosis and ingestion of cell fragments by the amoebae. Cells not near amoebae were undamaged. Their electron micrographs showed cytoplasmic damage in cells contacted by amoebae, but unlike E.histolytica, minute pinocytotic vesicles were formed along the area of contact. No surface lysosomes were seen but apparent cell fusion was noted, as in the present work.

A notable feature of amoebic lesions in man and experimental animals is the presence of numerous neutrophil leucocytes, many of them in a state of degeneration. The neutrophil granules appear to degenerate rapidly on cell contact with the amoebae, thus releasing lysosomal enzymes which destroy the leucocyte and probably damage adjacent tissue (Griffin, 1972). The chemotactic effect of amoebae upon leucocytes will accentuate this phenomenon.

It has been suggested by Villarejos (1972) that dying amoebae may liberate cytotoxic enzymes. While this mechanism may operate in experimental systems where amoebic suspensions are injected into tissue, it perhaps pushes the concept of protozoal self-sacrifice too far to suggest that it also operates in natural situations.

The gross pathology of amoebic lesions usually reveals extensive necrosis, with amoebae proliferating near the periphery. A gradient

of progressive anoxia and acidosis must exist between the normal tissue and the centre of the lesion. While the proliferating amoebae probably occupy the position most favourable to their metabolic requirements, there can be little doubt that host cells near the periphery of the lesion are damaged by these conditions. The role of devitalised tissue in pathogenesis will be discussed later when animal infections are being considered. The host's immune response to the amoebae must also contribute to lesion pathogenesis. Neutrophils have already been mentioned; but, in addition, lymphokines, vaso-active amines and other non-specific components of the inflammatory reaction must all damage tissue. In gut lesions, bacteria no doubt play a similar role but their presence cannot be considered essential to amoebic pathogenesis.

The studies of Takeuchi and Phillips (1975) have shown that the invasive mechanisms of E.histolytica are very similar and perhaps identical to those of amoebae in established lesions. Germ-free guinea pigs were inoculated intracaecally with trophozoites and their associated enteric flora. Cytoplasmic changes occurred in epithelial cells in contact with amoebae, such cells became shrunken and often detached from their basement membrane. The cells showed swollen mitochondria, a dilated endoplasmic reticulum and many lipid droplets. Leucocytes escaped from mucosal capillaries and sometimes crossed the epithelial basement membrane; they then degenerated rapidly and disrupted, especially when in contact with amoebae. Amoebae entered the lesion by active pseudopodial movement.

10.2. Amoebic Destruction by Host

Protective immunity to amoebic tissue invasion has been demonstrated in dogs (Swartzwelder and Avant, 1932), guinea pigs (Sato, 1957) and hamsters (Krupp, 1974); there can be little doubt that the human host responds in the same way. Not only may clinically evident bowel disease be self-limiting, but in pre-emetine days liver abscess patients sometimes recovered spontaneously following drainage through a hepato-bronchial fistula (Rogers, 1922). Furthermore, the frequency of amoebic antibody and skin sensitivity in subjects living in endemic areas, suggests that spontaneous recovery is the general rule rather than the exception.

Most textbooks state that there is little cellular response to amoebic invasion; apart from a local increase in neutrophils, which in gut lesions is usually attributed to bacteria. However, the material upon which this impression is based may be very biased as it derived mostly from autopsies or surgical specimens from patients with fulminant disease. In such patients the immune system has indeed failed, but it is wrong to infer from this that an inflammatory reaction does not often occur. The most chronic gut lesions, referred to as amoebomas, show quite extensive lymphocyte, plasma cell, monocyte and fibroblast infiltrations and similar cells may occur at the periphery of some liver lesions. Gilman and Prathap (1971), studying rectal biopsies in Malayan aborigines, noted that long standing or healing rectal ulcers showed a definite granulation tissue response. The absence of a cellular immune response is to be expected in weanling rats as this species does not become fully immunocompetent until 2 or 3 months of age; a similar situation prevails in kittens.

Experiment (4), described in Part 9.2., showed that antibody, at concentrations likely to be present in the tissues, did not inhibit the cytopathic effect of amoebae. In man most amoebic antibody belongs to the IgG class with smaller amounts of IgM in acute lesions; in addition the frequency of an immediate skin sensitivity to amoebic antigen, in diseased patients, strongly suggests that IgE antibody is present also. It is uncertain whether antibody alone can eliminate tissue invasion; liver abscess patients may have very high titres when measured by indirect immuno-fluorescence or indirect haemagglutination, but the disease process is often not halted. Immobilisation titres in man are generally low, furthermore amoebae may remobilise after contact with antibody (Biagi-F et al., 1966).

The role of cell-mediated immunity deserves more attention. Delayed skin hypersensitivity was found in convalescent South African Bantus and those with prolonged symptoms (Maddison et al., 1968a); and similar findings are reported from Thailand (Suvanat et al., 1973a). Recently blood lymphocytes, from patients with liver abscess, have been shown to transform in the presence of amoebic antigen (Suvanat et al., 1973b). Patients with acute liver abscess were found to have a diminished delayed skin response and macrophage migration inhibition to amoebic antigen; after treatment both tests were positive with the same antigen (Ortiz-Ortiz et al., 1975). Harris and Bray (1978) working in The Gambia have shown transformation not only in liver abscess patients, but also in some apparently healthy persons in a highly endemic area.

There are several immunological mechanisms that might operate through a T-cell response:-

(1) Transformed T-cells may release a skin reactive factor, thereby increasing the permeability of mucosal and sub-mucosal capillaries, and so allow antibody to escape. A similar effect might be produced by damaged neutrophils or mast cells degranulated by IgE mediated sensitivity.

(2) Sensitized T-cells may kill directly by binding specifically with surface receptors.

(3) Antibody coated amoebae might be subject to attack by cytotoxic killer cells.

(4) Antibody coated amoebae might become attached to phagocytes by opsonic adherence, or in the presence of complement by immune adherence.

(5) Transformed T-cells might produce macrophage activation either non-specifically or by a specific macrophage arming factor.

(6) Transformed T-cells might release cytotoxin directly toxic to amoebae.

Experiment (3) described in Part 8.2, showed that a lymphotoxin was released by spleen cells in the presence of antigen but this was not toxic to amoebae. A similar lymphotoxin was reported by Granger *et al.* (1969) using PPD and spleen cells from tuberculin-sensitised mice. Such substances may well cause local tissue damage even if they do not kill the micro-organism directly. Since macrophages have not so far been

implicated in natural amoebic lesions, mechanisms 2 and 3 appear the most likely explanation for the other experimental findings, although, as mixed spleen cell suspensions were used, macrophages may have been involved as well. The finding in Experiment (2) (Part 9.2.) that amoebal death was more rapid in the presence of antibody would support either mechanism 3 or 4. The destructive effect of spleen cells from mice given Freund's adjuvant alone, suggests non-specific macrophage activation, similar perhaps to the macrophage-mediated suppressive effect of BCG upon the growth and metastasis of hydatid infections (Rau and Tanner, 1975). Clearly several mechanisms may operate simultaneously and the system could be very complex.

While the immune rejection of invasive amoebae is fairly well established, the elimination of luminal forms from the gut by immune mechanisms is much more doubtful. Clinical experience suggests that patients with invasive disease, treated only with emetine, chloroquine or tetracycline, rarely lose their intraluminal infection, despite the presence of serum antibody. Studies to demonstrate IgA antibodies in serum have so far failed (Maddison et al., 1968b) but no definitive studies have been made to detect them in gut contents or faeces. It is possible that the brush border of the colonic epithelial cells becomes coated with IgA antibody (Tomasi, 1972) and that this prevents intimate contact between the amoeba and the enterocyte. The phenomenon of sterile immunity can be studied epidemiologically and this will be discussed later (Part 20).

10.3. Assay of Amoebicidal Drugs

When drug concentrations are plotted against the percentage mortality of an organism, a sigmoid-shaped dose-response curve is often obtained. The central portion of the curve often becomes more linear when the logarithm of the drug concentration is plotted. This phenomenon was observed in Part 7.4.2, when log.emetine concentration was plotted against amoebic count (Figures 6 and 7), or cytopathic loss caused by amoebae (Figures 10 and 11). Theoretically this finding is of considerable interest because it is possible to interpret such curves in several ways:

(A) The curve may reflect heterogenous drug susceptibilities among the test organisms.

If the individual susceptibilities are distributed in a normal (Gaussian) manner, then the dose-response line represents the cumulative mortality at each concentration. Hence when the percentage mortality is plotted against drug concentration a sigmoid curve is obtained with a mean equal to the $LD_{50}$ and a standard deviation related to the slope of the dose-response line. If the proportional mortalities are plotted on a probit scale, then the standard deviation is equal to the reciprocal of the slope. The observation that the original lines were nearly linear when log. doses were plotted would suggest that the susceptibilities of individual organisms were themselves distributed in a log. normal manner.

(B) The curve may reflect the random uptake of a drug by a homogeneous population.

If the uptake of drug by amoebae is random and proportional to the drug concentration, then after a given time interval, the frequency distribution of drug molecules per amoeba will be Poisson, with the mean equal to the variance. Now, if only a 'single effective hit' by a drug molecule is required to kill each amoeba, then the dose-response will be a negative exponential; and the response will be linear when log. survival is plotted against dose. Similarly, if 'multiple effective hits' are required and these act independently to kill an amoeba, then the main part of the dose-response will also be a negative exponential; however, in this case, when log. survival is plotted against dose, the initial part of line will be convex upwards before the linear descent begins.

A different situation would exist if a number of drug molecules needed to act cooperatively to kill an amoeba. In a homogenous amoebic population each amoeba would be susceptible to the same individual dose of drug molecules, and as the drug concentration of the medium was increased the amoebae would die as the critical value was reached. Because of the Poisson distribution of drug molecules, the amoebae would not all be killed simultaneously. The slope of the log. dose-survival curve in this situation would be related to the lethal number of molecules; higher numbers giving steeper slopes. The minimum slope value of the probit survival-log. dose plot would in fact be 2, corresponding to the situation where one molecule was lethal. Slope values less than 2 are incompatible with an hypothesis of drug molecules acting cooperatively upon a homogenous population.

It should be noted that all these models of drug action might give sigmoid-shaped log. dose-response curves. However, the models for molecules acting independently upon homogenous organisms, whether by a 'single effective hit' or 'multiple effective hits', would both give linear log. survival-dose curves. When the data presented here, in Part 7.4.2., was plotted in this way, the responses were not linear and the initial part of the curve was concave upwards. Furthermore, the slopes of the probit survival-log. dose plots were all less than 2, suggesting that drug molecules were not acting cooperatively upon an homogenous population.

When the heterogenous model was applied to the log. dose-response lines shown in Figures 6, 7, 8 and 9, and the standard deviation of drug susceptibility calculated from the probit slopes, the values were much higher than would be expected for genetic differences within one amoebic population. Selection in vitro for drug resistance appears to be very difficult with E.histolytica (Shaffer and Washington, 1952) and resistance has not appeared in vivo. Were genetic differences to explain the variation observed here, then selection should be easy. One likely form of non-genetic variation, that would explain the findings reported here, would be different drug susceptibilities during the division cycle.

Amoebicidal drugs are normally compared by measuring the minimal 100% lethal concentration, using the method described in Part 7.4.1. Now when the dose-response curve is not steep the inaccuracy will be considerable compared with measurement of the $LD_{50}$. Provided the slopes of the dose responses of two drugs are not too dissimilar, then comparison

of the $LD_{50}$ will give a more accurate comparison of lethal effects. The two methods developed in this thesis in Part 7.4.2, will allow such comparisons to be made. Considering the different principals underlying the two methods the concordance of the $LD_{50}$ is good. Using the first method, $LD_{50}$ values for emetine fell within the range log. 0.8 - log. 2.2, i.e. 6.3 - 158 mcg.ml after 3 hours drug exposure; after 48 hours the $LD_{100}$ was between 4.2 and 38 mcg.ml (see Part 7.4.1). The duration of drug exposure is important when $LD_{50}$ values are being measured, as exemplified by the results in Table 10. However, provided the time is standardized, valid comparisons may be made.

10.4. The Biological Forms of E.histolytica in vitro and in vivo

The lumen dwelling 'minuta' form of E.histolytica has always been recognised as being biologically different from the invasive 'magna' form. Besides its larger size, the 'magna' form is characterised by more active movements, larger food vacuoles containing no bacteria, a larger nucleus that is less well seen by phase contrast microscopy, the ability to readily ingest erythrocytes and fragments of tissue cells and its inability to encyst. Furthermore, cytochemical studies have shown that invasive amoebae within hamster livers have higher concentrations of acid phosphatase, non-specific esterase and NAD diaphorase, compared with 'minuta' forms growing with bacteria (Michel and Westphal, 1970).

In view of the many differences between the two forms, it is reasonable to speculate that they are the expression of different genetic codings; homologous perhaps with the biological forms of the Trypanosomatidae. If this is the case then one or more environmental triggers may induce the genetic expression of the other form; as occurs for example when Trypanosoma cruzi is cultured at different temperatures (Neva et al., 1961).

The question then arises as to which form do cultured amoebae correspond? Bacteria-associated cultures appear to be similar to the lumen dwelling 'minuta' form with regard to their morphology, infectivity to animals and their ability to encyst. In addition, such cultures require anaerobic conditions. Hurinsuta and Hurinsuta (1955) showed that an oxidation-reduction potential of -200 millivolts was required for multiplication. On a solid medium, Bulsmith and Bront (1954) found that 0.1% oxygen inhibited growth and 2% was lethal.

Crithidia-associated and axenic amoebae, however, are similar in several respects to the 'magna' form. They are larger than those grown with bacteria, they adhere readily to a glass substrate and they do not encyst. In TTY cultures amoebae grow readily in the upper, more aerobic part, of the culture tube (personal observation) and will also grow in the absence of an added reducing agent (see Part 5.5). Similarly Wittner (1968) showed that amoebae in axenic cultures multiply in the upper part of the culture tube, and would grow at an oxygen tension one tenth atmospheric. Cytochemically, crithidia-associated amoebae are similar to invasive forms in the hamster liver (Michel and Wratphal, 1970). However, unlike the situation with freshly obtained invasive amoebae, gut infections in rodents cannot be established with crithidia-associated cultures (personal observation), and axenic strains injected intracaecally do not infect guinea pigs or rats (Phillips et al., 1972). However, crithidia-associated cultures will infect hamster livers when injected intrahepatically (Raether, 1971), to produce typical amoebic abscesses. Similarly, large inocula of axenic amoebae will do the same when injected directly into hamster livers (Tanimoto et al., 1971; Diamond et al., 1973).

It has been commonly assumed that amoebae in tissues require physicochemical conditions similar to those of bacteria-associated cultures. In the present work, however, it has been shown that crithidia-associated amoebae can grow (see Parts 5.4 and 5.5) and exert a cytotoxic effect (see Part 6.3.3.c) at pH and redox levels similar to those of mammalian tissues. Furthermore, isolated trophozoites may quite often be seen some distance from a necrotic lesion and Wittner and Rosenbaum (1970) were able to culture amoebae from hamster livers showing no lesions, three weeks after the intraportal injection of axenic amoebae.

The status of MSF cultures, growing upon non-multiplying bacteria, is uncertain. They will readily infect rodent caeca; and Montalvo et al. (1971) have shown that they will metabolise glucose aerobically or anaerobically, depending on the presence of air.

On repeated subculture bacteria-associated amoebae often lose virulence quite rapidly (see Part 13), although it may sometimes be restored by liver passage. In contrast bacteria-free cultures appear to maintain some virulence to the hamster liver; thus the 3 strains studied by Phillips et al. (1972) had been in axenic culture for 7, 2 and 2 years respectively. Similarly the strain (LIGGINS) used in Parts 15 and 16 to produce liver abscess in hamsters had been in TTY culture for 1 -1¼ years. It should be noted, however, that Bos (1973) using crithidia-associated, and Wittner and Rosenbaum (1970) using axenic amoebae, found a decline in virulence after removal of bacteria.

In the present work it has been observed that all of the 16 typical strains established in TTY cultures, were cytotoxic to cell monolayers; as also was NIH: 200 strain which was originally axenised in 1965. Cytotoxicity did not decline during an 18 month observation period. It is not known whether all strains of typical E.histolytica can be established in bacteria-free cultures, but it appears likely that once established they will be cytotoxic. Similarly in man, it is quite possible that all strains may at times transform into the 'magna' form and become pathogenic.

The stimuli that trigger the conversion between 'minuta' and 'magna' forms have not been elucidated, but they are clearly of central importance in the understanding of pathogenic mechanisms. The hypothesis of Wittner and Rosenbaum (1970) that live bacteria transmit an episome to amoebae is attractive but the findings presented here do not support it, nor does the finding that axenic amoebae will produce liver abscess (Diamond et al., 1973). Viruses have been found in axenic amoebae (Diamond et al., 1972) and particles resembling rhabdo-viruses were present in the TTY cultures used in this thesis (see Plate 16). It is possible that virus infection of E.histolytica may account for the instability of certain strain characteristics, including virulence and ease of cultivation. The situation could be analogous to the lysogenic state in bacteria; for example, bacteriophage induced toxigenicity in Corynebacterium diphtheriae or altered enzyme patterns in phage infected strains of Mycobacterium (Juhasz et al., 1969). The finding by Honigberg and Read (1960) that virulence in Trichonomas gallinae could be transferred between strains by a cell-free homogenate could be explained in a similar way; as could the temporary hybridisation, achieved by Entner (1971) between typical E.histolytica and the LAREDO strain.

## 11. INTRODUCTION

### 11.1. Intestinal Amoebiasis

The susceptibility of different host species has already been briefly reviewed in Part 4.2.2. Age is a well recognised factor especially in rats; the weanling being much more susceptible than the adult. The strain of host is also important and several inbred rat strains have recently been compared by Neal and Harris (1975).

The relationship between the gut bacterial flora and amoebiasis has been studied by several workers. Concurrent infection with haemolytic streptococci or pneumococci (Spector, 1935) or Escherichia coli (Deschiens, 1937) increased the severity of lesions in cats; and killed Salmonella typhi or S.paratyphi (Deschiens, 1938) had the same effect. When germ-free guinea pigs were mono-contaminated with various non-pathogenic bacterial species the severity of amoebic lesions differed (Phillips and Gorstein, 1968). The latter is rather an artificial situation, however, and in general the exchange of bacterial associates between amoebic strains does not affect their pathogenicity (Neal, 1957); although Serkisian (1967) has reported enhanced pathogenicity when Clostridium perfringens is added to amoebic inocula.

When the rectal mucosa of cats was damaged with finely powdered glass, amoebic lesions developed more readily (Phillips, 1957). Mucosal damage probably also explains the effect in dogs of a canned salmon diet (Artigas and Beaver, 1961) and croton oil (Losch, 1875); Neuss

and Rappaport (1940) reported that the effect of croton oil in cats was enhanced by the presence of 'certain' associated bacteria. Possibly some of the reported effects of a high dietary cholesterol (Biagi et al., 1982; Das and Singh, 1985) are due to an irritant effect. In guinea pigs the administration of cortisone or hydrocortisone increased the size of caecal amoebic lesions and predisposed to perforation (Teodorovic et al., 1983).

Despite its potential importance host diet has been relatively little studied experimentally. Taylor et al. (1950, 1952) found that the outcome of infection in both rats and guinea pigs differed when two diets were compared. Hogner and Eskridge (1937) noted that rats given a high protein diet eliminated their amoebic infection, presumably E.muris. Gopal Rao and Padma (1971) while studying strain differences noted that rats on a low protein diet sometimes developed more extensive and severe caecal ulceration. In one study (Carrera et al., 1952) guinea pigs given protein deficient diets did not show a different susceptibility to infection or tissue invasion; however, the animals were only given the deficient diets for 8-11 days. Westphal (1970) noted that a high carbohydrate diet increased the susceptibility of mice to E.histolytica infection, but not to tissue invasion. Sadun et al. (1951) reported that Vitamin C deficiency in guinea pigs favoured tissue invasion. The synthetic diet used by Lynch (1957) to enhance virulence in guinea pigs contained gum arabic, potassium acetate and magnesium oxide; it was shown that the diet itself induced histological changes in the caecal mucosa.

In the present work the synergistic effect of two intestinal helminths, Trichuris muris and Schistosoma mansoni, has been studied in mice (Parts

12 and 13). In man these frequently occur as mixed infections with E.histolytica. In addition, the role of low protein and high carbohydrate diets has been investigated in detail in rats (Part 14).

11.2. Hepatic Amoebiasis

Macgraith and Harinasuta (1954b) showed that guinea pigs sensitized to amoebae by a pre-existing gut infection were more liable to develop liver abscess after intraportal inoculation of amoebae. Sensitisation via the cubital vein also has the same effect (Beltran-H. et al., 1968). Krupp (1958) showed in guinea pigs that hepatic injury caused by migrating larvae of the nematode Toxocara canis prolonged the persistence of Entamoeba histolytica in the liver following intracaecal inoculation, but did not result in abscess formation. High dietary cholesterol increased the number of liver abscesses in hamsters (Biagi-F. et al., 1962) but this effect might be partly due to enhancement of gut lesions. The high serum cholesterol reported in human patients (Biagi-F. et al., 1965) could be a non-specific response to liver damage and stress, rather than a predisposing factor. These workers also report an increased susceptibility in animals given cortisone, progesterone or testosterone (Biagi-F. et al., 1963).

Sections of colonic wall in human cases of amoebic dysentery not infrequently show thrombosis of the smaller mesenteric veins. Furthermore, early hepatic lesions in man have a periportal distribution and the appearances sometimes suggest that they begin at the site of small venous emboli or thromboses in situ. In order to determine whether necrotic hepatic tissue predisposes to amoebic growth, experiments were designed to investigate the behaviour of bacteria-free E.histolytica

in hamsters with liver injury (Part 15). Liver tissue was damaged either by the injection of small glass particles into the portal vein producing disseminated necrotic foci or by the ligation of one lobar branch of the portal vein producing hypoxaemia of that lobe. The use of bacteria-free inocula eliminated the effect of concomitant bacteria.

In addition, the possible effect of alcohol induced liver damage has been studied experimentally in hamsters (Part 16).

## 12. THE INTERACTION BETWEEN E.HISTOLYTICA AND TRICHURIS MURIS IN MICE

### 12.1. Method

Trichuris muris infection. Three-week old female TO mice weighing 15 to 18 g were given by gavage 12.5 mg piperazine citrate (Antepar) in 0.1 ml of water; this was repeated 5 days later. Two days after the second dose of piperazine about 80 eggs of T.muris (kindly supplied by Mr. J. E. D. Keeling, Burroughs Wellcome, Kent) suspended in 0.1 ml of water were given by gavage. The eggs, which were derived from gravid female T.muris, had been incubated in water at 27 C for 6 to 12 weeks; when used about 60% to 70% of the eggs were fully embryonated. One week after infection 2 mg of cortisone acetate was given subcutaneously in 0.1 ml of saline and the same dose was given again 3, 5 and 7 days later. Each mouse thus received 8 mg of cortisone acetate, equivalent to about 400 mg/kg body weight. The control mice, that would later be infected with E.histolytica only, received the same medication with piperazine citrate and cortisone acetate.

This infection schedule is based upon the findings of Keeling (1961) and Wakelin (1967), who have studied experimental T.muris infections in mice. Young female mice are the most susceptible and piperazine is necessary to eliminate any pre-existing intestinal helminthic infection which might interfere with T.muris. The use of cortisone minimizes the immunological rejection that occurs during the establishment phase of the infection.

Entamoeba histolytica infection. Mice were infected with E.histolytica 40 to 48 days after the Trichuris eggs had been given; at this time the worms were mature and eggs were easily detectable in the faeces. The mice were weighed before infection.

At laparatomy under ether narcosis 100,000 to 120,000 unwashed trophozoites, from a 48-hour Robinson's medium culture, were inoculated intracaecally in 0.2 ml of culture medium; the needle being inserted near the base of the caecum and pushed towards the caecal apex before the injection was made. Mice were infected in batches of 4 to 6 animals, each batch containing the same proportion of Trichuris-infected and control animals. Two amoebic strains were used: strain DAWSON isolated 6 months previously and strain ZOCKLING isolated 14 months previously.

Examinations at Necropsy. Mice were killed 7 or 13 days after inoculation of amoebae and weighed; the caecum and proximal colon were removed, opened and scored for damage using a ranking scale similar to that of Jones (1946). The wall was scored as follows: 0, normal; 1, appreciably thickened; 2, marked local thickening and contraction; 3, extensive thickening, contraction and visible ulceration; 4, very contracted and extensively ulcerated. The contents were scored as follows: 0, normal; 1, partly liquid; 2, definitely liquid with mucus; 3, mostly mucoid, some pus, little solid matter; 4, pus and mucus only with no solid matter. Material from the caecal mucosal surface was taken for culture using Robinson's medium (Robinson, 1968), and a wet microscopic preparation was examined immediately. Only large, active amoebic trophozoites containing erythrocytes were considered to be E.histolytica. The mouse colony used was not free of E.muris and small, non-hematophagous amoebae could not be identified with certainty. The cultures were maintained at 37°C, subcultured at 24 hours and then examined 48 hours and 72 hours later. E.muris does not grow in simple amoebic culture media at 37°C (Pruss, 1959).

12.2. Results

The experimental work involved 5 consecutive groups of mice. Groups 1 and 2 were infected with strain DAWSON (Experiment 1) and groups 3, 4 and 5 with strain ZOCKLING (Experiment 2). In groups 1, 2 and 3 the time interval between amoebic infection and autopsy was 7 days; while in groups 4 and 5 it was 13 days.

| Type of Infection | No. Mice | Mean Caecal Score | | Mean Worm Load | Proportion with | |
|---|---|---|---|---|---|---|
| | | Wall | Contents | | Haematophagous Amoebae | Positive Culture |
| | | | GROUP 1 | | | |
| E.histolytica | 7 | 0.8 | 0.8 | 0 | 1/7 | 3/7 |
| E.histolytica + Trichuris | 5 | 2.4 | 3.2 | 30 | 5/5 | 5/5 |
| | | | GROUP 2 | | | |
| E.histolytica | 13 | 0.5 | 0.85 | 0 | 7/13 | 10/13 |
| E.histolytica + Trichuris | 14 | 1.2 | 1.85 | 13.2 | 9/14 | 11/14 |

Table 16. E.histolytica and Trichuris infections in mice. Experiment 1, outcome of amoebic infection (strain DAWSON) in mice with and without Trichuris infection.

The results of Experiment 1 are shown in Table 16. In group 1 there was a clear difference in the caecal scores and the finding of haematophagous amoebae, in mice with and without Trichuris; the difference in culture was less clear cut. Although the mean worm load was lower in group 2, the rates for both haematophagous amoebae and a positive culture were similar to the controls, however there was a difference in

caecal scores. The high rates of amoebic infection and tissue invasion in the control animals in this group suggested that strain DAWSON was too virulent for the purposes of the experiment and for this reason strain ZOCKLING was used thereafter. The presence of Trichuris infection had little effect on body weight.

| Type of Infection | No. Mice | Mean Caecal Score | | Mean Worm Load | Proportion with | |
|---|---|---|---|---|---|---|
| | | Wall | Contents | | Haematophagous Amoebae | Positive Culture |
| | | | GROUP 3 | | | |
| E.histolytica | 10 | 0.2 | 0.2 | 0 | 1/10 | 3/10 |
| E.histolytica + Trichuris | 16 | 2.5 | 2.4 | 9 | 11/16 | 13/16 |
| | | | GROUP 4 | | | |
| E.histolytica | 10 | 0.2 | 0.3 | 0 | 1/10 | 1/10 |
| E.histolytica + Trichuris | 8 | 2.0 | 2.75 | 20.3 | 5/8 | 5/8 |
| Trichuris only | 5 | 0.0 | 0.4 | 33 | 0/5 | 0/5 |
| | | | GROUP 5 | | | |
| E.histolytica | 10 | 0.0 | 0.0 | 0 | 1/10 | 0/10 |
| E.histolytica + Trichuris | 8 | 3.0 | 2.25 | 21.8 | 7/8 | 8/8 |
| Trichuris only | 5 | 0.4 | 0.6 | 34.0 | 0/5 | 0/5 |

Table 17. Experiment 2, outcome of amoebic infection (strain ZOCKLING) in mice with and without Trichuris infection, and in mice infected with Trichuris only.

E.histolytica and Trichuris infections in mice.

Table 17 gives the findings in Experiment 2, using strain ZOCKLING. This strain showed a low infectivity and invasiveness in control animals but in those infected with Trichuris the rates for positive culture and the presence of haematophagous amoebae were higher, as were the caecal scores. The results suggest that the longer time interval between amoebic infection and autopsy in groups 4 and 5 gave a lower infection rate in control animals but not in those infected with Trichuris. Animals infected with Trichuris only were studied in groups 4 and 5 to determine whether the helminth infection itself affected the caecal score. Of the 10 mice studied, one, with an exceptional load of 81 worms, showed visible wall thickening of the caecal apex; and this animal, together with two others, had partly liquid caecal contents. With these exceptions, the caeca of mice infected with Trichuris only were macroscopically normal: bleeding was not seen at the sites of attachment. It is therefore likely that the amoebic infection was predominantly responsible for high caecal scores of animals with double infections. As in Experiment 1, changes in body weight were not great, although mice doubly infected with E.histolytica and Trichuris lost 1 to 4 g more. Weight loss correlated better with the caecal score or the presence of haematophagous amoebae than with the worm load, suggesting that amoebic tissue invasion might be responsible. E.muris trophozoites were seen in 5 of the 10 animals infected with Trichuris only, but there was no growth on culture.

In general, it will be noted that culture was a more sensitive method of detecting E.histolytica than the finding of haematophagous trophozoites, presumably this is partly because non-invasive amoebae will grow on culture. Of the 48 animals in which haematophagous amoebae were found,

all but one were positive on culture. The finding of haematophagous amoebae strongly suggests that the amoebae are invading tissue. It is just possible that erythrocytes, leaked into the bowel by feeding Trichuris, are ingested by intralumenal amoebae; however, the caecal contents of animals infected with Trichuris only showed very few erythrocytes. Furthermore, there was a strong correlation between a caecal wall score of 2 or more, which is suggestive of mucosal ulceration, with the finding of haematophagous amoebae.

Histological Findings (see Plates 17, 18, 19 and 20). Serial sections of caeca from mice with double infections showed that ulcerative lesions containing invasive E.histolytica trophozoites were often present in the mucosal tissue immediately adjacent to the heads of the Trichuris worms. The amoebic lesions were predominantly cytolytic. The heads of worms not associated with amoebic lesions showed no tissue necrosis, but there was some oedema and a mild eosinophil and lymphocyte cellular reaction. There was no evidence of bleeding at the site of attachment.

12.3. Discussion

The difference in rates for positive amoebic cultures in mice with and without Trichuris shows that the presence of this worm prolongs the persistence of an induced amoebic infection. The more frequent detection of haematophagous trophozoites and high caecal scores in animals with Trichuris, together with the histological findings, indicates that amoebic tissue invasion was taking place. In many of the animals

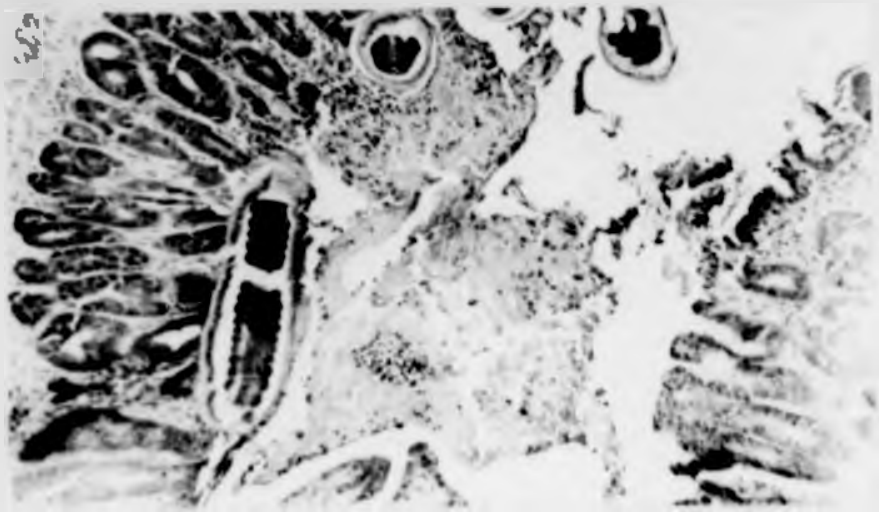


Plate 17. E.histolytica and Trichuris infections in mice. 1. Head of T.muris (sectioned longitudinally) embedded in caecal mucosa. Superficial to the worm head there is mucosal destruction with a fibrinous and inflammatory cell exudate that contains amoebae. (x 80)

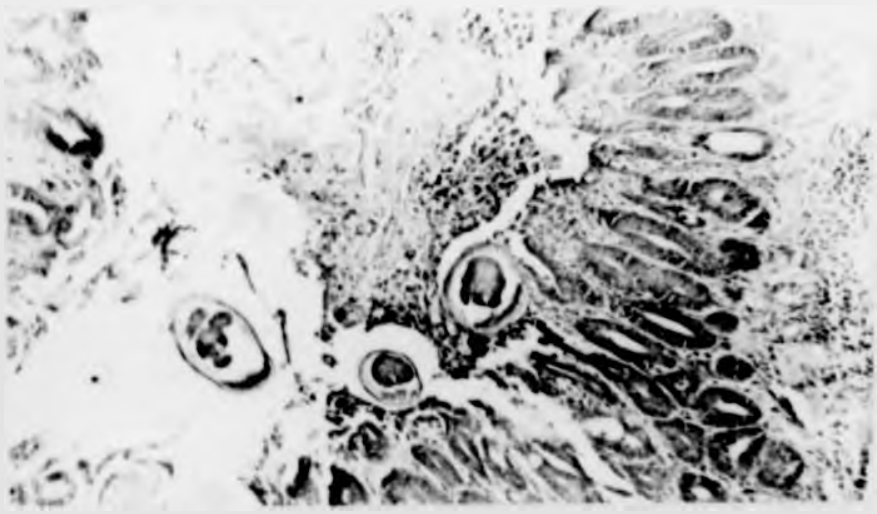


Plate 18. E.histolytica and Trichuris infections in mice. 2. Anterior parts of T.muris (sectioned transversely) lying on caecal mucosa which shows epithelial damage and inflammation. (x 80).

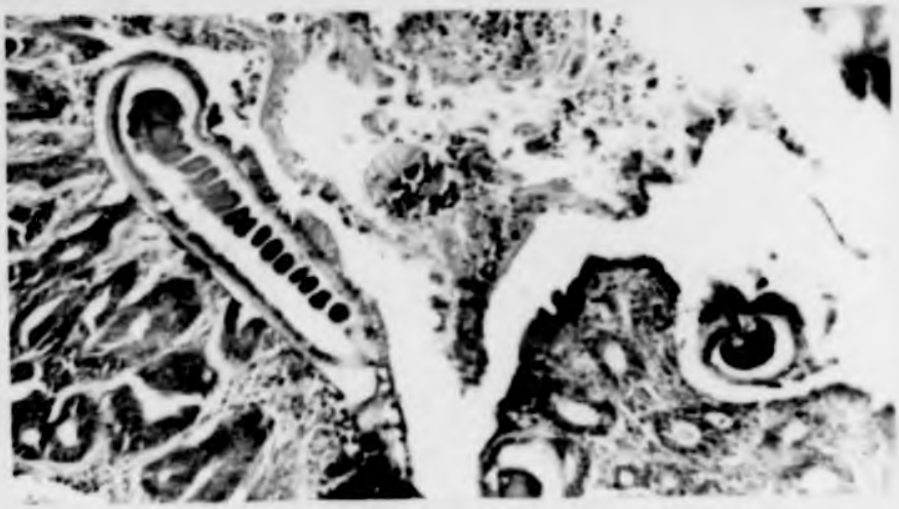


Plate 19. E.histolytica and Trichuris infections in mice. 3. Head of T.muris (sectioned longitudinally) embedded at site of damaged caecal mucosa. Amoebae are seen adjacent to the worm. (x 80)

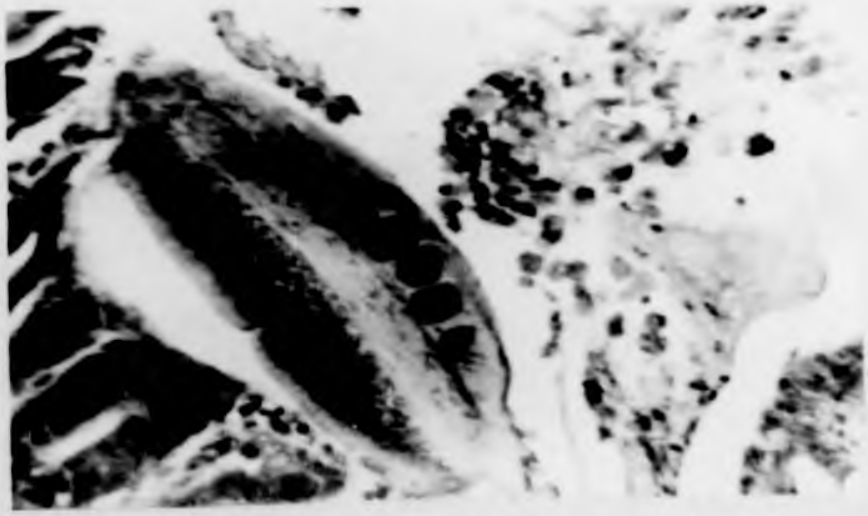


Plate 20. E.histolytica and Trichuris infections in mice. 4. Higher magnification of T.muris head (sectioned longitudinally) and amoebae within superficial layer of caecal mucosa. (x 320)

with mixed infections the very large number of amoebic trophozoites in the caecum was remarkable; most contained ingested erythrocytes. It is worth noting that whipworms, unlike hookworm species, digest ingested red blood cells within their gut lumen and do not release them into the host's caecum. Histological studies suggested that amoebic tissue invasion usually, but not always, occurred at the actual site of the worm attachment to the mucosa.

## 13. THE INTERACTION BETWEEN E.HISTOLYTICA AND SCHISTOSOMA MANSONI IN MICE

Light schistosomal infections were used as these represent a more realistic model of the host parasite situation as it occurs in man (Warren, 1963). The experiments were designed to determine the outcome of E.histolytica challenge at different stages of S.mansoni infection, the effect of different worm loads and also the effect of unisexual schistosomal infections. As strains of E.histolytica often lose their infectivity to mice relatively quickly it was necessary to use 3 different strains during this work.

### 13.1. Methods

Female TO mice weighing 20 - 25 g were used throughout. S.mansoni infections were established by the subcutaneous injection (Peters and Warren, 1969) of a known number of fresh cercariae (Puerto Rican strain, kindly supplied by Professor G. S. Nelson) suspended in 0.5 ml of distilled water. In the third experiment the cercariae liberated from a single snail were injected into different groups of mice in order to obtain some unisexual infections. However, since light infections were produced in all experiments, a few were unisexual whatever the source of cercariae.

At laparotomy under ether narcosis 100,000-150,000 unwashed trophozoites, from a 48-hour Robinson's medium culture, were inoculated intracaecally in 0.2 ml of culture medium. Mice were infected in batches of 4-6 animals, each batch containing the same proportion of schistosome-infected and control animals. On the 7th day after amoebic infection the mice were killed with sodium pentobarbital and the abdomen and thorax opened. The caecum was removed following the application of

a haemostatic clamp across the extreme distal end of the ileum and the proximal part of the ascending colon. The portal vein was then cut near the liver and the hepatic circulation perfused with 10-15 ml of heparinized saline (Duvall and DeWitt, 1967) injected into the left ventricle, the perfusate was collected and the number of worms counted under a dissecting microscope. Following the perfusion, the liver was removed and compressed between 2 thick pieces of glass so that the occasional worm which had not been flushed out by the perfusion could be detected and counted. The caecum was then opened, examined and cultured as described in Part 12.1. The total caecal score was taken as the sum of the two caecal scores (wall + contents).

13.2. Results

Experiment 1. In order to study the effect of the duration of the schistosome infection a group of mice was exposed to a mean of 28 cercariae each, and then infected with E.histolytica (strain LIGGINS isolated 8 weeks before the first inoculation) in 3 batches, 5, 10 and 13 weeks later.

| Duration of Schistosome Infection | 5 weeks | 10 weeks | | | 13 weeks | | |
|---|---|---|---|---|---|---|---|
| Schistosome-infected or Control | Infected | Infected Bi-sexual | Infected Uni-sexual | Control | Infected Bi-sexual | Infected Uni-sexual | Control |
| Caecal score ⩾ 2 | 0 | 13 | 1 | 1 | 9 | 0 | 0 |
| *E.histolytica* seen | 1 | 14 | 2 | 0 | 11 | 0 | 3 |
| *E.histolytica* cultured | 0 | 4 | 1 | 2 | 1 | 0 | 0 |
| *E.histolytica* seen or cultured | 1 | 14 | 2 | 2 | 11 | 0 | 3 |
| Number of mice | 9 | 21 | 4 | 7 | 15 | 1 | 14 |

Table 18. *E.histolytica* and *Schistosoma* infections in mice. Experiment 1, the effect of duration of schistosome infection upon outcome of amoebic infection (strain LIGGINS).

Table 18 clearly shows that at 5 weeks, that is near the end of the prepatent period of the schistosome infection, the mice were insusceptible to amoebic infections and none showed a caecal score of 2 or more. Because of the small size of the worms at this time it was not possible to determine accurately the worm load in these 9 mice; it is likely that most had bisexual infections but in their response to amoebic challenge they behaved as a control group would have done. At 10 weeks the schistosome infections were patent as shown by visible liver granulomata in the bisexual infections. 2 of the latter mice were moribund on the 4th day after amoebic infection and these were killed; both showed extensive caecal pathology but their worm load was not measured. 13/21 (62%) of bisexual infections showed significant pathology at 10 weeks; 3 weeks later the proportion was 9/15 (60%).

In order to exclude the possibility that the bacterial flora of the cultures were responsible for the lesions, 5 schistosome infected mice, 3 with bisexual infections, were inoculated at the 10th week with the supernatant from the amoebic culture. One week later all had caecal scores of 0 and none showed visible E.histolytica or a positive culture.

Experiment 2. The effect of worm load on the response to amoebic challenge was studied by exposing 3 groups of mice to 25, 50 and 100 cercariae respectively. A 4th group was kept as a control. 9½ weeks (66 days) later they were infected with one of two strains of E.histolytica; either strain LIGGINS as used in Experiment 1, which had by this time been maintained in culture for 6 months, or strain ZOCKLING isolated 10 weeks before the experiment. Analysis with respect to worm load will be given later but Table 19 shows the outcome of the experiment analysed as in Experiment 1, and comparing the two amoebic strains.

| Amoebic Strain used for Challenge | LIGGINS | | ZOCKLING | | |
|---|---|---|---|---|---|
| | | | Infected | | |
| Schistosoma-Infected or Control | Infected Bisexual | Control | Bisexual | Uni-sexual | Control |
| Caecal score ≥ 2 | 4 | 0 | 7 | 1 | 2 |
| *E.histolytica* seen | 2 | 1 | 9 | 1 | 3 |
| *E.histolytica* cultured | 4 | 1 | 8 | 1 | 5 |
| *E.histolytica* seen or cultured | 4 | 3 | 10 | 1 | 5 |
| Number of mice | 8 | 12 | 15 | 1 | 13 |

Table 19. Experiment 2, comparison of 2 amoebic strains (LIGGINS and ZOCKLING) on inoculation into schistosoma-infected (9½ weeks) and control mice.

The proportion giving either a positive culture or a caecal score of 2 or more is similar for both strains but the caecal scores were higher with strain ZOCKLING. The latter finding is consistent with the observations made 3 weeks before this experiment when these strains had been inoculated into weanling rats. Of 9 rats inoculated with strain LIGGINS only 2 gave a caecal score of 2 or more and 7 gave a positive culture; of 5 rats inoculated with strain ZOCKLING, 4 gave a caecal score of 2 or more and all gave a positive culture.

The findings of this experiment illustrate the delicacy of the balance between the 2 infections and shows how this is influenced by the strain of *E.histolytica* used. Thus with strain ZOCKLING 8/15 schistosoma-infected mice gave a positive culture compared with 5/13 in the controls; while with strain LIGGINS the corresponding figures were 4/8 and 1/12. Strain ZOCKLING therefore suggests no real difference in susceptibility while strain LIGGINS does.

Experiment 3. By injecting cercariae collected from a single snail into a group of mice, it was hoped to produce at least some mice with unisexual infections. Five groups of mice were infected in this way, giving in the different groups, between 40 and 60 cercariae to each mouse. In fact, 3 of the groups produced mainly bisexual infections, one produced heavy male unisexual infections and the last produced mainly female unisexual infections. The schistosome-infected mice together with an equal number of controls were infected with E.histolytica (strain ARNELL isolated 2 weeks previously) in 3 batches, 9, 10 and 11 weeks later with the 5 groups of schistosome-infected mice equally distributed in each batch.

| Duration of Schistosome Infection | 9 Weeks | | | 10 Weeks | | | 11 Weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| | Infected | | | Infected | | | Infected | | |
| Schistosome Infected or Control | Bi-sexual | Uni-sexual | Con-trol | Bi-sexual | Uni-sexual | Con-trol | Bi-sexual | Uni-sexual | Con-trol |
| Caecal score ⩾ 2 | 7 | 1 | 0 | 3 | 1 | 0 | 7 | 0 | 0 |
| E.histolytica seen | 5 | 3 | 5 | 3 | 0 | 1 | 8 | 1 | 1 |
| E.histolytica seen | 4 | 2 | 7 | 2 | 1 | 1 | 3 | 1 | 0 |
| E.histolytica seen or cultured | 6 | 3 | 9 | 3 | 1 | 1 | 9 | 1 | 0 |
| Number of mice | 9 | 5 | 15 | 3 | 2 | 5 | 13 | 4 | 17 |

Table 20. E.histolytica and Schistosoma infections in mice. Experiment 3. Comparison between bisexual and unisexual schistosome infections and control mice when infected with amoebae (strain ARNELL) 9, 10, 11 weeks later.

From Table 20 it will be seen that there is a progressive fall, from weeks 9-11, in the infectivity of this strain of E.histolytica to the control mice. This reinforces the observation made in Experiment 2 that using a strain of high infectivity it may not be possible to demonstrate a relationship between S.mansoni and E.histolytica based upon infection rate; however, when the infectivity is lower, as with the 11th week data on Table 20, there is clearly a significant difference with 0/17 controls infected compared with 9/13 with bisexual schistosome infections.

When the comparison is based upon the caecal scoring the difference between bisexual infections and controls is very evident whatever the infectivity of the amoebic strain used. Only 2/11 unisexual infections gave a caecal score $\geqslant 2$ despite the fact that in the 7 male infections the load was very high with a mean of 31 worms.

Histological Findings (See Plates 21, 22, 23 and 24).

In order to detect any anatomical relationship between schistosome ova and amoebic ulceration serial sections of caecal wall from mice with bisexual infections were examined. Ova surrounded by a well-developed granuloma which included a fibroblastic response did not appear to be related to amoebic ulceration (Plate 21). On the other hand, amoebic ulcers, with a clearly defined area of mucosal loss, were frequently found to be very close to ova surrounded by an acute eosinophilic inflammatory response (Plates 23 and 24).

13.3. Discussion

In order to study the effect of different worm loads, and unisexual infections, upon the outcome of the amoebic infection the results from

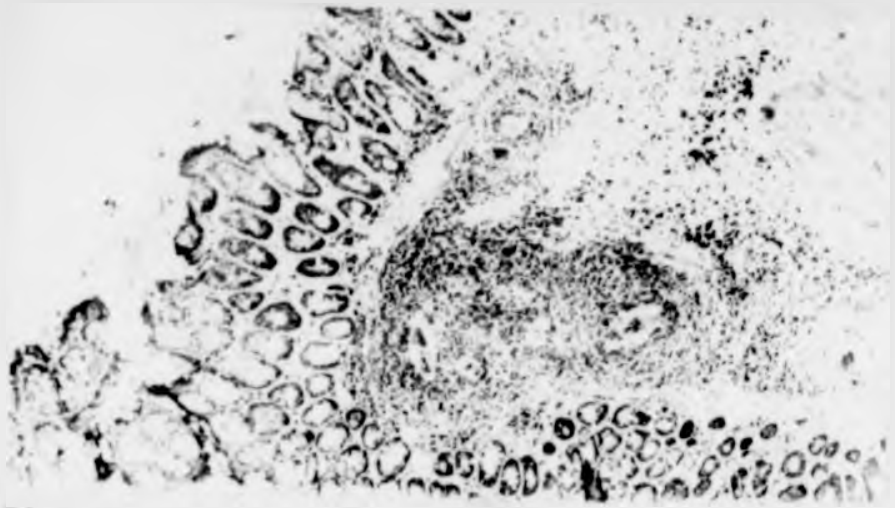


Plate 21. E.histolytica and Schistosoma infections in mice 1. Five S.mansoni eggs beneath caecal muscularis mucosa. The eggs are surrounded by concentric rings of fibroblasts and inflammatory cells. This is the granulomatous type of host response and the mucosa shows no evidence of amoebic damage. (x 80)

Plate 22. E.histolytica and Schistosoma infections in mice. 2. Two microabscesses containing S.mansoni eggs, beneath caecal muscularis mucosa. A polymorphonuclear reaction, predominently eosinophils, extends between the crypts towards the mucosal surface. (x 80).

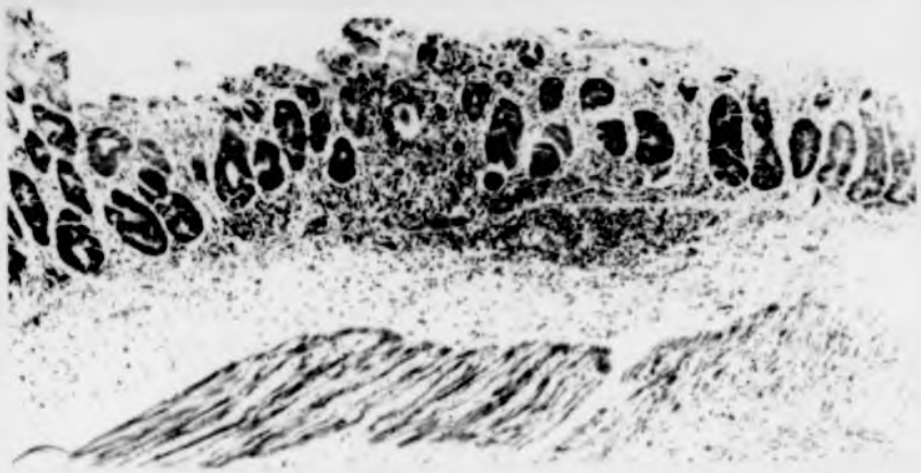


Plate 23. E.histolytica and Schistosoma infections in mice. 3. Extensive superficial amoebic mucosal erosions overlying two microabscesses situated within the lamina propria, and beneath the muscularis mucosa respectively. S.mansoni eggs are seen within the microabscesses. (x 80)

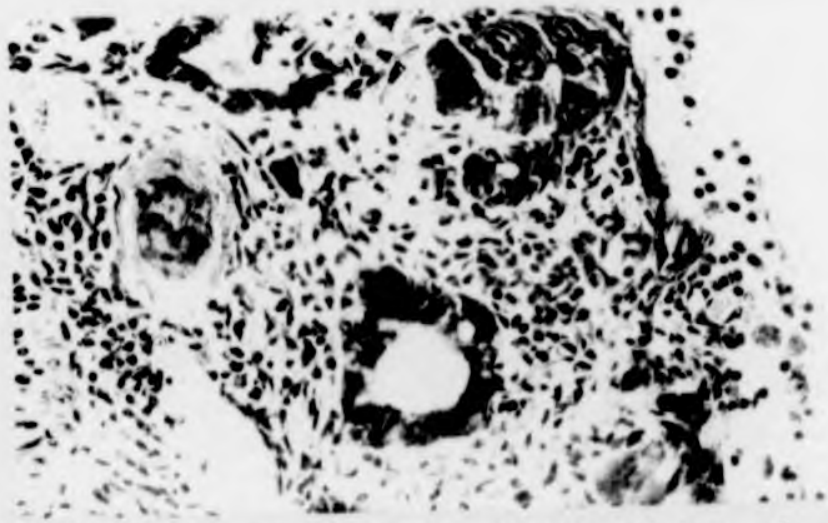


Plate 24. E.histolytica and Schistosoma infections in mice. 4. Higher magnification of S.mansoni egg surrounded by eosinophils, within the lamina propria. The superficial part of the mucosa is destroyed, and amoebae and inflammatory cells are seen at the surface. (x 320)

all the mice, apart from the 9 prepatent infections in Experiment 1 have been combined in Table 21.

| Schistosomes | E.histolytica detected | Caecal scores ≥ 2 | E.histolytica and caecal scores ≥ 2 | Number of mice |
|---|---|---|---|---|
| None (Control) | 23 (28%) | 3 (4%) | 3 (4%) | 83 |
| Unisexual | 8 (47%) | 4 (24%) | 4 (24%) | 17 |
| All bisexual* | 53 (63%) | 48 (57%) | 39 (46%) | 84 |
| 1 pair | 15 (57%) | 9 (32%) | 7 (25%) | 28 |
| 2 pairs | 10 (66%) | 10 (66%) | 7 (47%) | 15 |
| 3 and 4 pairs | 16 (73%) | 17 (77%) | 14 (64%) | 22 |
| 5 to 12 pairs | 10 (59%) | 10 (59%) | 9 (53%) | 17 |

Table 21. E.histolytica and Schistosoma infections in mice. Summary. Combined results from Experiments 1, 2 and 3 showing relation between type of schistosome infection, the detection of E.histolytica by culture or microscopy and the caecal score. Percentage of total number of animals given in parenthesis.

*Not prepatent infections in Experiment 1.

This clearly shows the differences in E.histolytica infection rates between the controls (28%) and the bisexual infections (63%); however, the infection rate does not alter significantly with different numbers of worm pairs. When caecal ulceration is considered the findings are even more striking as only 4% of the controls gave a caecal score ≥ 2 compared with 57% of the bisexual infections; in addition there is a significant difference ($p<0.01$) between mice with 1 worm pair and those with more

than 1 worm pair, although within the latter group the results are relatively uniform and not directly related to the worm load. The mean number of worm pairs in the 3 experiments were 2.38, 3.83 and 3.60 respectively; and the combined frequency distribution was a simple curve falling steadily from a mode of 1 pair (36% of the infections) to the 1 infection of 12 pairs; only 21% of the infections were of more than 4 pairs.

With regard to unisexual infections, Table 21 shows that both the infection rate with E.histolytica and the degree of caecal ulceration are intermediate between those of the controls and the bisexual infections. Unfortunately the number of animals is small and the worm loads cover a wide range; however, it appears that compared with controls, mice with unisexual schistosomiasis are more susceptible to amoebiasis. The third column of the Table showing the numbers of mice with a caecal score $\geq 2$ together with the finding of E.histolytica illustrates the close correspondence between these 2 parameters.

This correspondence can also be clearly seen in Figure 12 which shows the number of mice giving each caecal score together with the proportion of this number in which E.histolytica has been either cultured or seen microscopically. The caecal score shows a definite bimodal distribution and the proportion of mice with E.histolytica rises from 32% in those with a score of 0 to over 70% in those with a score of 3 or more. In fact the caecal score distribution curves for 1, 2, 3 or 4 worm pairs were all bimodal. A score of 2 or more evidently belongs to the second mode and presumably indicates amoebic tissue invasion; it is for this reason that this score has been selected when tabulating the results. The bimodal curve suggests that amoebic tissue invasion in rodent models probably takes the form of an all or none phenomenon, as it does in man.

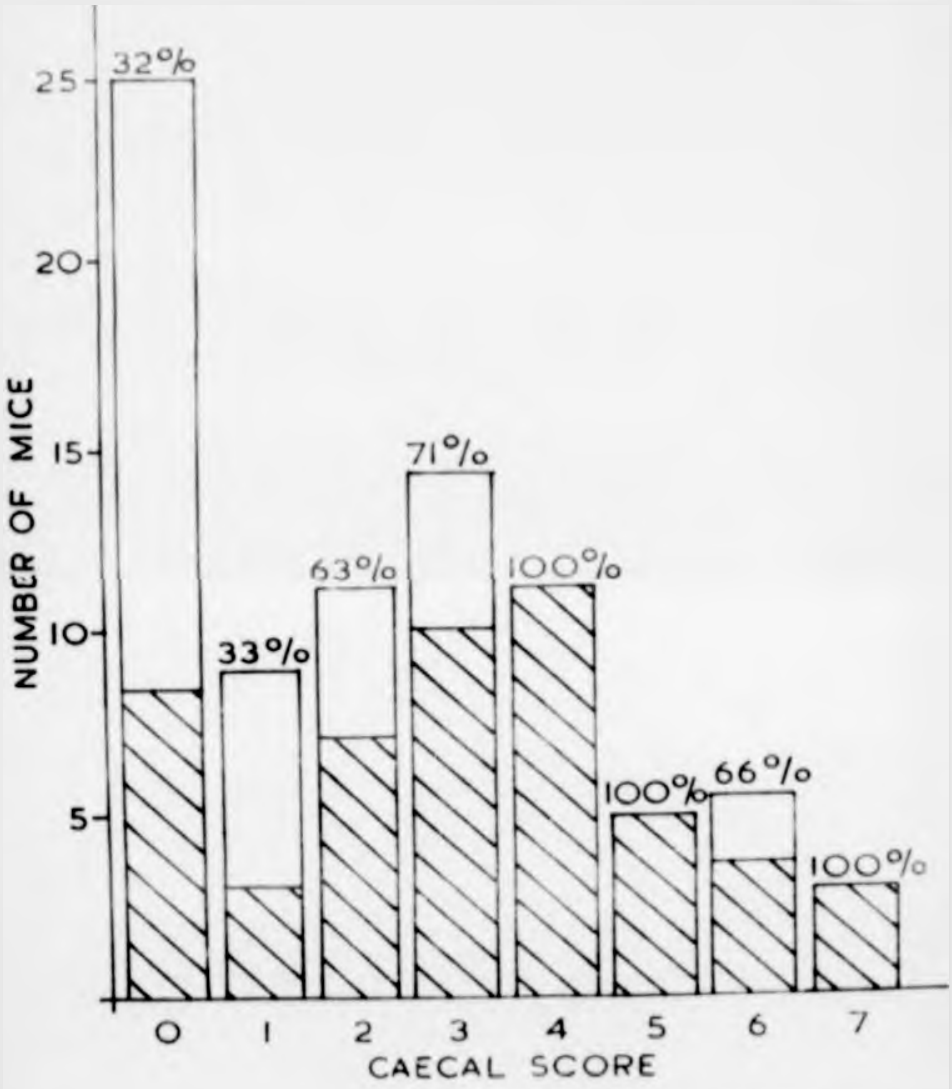



Figure 12. Frequency distribution of caecal scores in mice with E.histolytica and bisexual S.mansoni infections. Cross hatching denotes those with E.histolytica on microscopy or culture; the percentage positive is given for each score.

The results of culture for E.histolytica were sometimes rather disappointing, but the overall correspondence with haematophagous amoebae was highly significant ($p<0.001$) and it is thus justifiable to assume that amoebae identified in wet preparations as E.histolytica did in fact represent that species.

The evidence that it was amoebic ulceration and not simple caecal schistosomiasis which determined the caecal score is as follows: (1) The frequency distribution of number of worm pairs was unimodal, while that of the caecal scores was bimodal. (2) Amoebae were usually present when the caecal score was high. (3) Caecal scores did not rise in Experiment 1 between weeks 10 and 13, nor in Experiment 3 between weeks 9 and 11; a rise would have been expected if a given number of worms continued to oviposit and the eggs contributed to the caecal score. No mice died during these periods and the worm loads were similar. (4) At the time of the amoebic inoculation the caeca showed either normal appearances or the presence of some granulomas; no contraction of the caecum was seen.

## 14. DIETARY FACTORS AFFECTING THE PATHOGENICITY OF E. HISTOLYTICA IN RATS.

### 14.1. Methods

Wistar strain albino rats were used in all experiments. They were given 1 of 4 diets (see Appendix for composition):

Diet A. A 'balanced' diet, NDpCal 9.8% (Powdered).

Diet B. Low protein diet, NDpCal 5.2% (Powdered).

Diet C. Low protein, high calorie, NDpCal 4.5% (Powdered).

Diet D. A standard commercial diet (No. 86), NDpCal 8-9% (Pellets).

Diets A and B are the same as those used by Stewart and Sheppard (1971) in their studies on protein calorie deficiency in rats, and referred to as C - 10 and C - 5 respectively. The rats were allowed water and the allocated diet ad lib.

The rats were inoculated intracaecally with 100,000 - 150,000 unwashed trophozoites (strain ARNELL) in 0.2 ml of culture medium from a 48-hour Robinson's medium culture. Seven days later the rats were killed and the caecum removed, opened, examined and scored as in Part 12.1.

In all experiments individual rats were weighed at weekly intervals. The weight gains referred to in the Tables are up to the time of amoebic challenge and not to necropsy.

In Experiments 3 and 4 the redox potential was measured with a standard Cambridge meter and redox electrode. Caecal contents were gently emulsified in distilled water to give a final concentration of 1% weight/volume.

14.2. Results

Experiment 1. Twenty-two freshly weaned 3-week old rats weighing 52 - 56 g were randomly allocated to 1 of 3 diets: the 2 powdered diets A and B and the commercial pellet diet, Diet D. Three weeks later all were inoculated with E.histolytica.

| | Mean weight gain (g) | Mean caecal wall score | Caecal wall score $\geq 2$ | Mean caecal contents score | positive cultures |
|---|---|---|---|---|---|
| DIET A | 42.0 | 1.0 | 5/7 | 1.7 | 5/7 |
| DIET B | 23.5 | 2.0 | 6/8 | 1.0 | 7/8 |
| DIET D | 81.0 | 0.7 | 1/7 | 0.3 | 1/7 |

Table 22. Diet and E.histolytica infection in rats. Experiment 1, comparing outcome of amoebic challenge 3 weeks after starting respective diet at the time of weaning. A = 'balanced' B = low protein: D = standard.

The results are shown in Table 22. Although Diet A was designed to provide all the necessary nutritional requirements of the rat their mean weight on this diet at the end of 4 weeks was only 108.7 g compared with 152.3 g for those on Diet D. It was thus apparent that as both groups fed ad lib, the rats "preferred" the pellet diet and hence those rats on Diet A in effect received suboptimal amounts of a balanced diet. Combining the culture results of those that were malnourished, i.e. Diet A and B then 12/15 were positive compared with 1/7 on the standard Diet D ($X^2$ = 4.9, $0.0 < p < 0.05$). On direct microscopy 7 caecal smears showed amoebae considered to be E.histolytica, all of these were subsequently positive on culture.

Experiment 2. Thirty unweaned 2-week old rats with their mothers were used. There were 3 litters of 10 and each litter with their mother was randomly allocated Diet A, B or D. At the age of 3 weeks the litters were separated from their mothers and continued on the same diet to which it had been originally allocated. When weaned the rats weighed between 45 and 57 g.

The weight gains during the 4-week period were somewhat less than those in Experiment 1 but the gains on each diet relative to the others were similar (Table 23). Thus feeding the mothers on the diets during the last week of suckling had some effect on the subsequent growth of rats.

| | Mean weight gain (g) | Mean caecal wall score | Caecal wall score $\geq 2$ | Mean caecal contents score | Positive culture |
|---|---|---|---|---|---|
| DIET A | 27.5 | 0.9 | 3/10 | 2.1 | 7/10 |
| DIET B | 15.6 | 3.0 | 9/10 | 2.7 | 10/10 |
| DIET D | 50.9 | 0.1 | 1/10 | 0.1 | 2/10 |

Table 23. Diet and E.histolytica infection in rats. Experiment 2, comparing outcome of amoebic challenge 3 weeks after weaning. Respective diets also given to suckling mother for 1 week before weaning.
A = 'balanced' B = low protein D = standard.

The outcome of amoebic challenge in this experiment was similar to the last. Those on Diets A and B both developed high infection rates and counting these together 17/20 were infected compared with 2/10 of those on Diet D ($\chi^2$ = 9.3, $0.001 < p < 0.01$). Most workers using rat models

for amoebiasis sum the caecal wall and caecal content scores but in this study it was suspected that diet itself affected the consistency of the caecal contents; this was evidenced by observations at the time of amoebic inoculation and by the presence of several content scores of 1 in the control experiment (No. 4). Hence only caecal wall scores have been used in the analysis of results. Combining the data from Experiments 1 and 2, of the 52 rats 22 gave a wall score of 0, 7 a score of 1, 17 a score of 2 and 6 a score of 3; 22/23 of the rats with a score of 2 or 3 gave a positive culture for E.histolytica. Thus there is a bimodal distribution of caecal wall scores and it is justifiable to regard those animals with scores of 2 or more as having tissue invasion of the caecum, macroscopic ulcers were present in those with a score of 3. The proportion of infected animals with caecal scores of 2 or more were for Experiments 1 and 2 respectively: Diet A 3/5 and 3/7 and Diet B 6/7 and 9/10. This emphasizes that the protein deficient Diet B gives a very high proportion of ulcerated caeca in those animals that become infected. On direct microscopy 16 caecal smears showed amoebae considered to be E.histolytica; cultures from all of these caeca were positive.

Experiment 3. In this experiment Diet A was replaced by Diet C which represented a low protein, high carbohydrate intake. The experiment was designed to determine firstly whether by feeding the rats on the diets for varying periods of time there was any difference in the outcome of amoebic challenge and secondly whether infectivity of amoebic inocula from the same strain 1 week apart differed.

Thirty-one freshly weaned rats (Group 1) weighing 48.4 to 51.6 g were randomly allocated to 1 of the 3 diets, B (low protein), C (low protein, high carbohydrate) and D (standard pellet diet). One week later a further 29 freshly weaned rats (Group 2) weighing 48.2 - 51.7 g were randomly allocated to the same 3 diets. The rats were infected with E.histolytica in 2 batches 1 week apart; the first batch was given to groups 1 and 2 when they had been on their diets for 3 and 2 weeks respectively; the second batch was given to groups 1 and 2 when they had been on their diets for 4 and 3 weeks respectively. The design of the experiment is thus a 2 x 3 x 3 factorial with 2 batches of amoebae, 3 diets and 3 periods of diet (2, 3 and 4 weeks). The design is incomplete as each batch of amoebae was given to only 2 of the 3 periods on diet.

| | Mean weight gain (g) | Caecal wall score $\geq$ 2 | Redox potential (mV) (mean and S.D.) | Positive culture | Amoebic inoculum |
|---|---|---|---|---|---|
| DIET B | | | | | |
| 2 wks | 7.4 | 2/5 | +100 ± 13 | 2/5 | Batch 1 |
| 3 wks | 17.0 | 4/5 | - 9 ± 41 | 4/5 | Batch 1 |
| | 12.7 | 2/5 | - 11 ± 45 | 3/5 | Batch 2 |
| 4 wks | 18.0 | 2/5 | - 64 ± 30 | 2/5 | Batch 2 |
| DIET C | | | | | |
| 2 wks | 7.1 | 2/4 | + 69 ± 17 | 4/4 | Batch 1 |
| 3 wks | 16.8 | 5/5 | - 34 ± 21 | 5/5 | Batch 1 |
| | 9.4 | 2/5 | + 34 ± 35 | 4/5 | Batch 2 |
| 4 wks | 14.3 | 0/6 | + 54 ± 28 | 5/6 | Batch 2 |
| DIET D | | | | | |
| 2 wks | 50.8 | 2/5 | + 63 ± 29 | 2/5 | Batch 1 |
| 3 wks | 82.5 | 1/5 | + 95 ± 17 | 2/5 | Batch 1 |
| | 68.1 | 0/5 | + 10 ± 39 | 1/5 | Batch 2 |
| 4 wks | 97.2 | 2/5 | + 38 ± 21 | 3/5 | Batch 2 |

Table 24. Diet and E.histolytica infection in rats. Experiment 3, comparing outcome of 2 batches of amoebic inocula 2, 3 and 4 weeks after starting respective diet at time of weaning. B = low protein; C = low protein high calorie; D = standard.

There was little difference between the mean weight gains of the rats fed on Diets B and C for the same period of time (see Table 24); however, both were significantly lower in weight than those fed on Diet D. Comparing the first and second batches of amoebic inocula the respective infection rates were 19/29 (65%) and 18/31 (58%) and the respective numbers with caecal scores of 2 or more were 16/29 (55%) and 8/31 (25%). Thus the second batch gave somewhat lower values especially for caecal scores although even the latter difference is not significant at the 5% level. For none of the diets do the caecal scores of the rats on their diet for either 2 and 3 weeks (batch 1 amoebae) or for 3 and 4 weeks (batch 2 amoebae) show any real difference; the infection rates, which are similar in the 2 batches of amoebae, are nearly identical at 2 and 4 weeks. Thus length of time on diet, within the range of 2 to 4 weeks, did not appreciably affect the outcome of amoebic challenge.

With respect to the infection rates for the 3 diets: Diet B (11/20) was greater than Diet D (8/20) and Diet C (18/20) was considerably greater than Diet B ($X^2$ = 3.34, $0.05 < p < 0.1$) and significantly greater than Diet D ($X^2$ = 7.4, $0.001 < p < 0.01$). There were interesting differences in the proportion of infected animals on each diet giving a caecal score of 2 or more; thus for Diet B 10/11 (91%) gave this score compared with 9/18 (50%) on Diet C. Unfortunately, the numbers of animals are small and this difference does not quite reach statistical significance at the 5% level ($X^2$ = 3.48); however, similar high figures were found in Experiment 1 (6/7) and in Experiment 2 (9/10) for Diet B so that the much lower figure (9/18) in this experiment for

Diet C strongly suggests that although Diet C greatly increased the susceptibility to infection there was not a corresponding increase in susceptibility to tissue invasion.

The redox potentials of the caecal contents showed a wide range but there was a correlation with both the positivity of culture and the caecal score. Thus the mean potential was 58.5 $\pm$ 18/mV in those with a negative culture and 10.0 $\pm$ 11/mV in those with a positive culture; this difference is significant ($t = 2.82$, $DF = 58$, $p < 0.01$). Of the animals with a positive culture and a caecal score of 2 or more the mean redox potential was 4.1/mV compared with a value of 37.4/mV in those with a positive culture and a caecal score below 2; thus it is clear that a low redox potential is strongly correlated with caecal ulceration.

Histological sections of non-ulcerated caeca of rats fed on the diets for 4 weeks showed that villous height in those on Diets B and C measured about 240 $\mu$m compared with 330 $\mu$m in those on Diet D; in the former the villi were broader and contained more lymphocytes. There were fewer goblet cells relative to epithelial cells in the mucosa of rats on Diets B and C so that in absolute terms the goblet cell population was much reduced.

Experiment 4. This experiment was designed to study the effects of Diets B, C and D on the characteristics of the caecal wall and the redox potential of the caecal contents, and also to determine whether E.muris could be cultured using the standard technique used in previous experiments. Thirty rats were randomly allocated to Diets B, C and D. They received no intracaecal injection and were killed after 3 weeks.

| | Mean weight gain (g) | Mean caecal wall score | Redox potential (mV) (mean and S.D.) | Positive culture |
|---|---|---|---|---|
| DIET B | 14.8 | 0 | +101 ± 18 | 0/10 |
| DIET C | 18.9 | 0 | +119 ± 13 | 0/10 |
| DIET D | 80.6 | 0 | + 33 ± 16 | 0/10 |

Table 25. Diet and E.histolytica infection in rats. Experiment 4, comparing effect of 3 diets given for 3 weeks from time of weaning. No amoebic inoculum given. B = low protein; C = low protein high calorie; D = standard.

Table 25 shows that E.muris did not grow despite the fact that this organism was seen in 21 of the rats. The redox potentials varied considerably but the mean potential of the rats on Diet D was significantly lower than those on Diets B or C ($p<0.001$). Weight gains were similar to those of the rats in Experiment 3 given their diets for 3 weeks. The histological changes were similar to those of the non-ulcerated caeca of the rats on the same diets in Experiment 3.

14.3. Discussion

The first 3 experiments clearly show that rats fed on protein deficient diets are more susceptible to infection with E.histolytica than those fed on a stock diet. Diet A must be regarded as suboptimal as rats grew consistently less well on it than on the pellet Diet D, despite the fact that the NDpCal% values are similar. Experiments 1 and 2 show that infection rates were highest on Diet B and lowest on the pellet diet, while Diet A gave an intermediate value. The proportion of

infected animals that developed caecal ulceration was much higher with Diet B than the pellet diet. Feeding the appropriate diet to the mother rats for 1 week prior to weaning (Experiment 2) reduced the subsequent growth of the weanlings but the response to amoebic challenge was almost the same as in Experiment 1. Experiment 3 confirms the high degree of caecal ulceration in those rats on Diet B that became infected; it also strongly suggests that carbohydrate supplementation of a low protein diet (Diet C) further increases the susceptibility to infection but that a smaller proportion of those infected develop caecal ulceration. Thus the greater carbohydrate intake may in fact be protecting the host from ulceration, when it does become infected.

## 15. THE EFFECT OF HEPATIC INJURY UPON THE DEVELOPMENT OF AMOEBIC LIVER ABSCESS IN HAMSTERS

### 15.1. Method

Male golden hamsters, 6 - 8 weeks old and weighing between 90 and 110 g were used. Animals were caged separately and given standard Diet No. 86 (Diet D in Part 14.1). Strain LIGGINS growing monoxenically with crithidia in TTY medium was used throughout (see Appendix 1.C).

Animals were anaesthetised by ether inhalation and a longitudinal incision, approximately 1 - 2 cm long was made through skin, abdominal wall and peritoneum. The incision for animals receiving injections into the mesenteric or caecal vein was in the lower abdomen and in animals having a hepatic branch of the portal vein ligated, it was in the upper abdominal wall. After operation, the peritoneum, muscular wall and skin were closed with continuous silk suture. The caecum was brought to the surface and the caecal tributary of the portal vein exposed. This branch drains the terminal ileum, the caecum and the first part of the colon and lies in a mesenteric fold between ileum and caecum. The injection of amoebae was performed with a short gauge 22 needle, bent at the base, and attached to a 1 ml syringe. The inoculum of 100,000 or 200,000 amoebae, from a 48-hour culture in 0.15 ml, was injected slowly.

To damage the liver 2 methods were used: (1) The intraportal injection of 10 mg amounts of sterile glass particles 75 - 125 µm in diameter, suspended in 0.2 ml normal saline and injected through a gauge 18 needle. Bleeding sometimes occurred owing to the larger size of the needle; it was controlled with gelatin sponge. (2) Ligation of the branch of the portal vein leading to the right medial lobe of the liver. This was performed with fine silk (Five 0) in a small round semilunar needle.

A few animals died during the operation. Surviving animals were killed with ether between the 8th and 14th day after operation. Autopsies were performed immediately. Animals which died during the period of observation were examined as soon as possible after death.

At autopsy the abdominal and thoracic cavities were opened and the liver examined. Smears emulsified in saline were taken from suspicious areas and were directly examined for E.histolytica; liver specimens were also taken for culture in Robinson's medium. Cultures were subcultured after 24 hours and examined 2 and 3 days later.

The macroscopic lesions of the liver were scored using the criteria suggested by Jarumilinta and Maegraith (1962).

0 = no gross lesions.

1 = 1 or 2 tiny abscesses, not more than 2 mm in diameter.

2 = 1 abscess 2 - 5 mm, or many (less than 10) small abscesses 1 - 2 mm in diameter.

3 = 1 big abscess involving about half a lobe or 2 or 3 abscesses 3 - 5 mm in diameter or many (more than 10) small abscesses 1 - 2 mm in diameter.

4 = big abscess involving more than half a lobe.

On macroscopic examination amoebic abscesses were yellow in colour, the lobes were enlarged and their consistency was harder than in the simple infarctions. The latter were light brown in colour and the involved lobe diminished in size. The final distinction between abscess and infarction could be made on microscopy and by culture.

Animals were studied at two dosage levels of amoebic inoculum (Experiments 1 and 2), in both there were 3 groups:

Group A (control). Inoculation of E.histolytica trophozoites into the caecal vein.

Group B. Injection of glass particles into the mesenteric vein shortly after inoculation of amoebae into the caecal vein.

Group C. Ligation of one hepatic branch of the portal vein immediately after inoculation of amoebae into the caecal vein.

15.2. Results

Experiment 1. In all 3 groups of animals approximately 100,000 amoebae were inoculated.

Group A. In 5 animals intracaecal inoculation of 100,000 amoebae produced no macroscopic changes in the liver 8 or 14 days after inoculation. Microscopic and cultural examinations did not reveal the presence of E.histolytica.

Group B. In 10 animals the injection of 10 mg of glass particles after inoculation of 100,000 E.histolytica trophozoites did not lead to the development of amoebic lesions in the liver. Minute grey areas throughout the liver substance were due to small infarctions produced by the glass particles; these were similar in appearance to those seen in the 2 control animals injected with glass particles only (Plate 25). Microscopic and cultural examinations showed no evidence of the presence of E.histolytica. Four animals died within the first 24 hours as a result of haemorrhage from the site of glass injection.

Group C. All 5 animals developed necrosis of the ligated lobe, but no changes due to amoebae could be found. Microscopic and cultural

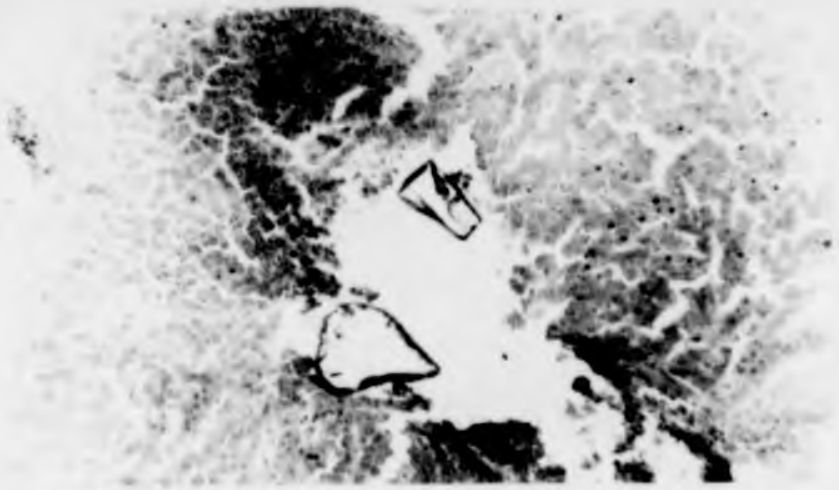


Plate 25. Hepatic lesions in hamsters. 1.
Localised necrotic lesion in vicinity of glass particles that had been injected intraportally 4 days previously. This hamster received no amoebic inoculum.
(x 80)

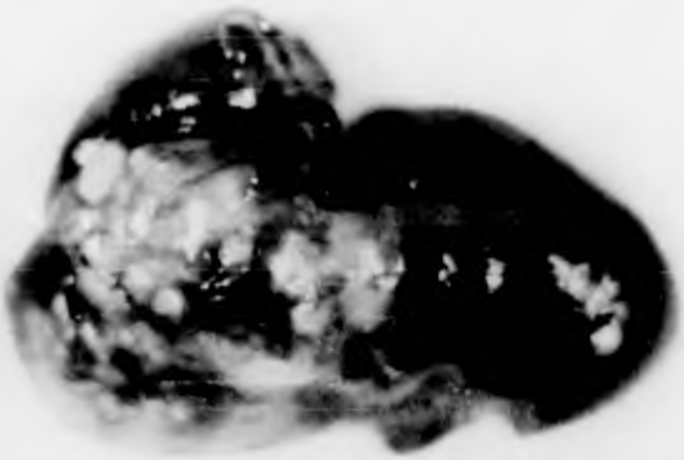


Plate 26. Hepatic lesions in hamsters. 2.
Disseminated amoebic abscesses 10 days after intraportal injection of glass particles together with 200,000 E.histolytica. (x 3).

examinations of E.histolytica were negative. Four animals died during the observation period, mostly on the fourth day, presumably because of liver necrosis.

In order to confirm the viability and potential virulence of the inoculated amoebae, live Escherichia coli were added to some TTY amoebic cultures 12 hours before inoculation into 2 animals; 1 of Group A and 1 of Group B. Both animals developed big abscesses over all liver lobes (grade 4) with numerous E.histolytica trophozoites present on microscopy and culture.

Experiment 2. In all 3 groups of animals approximately 200,000 amoebae were inoculated.

Group A. In 11 of 13 animals, injection of 200,000 amoebae produced no changes in the liver. Microscopic and cultural examinations were negative. Two animals developed small but visible lesions of Grade 1 and gave a positive culture; both were negative on direct microscopy.

Group B. In 12 of 15 animals, in which glass particles were injected after inoculation of 200,000 amoebae, amoebic liver abscesses were observed (Grade 1 in 8, Grade 3 in 2 and Grade 2 in 2). The microscopic or cultural examinations were positive for E.histolytica in 11 of these animals. Three of the 4 animals that were negative by microscopic examination had died within 24 hours of operation and it could be that the operations were themselves partly responsible for these early deaths.

Macroscopically the abscesses were widely disseminated throughout the liver (Plate 26). Most of the lesions were small (1 - 3 mm in diameter) but some reached 1.5 cm in size. Their content was finely granular; no liquification was noted.

Microscopically the abscesses were irregular in shape and chiefly periportal (Plate 27). There was a necrotic centre surrounded by a zone in which early tissue damage and a few amoebae were associated with a low grade of inflammatory response composed of lymphocytes, granulocytes and macrophages. The outermost zone consisted of normal tissue and here numerous amoebae were found. There was no evidence of fibrotic reaction.

Group C. In 10 animals the lobar branch of the portal vein leading to the right medial liver lobe was ligated immediately after the injection of 200,000 trophozoites. Seven animals developed 1 large abscess (Grade 4), involving the whole ligated lobe, but never spreading to other parts of the liver (Plate 28). In all 7, the microscopic or cultural examinations were positive for E.histolytica.

The microscopic pathology showed in some cases liquification in the centre of the necrotic lesions. The demarcation from healthy tissue was clearly defined and there was no evidence of scar-tissue formation.

15.3. Discussion

The results in Experiment I show that in hamsters with or without liver injury the intraportal infection of 100,000 E.histolytica trophozoites did not result in abscess formation. Autopsies 8 - 14 days after inoculation did not reveal any macroscopic changes due to amoebae and microscopical and culture examinations were negative. Association of the same number of amoebae with Escherichia coli for 12 hours before inoculation into 2 animals resulted in big abscesses (Grade 1) confirming

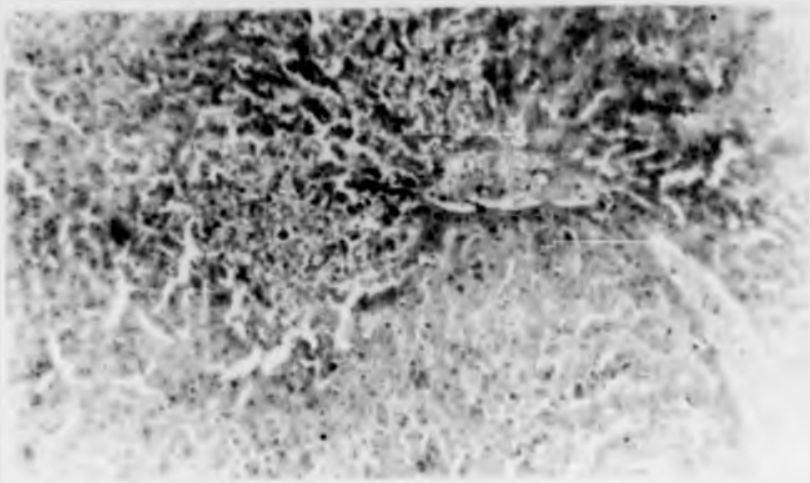


Plate 27. Hepatic lesions in hamsters. 3.
Minute amoebic abscess adjacent to portal vein radicle in a hamster that had been injected intraportally with glass particles and 200,000 E.histolytica. (x 80)

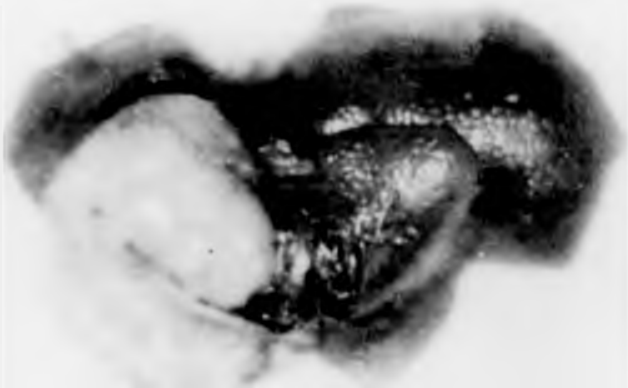


Plate 28. Hepatic lesions in hamsters. 4.
Amoebic abscess involving the whole of the right medial lobe. The portal vein branch to this lobe had been ligated immediately after the intraportal injection of 200,000 E.histolytica; the other lobes show no amoebic lesions.
(x 3)

that the amoebic inoculum was infective and that the virulence of the amoebae could be enhanced by a microbial associate.

In Experiment 2 carried out under the same conditions 200,000 amoebae were inoculated. In animals without liver damage no abscess could be observed and all direct microscopic examinations for E.histolytica were negative; however, very small lesions were seen in 2 animals and these were positive for E.histolytica on culture. In both groups with damaged livers, produced either by glass injection or ligation, big liver abscesses, positive for E.histolytica on microscopy or culture, developed in most animals. The abscesses started to appear from the second day after inoculation and became quickly so severe that several animals died before the end of the observation period.

An important observation was that in animals with glass injection, the abscesses were disseminated all over the liver without any preference to one lobe. Their distribution presumably was similar to the multiple minute necrotic foci seen in control animals in which only glass was injected.

Several of the animals in the group with ligation developed one big abscess in the ligated lobe. Spread to adjoining lobes of the liver was never observed. Microscopic and cultural examination from tissue from the affected lobe gave positive results but control smears from non-affected tissue showed no evidence of E.histolytica (2 animals were studied). This observation, as in the case of glass injection, indicates that healthy liver tissue did not normally provide a favourable medium for the survival and proliferation of injected amoebae.

16. THE EFFECT OF ALCOHOL UPON THE DEVELOPMENT OF AMOEBIC LIVER ABSCESS IN HAMSTERS

16.1. Method

Adult male hamsters weighing 81 to 157 g were given 4 - 7 daily intraperitoneal injections of 10 or 15% ethanol in normal saline. Control animals received normal saline. The animals were then infected intraportally with 200,000 or 300,000 strain LIOGINS amoebae from 48-hour TYI-S cultures using the methods described in Part 15. Animals were studied in 5 batches: numbers 1-6, 7-12, 13-18, 19-24 and 25-30. Animals 1-6 were infected 24 hours after the last ethanol injection, the others 4 or 6 hours afterwards. Surviving animals were killed with ether 9 or 10 days later.

16.2. Results

(See Table 26).

16.3. Discussion

The tabulation of results shows that the alcohol medication had no appreciable effect upon susceptibility to liver abscess, since of the surviving animals, 5/14 given ethanol developed abscess compared with 5/13 controls. 3/15 of the animals receiving 200,000 amoebae (nos. 1 to 18) developed abscesses, compared with 7/12 of those receiving 300,000 amoebae (nos. 19 to 30).

At the dosage given, ethanol had a narcotic effect upon the hamsters, which slept peacefully for 2 - 4 hours after each medication. At the time of infection it was noted that the livers of several of the treated animals were somewhat enlarged and mottled in appearance,

| Animal No. | Ethanol doses per 100 g body wt | Autopsy in d ys | Hepatic abscesses | Microscopy | Culture |
|---|---|---|---|---|---|
| 1 | None | 10,S | 0 | - | - |
| 2 | None | 10,S | 0 | - | - |
| 3 | None | 1,D | 0 | n.d. | n.d. |
| 4 | 2 ml 10% x 5 | 10,S | 0 | - | - |
| 5 | 2 ml 10% x 5 | 1,D | 0 | n.d. | n.d. |
| 6 | 2 ml 10% x 5 | 10,S | 0 | - | - |
| 7 | None | 10,S | 0 | - | - |
| 8 | None | 10,S | 1 large | + | + |
| 9 | None | 10,S | 0 | - | - |
| 10 | 3 ml 10% x 5 | 8,D | 4 large | + | + |
| 11 | 3 ml 10% x 5 | 10,S | 0 | - | - |
| 12 | 3 ml 10% x 5 | 10,S | 0 | - | - |
| 13 | None | 1,D | 0 | - | - |
| 14 | None | 10,S | 1 large | + | + |
| 15 | None | 10,S | 0 | - | - |
| 16 | 2 ml 15% x 4 | 10,S | 0 | - | - |
| 17 | 2 ml 15% x 4 | 10,S | 0 | - | - |
| 18 | 2 ml 15% x 4 | 10,S | 0 | - | - |
| 19 | None | 9,S | 0 | - | - |
| 20 | None | 9,S | 3 large | + | + |
| 21 | None | 8,D | Multiple small in 4 lobes | + | + |
| 22 | 2 ml 15% x 6 | 5,D | Large in 4 lobes | + | + |
| 23 | 2 ml 15% x 6 | 9,S | 0 | - | - |
| 24 | 2 ml 15% x 6 | 9,S | 0 | - | - |
| 25 | None | 9,S | 0 | - | - |
| 26 | None | 9,S | 0 | - | - |
| 27 | None | 9,S | Large in 2 lobes | + | + |
| 28 | 2 ml 15% x 7 | 9,S | 5 mm diam. in 2 lobes | + | + |
| 29 | 2 ml 15% x 7 | 9,S | 3 mm diam. in 1 lobe | + | + |
| 30 | 2 ml 15% x 7 | 9,S | Large in 2 lobes | + | + |

Table 26. Amoebic liver abscess in ethanol treated hamsters.
The outcome of intraportal amoebic infection in control and ethanol-treated hamsters. S = sacrified; D = died
n.d. - not done.

the latter sometimes persisted until autopsy. Histology of the livers of 2 treated but non-infected animals showed patchy vascular congestion and cell oedema but no tissue necrosis and no deposits of Mallory's hyaline.

The doses of ethanol used were 200 or 300 mg per 100 g body weight, which is equivalent to 2 or 3 g per kg. In man, such an intake, if continued for 4 - 7 days, would correspond to quite heavy 'spree drinking'. It is certainly possible that if hamsters were given higher doses or more prolonged medication, an effect upon liver abscess susceptibility might occur, especially if liver cell necrosis had been induced.

## 17. GENERAL DISCUSSION OF ANIMAL EXPERIMENTS

In both T.muris and S.mansoni infections of the mouse, amoebic ulceration occurred in the immediate vicinity of the worm parasites. Possibly the worms induce physiological changes within the caecum that are favourable to amoebic proliferation. During feeding, the head of Trichuris must produce mucosal damage. Furthermore, a cell mediated immune response occurs locally around the worm heads (Wakelin, 1967); although in these experiments this was partly suppressed by cortisone. Similarly in S.mansoni infection there is a vigorous cell mediated immune response as the eggs pass across the mucosa to the lumen of the caecum. These localized areas of tissue damage may allow amoebae to invade; perhaps because of anaerobiosis, a reduced redox potential or the provision of suitable food materials.

Host immunosuppression is the alternative explanation in schistosomiasis, and may be the only explanation in unisexual infections. S.haematobium causes a depressed cell mediated response in man (Wilkins and Brown, 1976). Several of the reported synergistic relationships reported in experimental S.mansoni infections probably operate in this way. For example, Salmonella and Listeria infections (Collins et al., 1972), hepatitis MHV3 infections (Warren et al., 1969) and a carcinogen (Domingo et al., 1967) in mice.

The mechanisms by which diet affects the outcome of an amoebic challenge are uncertain but Experiment 4 (in Part 14.2.) shows that diet alone can affect caecal histology and the redox potential of the caecal contents. Lynch (1957) has previously shown how diet itself

may affect histology. Reud (1950) and Lin (1971) have shown histologically that amoebae colonize chiefly the peripheral portion of the caecal contents. It is in this region that the contents move most slowly and where the pH is near neutrality lying between the alkaline mucus secretion and the acid bacteria-fermented food residue of the caecal contents; furthermore the redox potential is suitable for amoebic growth, providing microaerophilic conditions. In vitro E.histolytica have been shown to grow best with bacteria at a redox potential between -200 and -300 mV (Harinasuta and Harinasuta, 1955). The observation in Experiment 3 (in Part II.B.) that redox potentials were lower in those caeca that were ulcerated (caecal score 2 or more), may merely indicate that ulceration per se reduces this potential by pouring an exudate of necrotic tissue and cells into the caecal lumen; however, it is possible that rats having a lower caecal redox potential initially, develop ulceration and this further reduces the redox value. The optimum pH for in vitro growth has been mentioned already in Part 5.

Protein deficiency affects gastro-intestinal structure and function in several ways (Stewart, 1970). There is a reduction of epithelial cell proliferation leading to shortening of villi and in the small bowel a lack of mucosal enzymes such as disaccharidases. In addition, the production of pancreatic enzymes is reduced. The net result is that an increased amount of undigested polysaccharide, together with unabsorbed disaccharide and monosaccharide reaches the large bowel; these may provide ideal nutrients for intraluminal amoebae. Local factors in the caecal mucosa, such as a reduced production of alkaline mucus may allow amoebae to come into closer contact with the mucosa by shifting the

optimum pH towards the epithelial surface; the reduction in the number of goblet cells in the caeca of the malnourished rats, noted in Part 14.2., certainly suggests that mucus production may be impaired. In addition, if the mitotic rate of the caecal epithelial cells is reduced then an impaired ability of the mucosa to repair minor defects in its surface, might allow tissue invading amoebae to establish themselves.

The results obtained with hamsters in Part 15 suggest very strongly that localized liver damage favours the establishment of a progressive amoebic liver lesion. Intraportal injection is the most physiological way of administering amoebae to the liver in experimental animals. Direct inoculation (Reinertson and Thompson, 1951) inevitably causes some liver damage. Intraperitoneal injection (Jarumilinta and Maegraith, 1962) or the insertion of infected gelatin sponge between the hepatic lobes (Jarumilinta, 1968) obviates this difficulty; however, when bacteria-associated cultures are used, the bacteria themselves must cause some liver damage.

In man, amoebic abscesses are normally sterile. Bacteria-free lesions in animals have been produced by serial liver passages initiated with human liver abscess material (Wiles et al., 1963), or the intrahepatic injection of crithidia-associated (Raether, 1971) or axenic cultures (Tanimoto et al., 1971). The latter authors noted a marked dose related effect; this was also observed in the present work with hamster liver abscess in Parts 15 and 16.

Maegraith and Harinasuta (1954a) using guinea pigs have shown that micro-infarcts, produced by embolism of an intraportally injected amoebic suspension, may be the primary site of liver lesions. It may be that the predisposition of sensitised guinea pigs to liver abscess formation, is due to vascular lesions of the Arthus type occurring in the portal vein radicles within the liver.

## 16. HOST FACTORS AFFECTING TISSUE INVASION IN MAN

Unless infection rates are known it is difficult to assess from clinical data the effect of age, sex and race upon susceptibility to invasive amoebiasis. However, it is clear that in many endemic areas, children under five are especially vulnerable, and that after the age of puberty males are much more likely to develop liver abscess.

### Local Bowel Disease

In Durban, Shigella were isolated more commonly among amoebic dysentery patients than from matched controls (Powell, S.J., personal communication). Many cases of amoebiasis have been recognized during the recent S.dysenteriae pandemic in Central America. Amoebae may invade a damaged bowel more readily.

Non-specific ulcerative colitis may be difficult to distinguish from amoebic disease, and perhaps in some patients the latter is superimposed upon latent colitis. Patient LIGGINS (see Appendix 2), for example, clinically relapsed twice after medical treatment and sigmoidoscopic findings never returned to normal despite eventual parasitological cure.

Colonic carcinomas not uncommonly have a superimposed invasive amoebic lesion (Albores-Saavedra et al., 1968).

Following the demonstration of a synergistic relation between S.mansoni and E.histolytica infection in mice, a collection of sera made by Dr. Omer at Gezira in Sudan, was tested for amoebic antibody in collaboration with Dr. C. C. Draper, using strain DKB as antigen and the methodology in Appendix 3.

| Age | Number | % S.mansoni eggs | % E.histolytica titre ⩾ 1/64 | % S.mansoni positive at E.histolytica titre ⩽ 1/16 | ⩾ 1/64 |
|---|---|---|---|---|---|
| 0-4 | 66 | 21.2 | 30.3 | 19.5 | 25 |
| 5-9 | 69 | 69.6 | 50.7 | 64.7 | 74.3 |
| 10-14 | 60 | 91.7 | 51.7 | 89.7 | 93.5 |
| 15-19 | 55 | 89.1 | 40.0 | 87.9 | 90.9 |
| 20+ | 89 | 52.8 | 24.7 | 52.2 | 54.5 |
| Total | 339 | 62.8 | 38.3 | 57.9 | 70.8 |

Table 27. Relation between amoebic serology and S.mansoni infection in man.
The prevalence of S.mansoni infection and E.histolytica (FAT) titres ⩾ 1/64, together with the S.mansoni prevalence at different E.histolytica titres. Data from Gesira, Sudan.

It can be seen from Table 27 that the prevalence of schistosome infection and a significant amoebic titre (⩾ 1/64) run parallel in different age groups. In each age group there were more high amoebic titres among those with demonstrable eggs; overall, the association was statistically significant ($X^2 = 5.15$ $p < 0.05$).

An association with T.trichiura and possibly Strongyloides, which may damage the colon during autoinfection, could be looked for in the same way. Among children in New Orleans, Jung and Beaver (1951) found a strong correlation between amoebic disease and Trichuris egg count; there was no association with Ascaris. In Durban, patients with amoebic dysentery were noted to be frequently infected with Trichuris (Beaver, 1958).

Rectal administration of corticosteroids may greatly worsen amoebic dysentery (Mody, 1959).

Systemic Conditions

Lewis and Antia (1969) in a clinical study at Ibadan, reported a strong association between amoebic disease and the second trimester of pregnancy and the puerperium. This relationship has also been noted in case reports by De Silva (1970) and Rivera (1972), and by Abioye and Edington (1972) in a necropsy series of 135 patients in Ibadan. The Nigerian studies also suggested that Hodgkin's disease and other malignancies, tuberculosis, pneumonia and typhoid were similarly related. Lewis and Antia (1969) mention 2 patients on cytotoxic therapy. Systemic steroids may also precipitate amoebic disease but cases are perhaps not often reported. Ejsent et al. (1959) noted this in a patient with pemphigus and in two personally studied cases, steroids had been given for suspected ulcerative colitis (Kanani and Knight, 1969a) and dermatitis herpetiformis (Kanani and Knight, 1969b).

Amoebic disease is well known among fighting troops, for example, Gurkhas in Burma and Frenchmen in Indo-China. Physical stress and exhaustion have been incriminated.

A factor common to many of these conditions, in particular pregnancy and cytotoxic and steroid therapy, is depression of cell mediated immunity. The important role that this may play in the rejection of amoebic invasion has been discussed in Part 10.2.

Diet

Elsdon-Dew (1949) considered that in Durban a low protein, high carbohydrate (corn meal and soda) diet favoured amoebic disease. However, in Cali, Columbia, Faust and Read (1959) believed that the high undigested starch content of the colon provided amoebae with nutrients and so mitigated against tissue invasion. Alexander and Meleney (1935) compared the diets of two Tennessee communities, both with adequate protein; the one taking a higher calorie and more varied diet showed less disease.

The experiments in Part 14 support the hypothesis that protein deficiency favours tissue invasion; they also show how carbohydrate supplementation of a low protein diet favours higher infection rates but reduces caecal ulceration.

Pre-existing Hepatic Damage

The experiments in Part 15 show how hepatic trauma favours amoebic abscess formation. In tropical countries liver damage in man is frequent and often recurrent; for example, viral hepatitis, dietary mycotoxins and pyrrolizidines, S.mansoni ogg granulomas, and in children reactions around migrating Ascaris larvae.

The association between 'tropical liver abscess' and alcohol intake was recognized in India, even before the role of amoebae was appreciated. Available evidence is circumstantial but nevertheless convincing. Focal necrosis may be necessary but this is known to occur in true alcoholic hepatitis. Relatively mild damage, as was studied experimentally in Part 16, may not be sufficient.

19. THE RELATIVE ROLES OF HOST AND PARASITE

The strongest evidence for differences in strain virulence comes from the comparison of isolates from invasive disease and from carriers. Such comparisons may not, however, be entirely valid as they are comparisons between isolates from 'magna' trophozoites and 'minuta' cysts. The decline of virulence in 'magna' isolates maintained *in vitro* may be due to a reversion to the 'minuta' form in the absence of environmental triggers, or alternatively a reduced enzyme activity in the absence of inducers. A less likely explanation would be a genetic drift under unphysiological cultural conditions.

Virulence is made up of at least two components, 'magna' transformation and cytotoxicity; both are likely to be genetically determined but they need not necessarily be correlated. Strains may differ in their ability to transform into 'magna' forms, or this may occur at different stimulus thresholds. This would explain the finding by some workers that long established bacteria-associated strains, and also some strains from healthy carriers, are difficult to grow monoxenically with *Crithidia*.

The present work has shown that all the strains tested, apart from the atypical LAREDO and HUFF, were cytotoxic *in vitro*. There were reproducible strain differences but these were rarely more than threefold. It is possible that all strains of true *E.histolytica* are cytotoxic, once they are in the 'magna' form.

The frequency of tissue invasion among symptomless persons is still disputed. The strongest evidence comes from seropositivity rates in endemic areas, for example those given in Table 27. In areas of high

high and low endemicity, rates of seropositivity are higher among carriers than uninfected persons, the difference varying with the sensitivity of the technique.

Furthermore, the observations that some carriers have abnormal sigmoidoscopic appearances (Morton et al., 1951) or demonstrable lesions at necropsy after accidental death (Faust, 1941) cannot be ignored There is also the teleological argument that if tissue invasion is an accidental phenomenon of no biological advantage to the species, then why is it so frequent with E.histolytica but absent in other species like E.hartmanni.

There is no good epidemiological evidence of significant strain differences. Boyd (1961) has analysed in detail some epidemics of amoebiasis, for example, Chicago 1933 and 1934 and South Bend, Indiana, 1953; he argues persuasively that enteropathogenic bacteria may have unmasked latent amoebic infection. In Nigeria, Nnochiri (1965) has indicated how severe invasive amoebiasis in children is usually derived from symptomless maternal infections. The apparent rarity of invasive disease among persons, or their contacts, after entering temperate countries from the tropics, can be interpreted in several ways. Firstly, the attack rate per new infection may in fact be no lower than that of highly endemic areas. Kessel et al. (1965) compared two groups of symptomless carriers in California and showed that 82% of patients in a mental institution had positive indirect haemagglutination titres compared to 13% of university students, suggesting that tissue invasion was more likely in communities where reinfection was common. Alternatively, various host susceptibility factors may diminish outside the tropics.

Lastly, disease may occur mainly in new infections and most infected migrants may have passed this stage when they arrive.

Host susceptibility factors in man (Part 18) and experimental animals (Part 11) have already been discussed. They are not dissimilar to those relating to other pathogenic lumen dwelling protozoa (Part 3). The apparently sporadic occurrence of disease among seemingly homogenous human and animal populations cannot easily be attributed to host differences, especially when it is suspected that a different result would be obtained if the observations were repeated. In the experimental caecal infections (Parts 12, 13 and 14) it was noted that caecal scores followed a bimodal distribution suggesting that tissue invasion was an all or none phenomenon. A stochastic process may be the most valid way of interpreting such findings. In man, the probability of amoebic disease in infected persons is likely to differ in groupings based upon age, nutritional status and intercurrent disease; but within each group it may not be necessary to invoke hypothetical host differences.

It is, however, possible that fluctuations in immune status might explain some short-term differences in susceptibility within a particular host. It is now recognized that parameters of cell mediated immunity such as lymphocyte transformation to phytohaemagglutinin may be influenced by drugs, minor intercurrent illness, stress and trauma (Opelz et al., 1973; Kapunol et al., 1974). If mild amoebic tissue invasion really is a frequent phenomenon, then the proximate determinant of disease progression may be local cellular immunity.

## 20. THE USE OF EPIDEMIOLOGICAL DATA IN THE STUDY OF PATHOGENICITY

In population studies persons can be classified according to three parameters: luminal infection (I) as evidenced by cysts or non-haematophagous trophozoites in the stool, seropositivity (S) at a significant titre, and morbidity (M) denoted by clinical parameters or haematophagous trophozoites in the stool. Within a population the frequency of these parameters and their degrees of overlap can be represented by a Venn diagram with a total of 8 categories (Figure 13).

Each of the circles for I, S and M is made up of 4 component categories. For example, the circle for morbidity (M) comprises infected seronegatives, infected seropositives, non-infected seropositives (having lost luminal infection but not seropositivity), and finally uninfected seronegatives. The last two categories will be small but might include cases of post-dysenteric colitis or irritable bowel syndrome, strictures and fistulae, and constrictive pericarditis. Different populations should be studied and the relative size of the categories compared.

In practice morbidity rates are often too low and morbidity ascertainment too insensitive for population studies; furthermore, diagnosis founded upon seropositivity will inevitably be biased. As, however, it is believed that seropositivity only occurs with tissue invasion, we may use seropositivity as an indicator of current or recent tissue invasion. In practice most epidemiological studies will consider only faecal microscopy for cysts and serological status. An important variable in stool microscopy is the diagnostic sensitivity (p), which may be defined as the probability that an infected person will be detected at one examination. Methods for estimating p have been

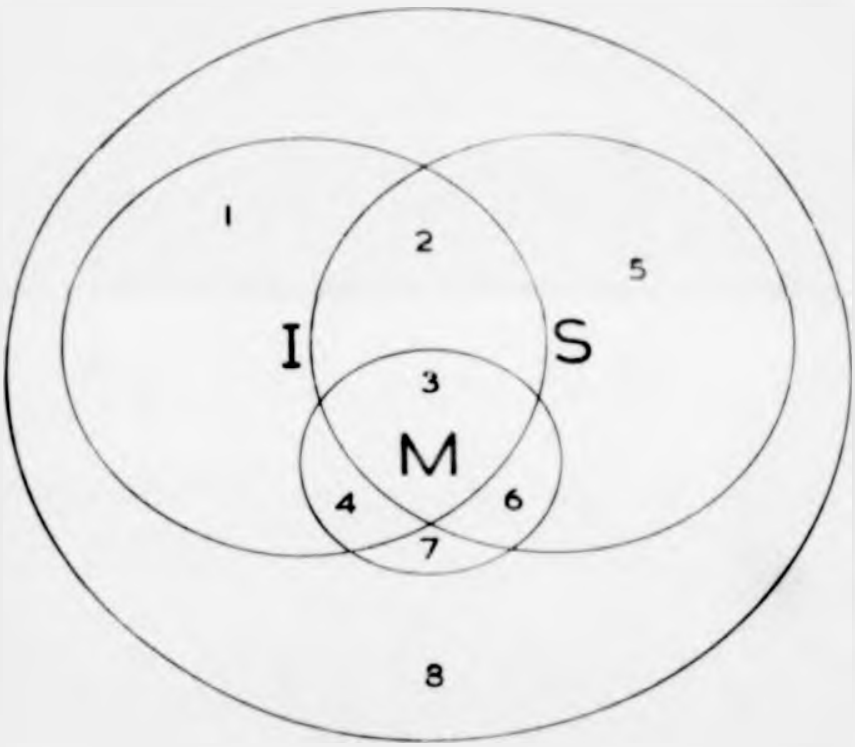



Figure 13. Venn diagram showing host-parasite relationship of schistosomiasis within a human population. Each of the 3 parameters, infection (I), seropositivity (S) and morbidity (M) is represented by a circle lying within the study population (outermost circle). The population is thus classified into 8 categories.

discussed in detail elsewhere (Knight, 1975); the formol-ether sedimentation and zinc sulphate flotation methods normally give values between 0.8 and 0.9.

If I and S are known for several communities then the ratios S/I may be compared. It is still uncertain whether or not the notorious morbidity centres, such as Mexico City, Freetown, Durban and Rangoon have particularly high ratios. A high ratio might indicate either a high virulence of local amoebic strains or a susceptible host population. When a relationship with another disease, such as shigellosis or typhoid, is being looked for, the S/I ratios of those with and without the second pathology may be compared. When there is no *a priori* reason to suspect double infection from common exposure, then a preliminary study can compare seropositivity in persons with and without the second condition. The use of this method is illustrated in Table 27, in relation to *S.mansoni* in man.

Longitudinal studies of these two parameters can also give useful interpretations. We can represent the dynamic relation between the categories of non-infected seronegative $X_1$, infected seronegative $X_1$, infected seropositive $X_2$ and non-infected seropositive $X_3$, by the vector diagram shown in Figure 14.

The size of each square represents the number of persons in each category, as a proportion of the total population. Seven rate constants connect the categories; each is defined as the probability that a person in the donor category will move to the adjacent category in unit time. Only one rate constant connects $X_3$ and $X_4$ because seropositivity can only occur during an infection. In many populations the sizes of the four categories remain more or less constant with time so that there is

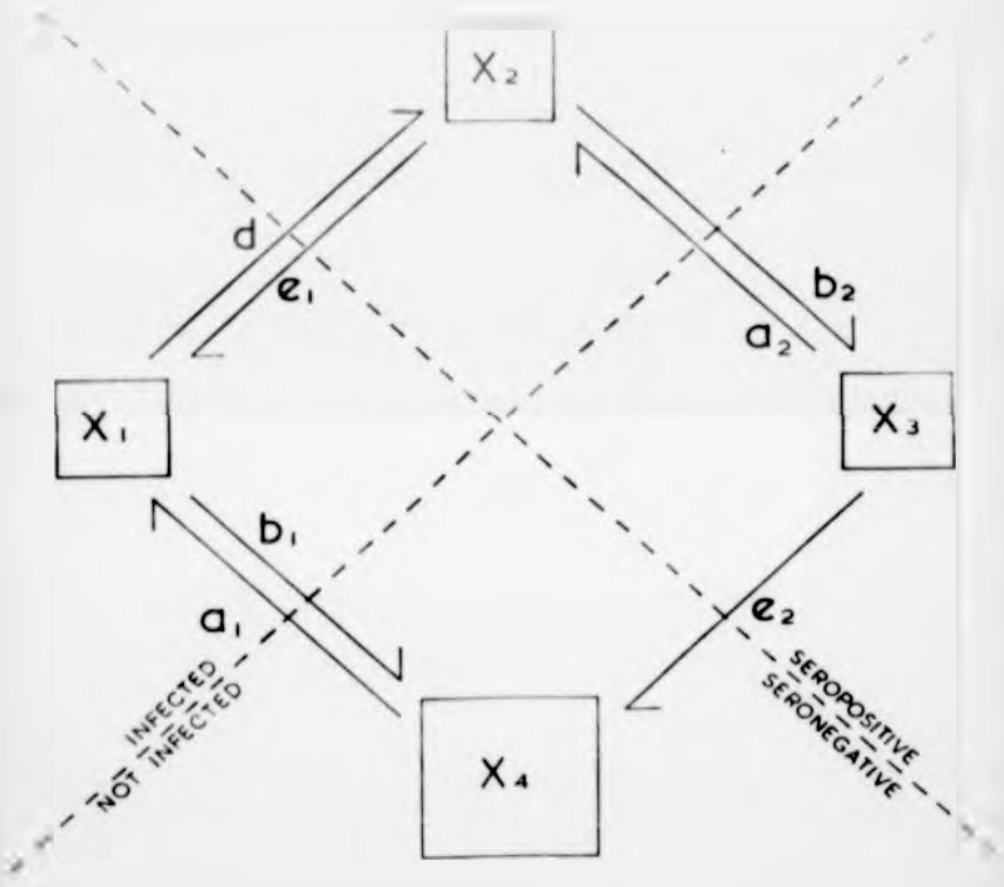



Figure 14. The dynamics of amoebic infection and seropositivity. Using these 2 parameters the population comprises 4 categories: $X_1$, $X_2$, $X_3$ and $X_4$. The 7 rate constants connecting the categories are labelled.

a dynamic equilibrium with a net clockwise rotation of $X_3e_2$ between adjacent categories. Thus, if the net rotation is known, together with one of a pair of rate constants, the counteracting rate constant may be inferred.

The following applications may be useful:

(1) Seroconversion rates (- to +) of infected persons (i.e. d in Figure 14) could be followed in pregnant women, persons undergoing surgery, or those receiving corticosteroid or antimetabolite therapy, to determine the frequency of the 'reactivation type' of tissue invasion.

(2) An immmnological rejection of luminal infection would be suggested by a higher rate of loss of stool positivity amongst the seropositive, i.e. $b_2 > b_1$ (Figure 14).

(3) If infected persons are liable to repeated tissue invasion then loss of seropositivity would be lower in those that remained infected, i.e. $e_1 < e_2$ (Figure 14).

(4) If seropositive persons are partly protected against reinfection then the stool conversion rate (- to +) would be lower in the seropositive, i.e. $a_2 < a_1$ (Figure 14).

(5) The concept of a protective effect by a resident non-invasive infection upon a potentially invasive super-infection (see Part 8.3.), would be supported if seroconversion rates (- to +) were lower in those initially stool positive compared with those becoming stool positive.

(6) In order to study incubation periods, the frequency distribution of the time of seroconversion (- to +) among those becoming stool positive could be studied. Many infections show a log normal distribution and amoebiasis may behave likewise. Sartwell (1950) has analysed the

1933 Chicago outbreak and gives a median of 21 days with 16% of the illnesses occurring after the 44th day; the infective dose possibly affects the median value and may have been high in this epidemic. The alternative, less likely hypothesis is that following a short latent interval, tissue invasion and illness have the same probability of occurrence throughout the duration of the infection.

Rate constants of gain or loss of infection, or serologic status should preferably be estimated from cumulutive data in frequently sampled cohorts.

For example, if the incidence rate (A) is defined as the probability that one susceptible (i.e. non-infected person in a population where the prevalence of infection is P) will become infected in unit time. Now in a cumulative study, loss of infection can be ignored so that:

$$\text{Change in prevalence } \frac{dP}{dt} = A\,(1 - P)$$

$$\text{so that} \quad P = 1 - e^{-At}$$

$$\text{and} \quad -\log_e (1 - P) = At$$

When P is plotted on reverse log scale against time, the gradient is A. log 2 cycle by arithmetic paper is used and the method is similar to that of Draper et al. (1972) for the analysis of serological data in malaria. No correction will usually be necessary for infections lost provided the time interval between examinations is short. The rate of loss of infection (R) usually has a value of about 0,2 per year so that if examinations are repeated at 10-week intervals, the number of infections lost will be the same as would occur if all the new infections occurred

at the midpoint, i.e. 5 weeks; hence infections lost = $0.2 \times \frac{5}{52} \simeq 2\%$, a negligible amount. Values of P must, however, be corrected for diagnostic sensitivity p.

An alternative method of estimating A and also B (the rate of loss of infection) is by the analysis of the plotted curves of either simple age prevalence data, or those of longitudinal prevalence rates among persons known initially to be infected or non-infected. The method involves using a simple deterministic model of amoebic infection with two important assumptions:

(1) That the rates of gain and loss of infection can both be represented by simple rate constants, A and B, which apply to both sexes and to all ages.

(2) Superinfection may occur but this does not affect the duration of infection, see Part 8.3.

Now incidence of infection $= A(1 - P)$

and loss of infection $= BP$

hence change in prevalence with time, $\frac{dP}{dt} = A(1 - P) - BP$

which on integration gives $P = \frac{A}{A + B} + c.e.^{-(A + B)t}$

If P = 0 when t = 0, i.e. at birth or at the beginning of exposure, then the expression becomes:

$$P = \frac{A}{A + B}(1 - e^{-(A + B)t}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Similarly, if we are interested in the loss of infections by persons known to be infected we may substitute P = 1 when t = 0 in the expression giving:

$$P = \frac{A}{A + B} + \frac{B}{A + B} e^{-(A + B)t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

If persons losing infection are not re-exposed to infection,

then $\frac{dP}{dt} = BP$ and $P = e^{-Bt}$ ..... (3)

The general form of these functions is shown in Figure 15. It will be seen that for expression (1), i.e. simple age prevalence data, the prevalence rises with time and reaches an asymptote or equilibrium value at which the gain of new infections is exactly balanced by those lost. This limiting prevalence can be termed $P_L$.

At the limit $\frac{dP}{dt} = 0 = A(1 - P) - PB$

so that $P_L = \frac{A}{A + B}$ ..... (4)

which indicates that prevalence rates can never reach 100% with this model.

An analytical method of estimating A and B from such curves is given in detail by Muench (1959), together with the appropriate nomograms. In the example shown in the figure, A and B have been given the respective values 0.06 and 0.14 so that $P_L = 0.3$.

An alternative method of analysis is as follows:

From (1) and (4) $P = P_L (1 - e^{-(A + B)t})$

Hence $1 - \frac{P}{P_L} = e^{-(A + B)t}$

and $-\log_e (1 - \frac{P}{P_L}) = (A + B)t$

so that if the proportion $\frac{P}{P_L}$ is plotted on a reverse log scale against time or age then a linear plot will be obtained having a gradient (A + B). This method has the advantage of speed and it is easy to see by inspection whether the plot is nearly linear. Another important advantage is that the proportion $\frac{P}{P_L}$ is not influenced by the diagnostic sensitivity as both values require the same correction. Hence, uncorrected data can be

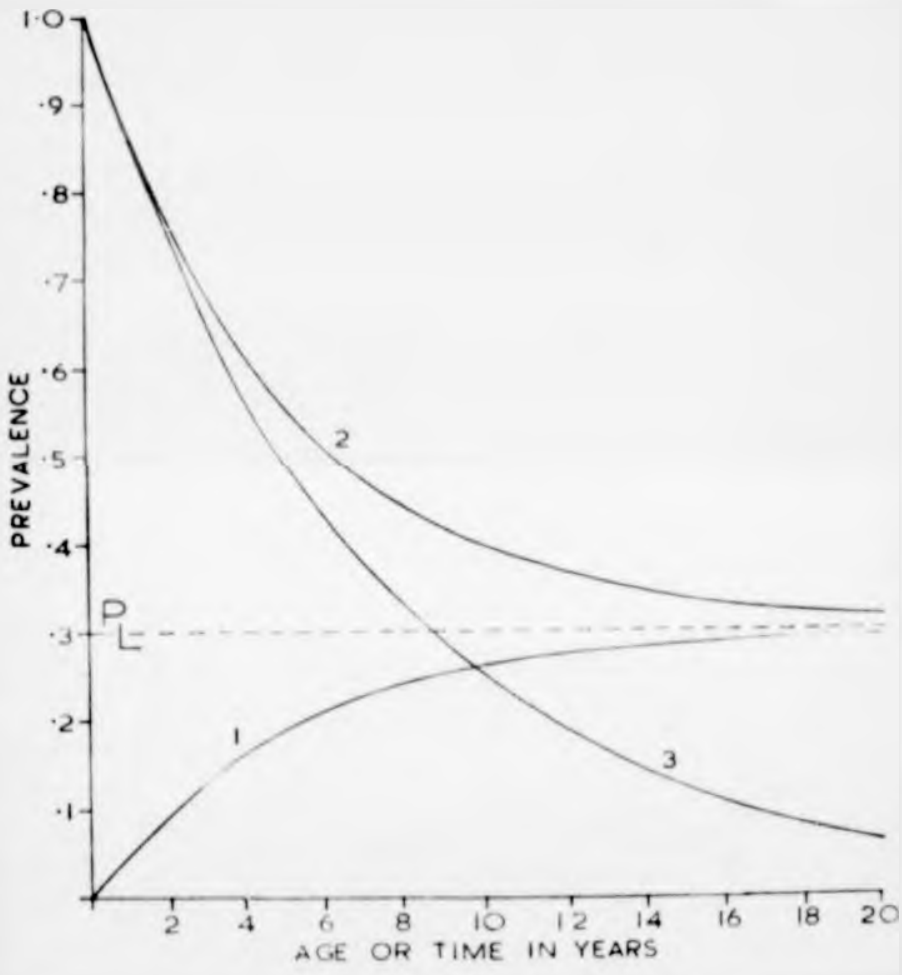



Figure 15. Age prevalence or time prevalence curves, as predicted by deterministic epidemiological model. The values $\lambda$ (annual incidence) = 0.08 and R (annual loss) = 0.14 have been substituted. Curve 1 shows rate of increase in prevalence among persons initially non-infected (expression 1). Curve 2 shows rate of fall in prevalence among persons known to be infected at zero time, who continue to be re-exposed (expression 2). Curve 3 shows rate of loss of infection in the absence of re-exposure (expression 3). It will be seen that curves 1 and 2 approach the equilibrium value of $P_L$ (limiting prevalence) = 0.3.

plotted to give the true value of (A + B) and this multiplied by the corrected value of $P_L$ will give A; B is then obtained by subtraction. This method is equivalent to taking $P_L$ as 1 and expressing P as a proportion of this. When it is difficult to assign a value of $P_L$ to the data, because of unstable values near the limit, then the analytical methods of Muench should be used.

Application of this expression to published data (Knight, 1975), gives values of A between 0.08 and 0.14 per year; and values of B between 0.11 and 0.22 per year; the latter imply median durations of infection ($\frac{\log .5}{\log (1-B)}$) between 6.3 and 3.2 years, and mean values ($\frac{1}{-\log (1-B)}$) of 8.6 and 4.0 years.

It will be of great interest to compare these constants in different communities and to relate them to the prevalence of seropositivity and the incidence of invasive amoebic disease. If most tissue invasion occurs soon after infection then the prevalence of seropositivity should be proportional to incidence of infection (A) rather than prevalence P. Similarly, if seropositivity encourages loss of infection then B will vary directly with seropositivity in different populations.

When seropositivity is plotted against age, it is possible to estimate the rates of gain and loss, using the same mathematical expressions as those used for infection. Hence the ratio of seroconversion rate (- to +) to incidence of infection (A) can be compared in different communities. If the rate of loss of seropositivity is known then the median and mean duration of positivity can readily be calculated.

The consequences of control measures

The simple deterministic model, derived here, can also be used to predict the outcome of control measures. Thus, if the rate constants for infection are known, one may calculate the effect of a reduced transmission rate or mass chemotherapy. The transmission constant k may be defined as the probability that one infective will infect one susceptible in unit time; uniform mixing of infectives and susceptibles is assumed. Incidence of infection (A) will now equal kP, so if this is substituted in the general expression $\frac{dP}{dt} = A(1 - P) - BP$, we obtain:

$$\frac{dP}{dt} = kP(1 - P) - BP = (k - B)\,P - kP^2$$

which on integration gives

$$P = \frac{k - B}{k\left\{1 - (1 - \frac{k - B}{kP_0})\, e^{-(k - B)t}\right\}} \qquad \ldots\ldots (5)$$

where $P_0$ is prevalence at zero time and P is the value after time t. At equilibrium,

$$\frac{dP}{dt} = 0, \text{ hence } P = \frac{k - B}{k} \qquad \ldots\ldots (6)$$

Now B, the rate of spontaneous loss of infection, probably has a similar value in most populations and cannot be altered except possibly by immunization if it is true that $b_2 > b_1$ (Figure 14). The transmission constant k can be reduced by the provision of clean water supplies, latrines, fly control, and education in personal hygiene. Expressions (5) and (6) show that if $B > k$, then P will tend to zero; hence $k = B$ is the break point for transmission and sanitary measures need only reduce k to this value. For example, if in a hypothetical population

P = 0.5 and B = 0.2, then k = 0.4. If this value of k is halved, then amoebic infection will eventually disappear. A reduction of 25%, i.e. k = 0.3, will give a final prevalence of P = 0.33, which is a 34% fall. However, this value will be reached slowly, for, from expression (5), the prevalence rates after 1, 5 and 10 years will be 0.48, 0.12 and 0.38. In reality, k will vary between persons and will be higher in some parts of the community and in certain families; however, provided the mean value is less than B, no general transmission will occur. This is the situation in Britain now, so that despite the entry of new infectives from overseas, no significant transmission occurs. An exception is where an infective enters a local situation where k is potentially high, as in an institution for the mentally subnormal, and produces an epidemic.

Successful mass chemotherapy will reduce P to $P_0$ from which prevalence will steadily rise and finally reach its former level, provided k remains unchanged. Referring again to our hypothetical example, if P is reduced from 0.5 to 0.25, then from expression (5), it will reach 0.27 after one year, 0.37 after five years, and 0.44 after 10 years. Compared to sanitary measures, chemotherapy would appear to have good long-term effects; because incidence rates are usually low, retreatment programmes need not be frequent. To break transmission, however, k must be reduced. If chemotherapy is to be selective, then persons with the highest value of k should be treated and these would include food handlers and mothers with young children.

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NOTE ON COLLABORATIVE WORK

During the course of an ongoing research programme on the pathogenesis of amoebiasis, the author took the opportunity to collaborate with four other workers. In each instance the collaborative work involved one separate aspect of the main enquiry.

Dr. Kenneth Warren of the Department of Medicine, Case Western Reserve, Cleveland, Ohio, shared with myself an interest in the synergistic interaction between infections. The experiments in Part 13 involving S.mansoni and E.histolytica were designed in collaboration; the greater part of the experimental work was done by myself and the conclusions were made jointly.

Dr. Richard Bird, Head of the Subdepartment of Electron Microscopy at the London School of Hygiene and Tropical Medicine, kindly offered to help the author with the processing and examination of his specimens (the findings are reported here in Part 6.4). All the procedures as far as osmium fixation were carried out by myself. Dr. Bird undertook the embedding, sectioning and microphotography; in many instances specimens were examined jointly with the electron microscope, before photographs were taken. The interpretation of the photomicrographs was made jointly.

Dr. Graeme Ross worked in the Department of Clinical Tropical Medicine, London School of Hygiene and Tropical Medicine, for six months during 1972/73. The work described here in Part 14, on diet and amoebic pathogenicity in rats, was done jointly with him. The author was responsible for the general planning of the experiments and

carried out a large part of the experimental work including the inoculations, the reisolation of strains and the measurement of redox potentials. Dr. Ross was responsible for the day to day management of the rats and their dietary; he also made bacteriological studies and measurements of caecal pH but these findings are not reported here.

Dr. Harold Gogler worked in the Department of Clinical Tropical Medicine, London School of Hygiene and Tropical Medicine, for six months during 1972/73. The work described here in Part 15 on the effect of hepatic injury upon the development of liver abscess in hamsters was done jointly with him. The methods of producing liver damage had been developed by the author who was most fortunate to find a colleague whose surgical skills could be successfully applied to the delicate mesenteric vasculature of the hamster. Most of the surgical procedures and post mortem examinations were carried out together.

The work done with Dr. Ross and Dr. Gogler was carried out under the close supervision of my supervisor, Professor A. W. Woodruff, and the experimental designs and interpretations were discussed carefully with him.

With the exception of the electron microscopy all the collaborative work was carried out in Professor Woodruff's department, using departmental equipment and animal stocks. The amoebic methodology was all developed by the author, who also isolated and maintained all the amoebic strains used.

PUBLICATIONS ON AMOEBIASIS

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APPENDIX 1

CULTURE METHODS FOR ENTAMOEBA HISTOLYTICA

A. Robinson's Medium (Robinson, 1968).

Amoebae are grown in a polybacterial, but predominantly Escherichia coli-associated, diphasic medium.

Components

1. Saline agar slopes - Agar powder 1.5% is made up in 0.7% sodium chloride, heated, distributed in 2.5 ml amounts in Bijou bottles and sloped after autoclaving (15 lbs for 10 minutes).
2. Erythromycin solution - 0.25 g of the base is suspended in 1 ml of 70% ethanol. Two hours later this is diluted in 49 ml of sterile water. The 0.5% solution is stored at 4°C.
3. Bactopeptone - dissolved 20% in water and autoclaved.
4. Rice starch (British Drug Houses) - used aseptically.
5. Phthalate buffer - 20.4 g of potassium phthalate is dissolved in 100 ml of 40% sodium hydroxide and made up in water to 2 litres. The pH is adjusted to 6.3 and the solution is distributed in 10 or 20 ml volumes and autoclaved.
6. Defined medium "R" for growing Escherichia coli - stock solution contains 125 g sodium chloride, 50 g citric acid monohydrate, 12.5 g potassium dihydrogen phosphate, 25 g ammonium sulphate, 1.25 g magnesium sulphate heptahydrate and 100 ml lactic acid (British Drug Houses, 90.08%) in 2.5 litres of water. For use, 100 ml with 7.5 ml 40% sodium hydroxide and 2.5 ml 0.04% bromthymol

blue solution is diluted in water to 1 litre, adjusted to pH 7 and autoclaved. Stock is used over 4 weeks old to avoid change of pH on autoclaving.

7. Basal medium "BR"-Escherichia coli, strain B, is grown at 37 C for 48 hours in 100 ml of medium "R" in 200 ml flat bottles. Store at room temperature.

8. Complete basal medium "BRS" - An equal volume of horse serum (heat inactivated, Wellcome No. 5) is added to "BR" and the mixture incubated at 37°C for a further 24 hours. Store room temperature.

Method

To initiate a culture - inoculation is made into 1.5 ml of "BR", 10 mg starch and 4 drops of erythromycin. After 24 hours the supernatant is removed and replaced by 1.5 ml of phthalate and "BRS" (1:1), 2 drops bactopeptone and 2 drops erythromycin.

To maintain culture - subculture into 3 ml phthalate and "BRS" (3:1 or 4:1) with 2 drops erythromycin, 2 drops bactopeptone and 10 mg of starch.

For preparation in bulk the following mixture may be used:

| | |
|---|---|
| Phthalate buffer | 70 ml |
| "BRS" | 25 ml |
| Erythromycin solution | 1.5 ml |
| Bactopeptone | 1.5 ml |
| Starch | 300 mg |

Subcultures are made from the deposit, every 3rd or 4th day; but some amoebae usually remain viable for up to 14 days.

Note:

The strain B of Escherichia coli was kindly donated by Dr. G. L. Robinson.

B. Modified Shaffer-Fryo Medium (MS-F) (Reeves et al., 1957).

Amoebae are grown monoxenically with penicillin inhibited Bacteroides symbiosus in a monophasic liquid medium.

Components

1. Mercaptosuccinic acid solution. 15 g of the acid is dissolved in 50 ml of water, and the pH adjusted to 7.0 with 6N sodium hydroxide. Water added to 100 ml. Stored frozen at -20°C.

2. Basic medium -

| | | |
|---|---|---|
| Distilled water | 485 | ml |
| Mercaptosuccinic acid (see above) | 5 | ml |
| Trypticase (BBL) | 10 | g |
| Glucose | 5 | g |
| Dipotassium hydrogen phosphate, $K_2HPO_4$ | 0.785 | g |
| Sodium chloride | 1.25 | g |
| Yeast extract (BBL) | 1.0 | g |

Dissolve solids by boiling, distribute in 12 ml amounts in 125 x 16 mm, screw cap tubes. Autoclave at 15 lbs for 10 minutes.

3. Horse serum - heat inactivated (Wellcome No. 5).

4. Penicillin G solution - at 10,000 units per ml.

5. Bacteroides culture - the organism is grown for 24 hours in medium without serum or penicillin.

Method

Just prior to inoculation the following are added to each culture tube:

0,5 ml horse serum

0,5 ml penicillin solution

2 ml *Bacteroides* culture.

Subcultures are made every 3rd or 4th day. Medium is decanted and replaced by a few ml. of ice-chilled fresh medium, gentle shaking removes the amoebae from the glass. Cultures are sloped at 15°.

Note:

A culture of *Bacteroides* was kindly donated by Dr. E. Mourovitch.

C. Tryptone Trypticase Yeast Medium (TTY) (Diamond, 1968a).

Amoebae are grown monoxenically with a *Crithidia* sp. in a monophasic liquid medium.

Components

1. Basic medium -

| | |
|---|---|
| Tryptone (Difco) | 8 g |
| Trypticase (BBL) | 8 g |
| Yeast extract (BBL) | 8 g |
| Glucose | 1 g |
| L-Cysteine monochloride | 0,8 g |
| Ascorbic acid | 0,32 g |
| Sodium chloride | 4 g |
| Dipotassium hydrogen phosphate, $K_2HPO_4$ | 1,99 g |
| Potassium dihydrogen phosphate, $KH_2PO_4$ | 1,28 g |
| Distilled water | up to 1 litre |

Tryptose is dissolved in water by heating, and the other substances serially added. The pH is adjusted to 6.75 - 6.8 with 1-Normal sodium hydroxide and autoclaved at 15 lbs for 10 minutes. Store at 4℃.

2. Defibrinated rabbits blood. Store at -20℃.

3. Antibiotic mixture - 1 g streptomycin and 500 mg of ampicillin are dissolved in 10 ml distilled water and stored at -20℃. Normally used at 0.5% to give a final concentration of streptomycin 500 mcg ml and ampicillin 250 mcg ml.

4. Horse serum - heat inactivated (Wellcome No. 5).

5. Crithidia suspension - the organism is grown for 48 hours in lightly capped culture tubes half-filled with TTY medium, with 2 additional drops of rabbit blood. Tubes are incubated vertically at 25℃.

Method

The components are mixed as follows:-

| | |
|---|---|
| Basic TTY medium | 95 ml |
| Horse serum | 5 ml |
| Defibrinated rabbit blood | 0.25 ml |
| Antibiotic mixture | 0.5 ml |
| Crithidia suspension (from uppermost part of culture tube) | 0.25 ml |

and decanted into 125 x 16 mm screw-capped tubes, to within 1 cm of the tube shoulder. Cultures are sloped at 15° during incubation.

Subcultures are made by chilling the tubes in ice water, inverting several times and centrifuging lightly. The inoculum is taken from the deposit. If excess crithidial debris collects in a culture tube, this is

carefully decanted and replaced with fresh chilled medium before proceeding with the subculture. Subcultures normally made every 3rd or 4th day.

Notes:

1. The basic medium has been slightly modified to give an osmolarity of 320 milliosmoles and a pH of 6.8. This makes the medium more compatible with mammalian tissue cells.
2. The strain of Crithidia sp., derived originally from Dr. L. S. Diamond, was kindly donated by Dr. E. Meerovitch.
3. Strains of Crithidia fasciculata and Strigomonas oncopelti (both kindly donated by Dr. B. E. Brooker, Nuffield Institute, London) also supported good amoebic growth in this medium; but they were not used routinely.
4. When growing the atypical strains HUFF and LAREDO at 25°C, very small crithidial inocula must be used or profuse overgrowth of Crithidia sp. will occur. This also applies to E.invadens. These three organisms can also be grown axenically in this medium.

APPENDIX 2

ORIGIN OF PROTOZOAL STRAINS USED IN THIS WORK

The table gives the patient's name or code number, the hospital number (with prefix T for Hospital for Tropical Diseases, London and G for Seamans Hospital, Greenwich), the country in which the infection was probably acquired, the date of isolation and lastly the mode of isolation (HT denotes haematophagous trophozoites).

A. Entamoeba histolytica

Liver abscess patients

| | | | | | |
|---|---|---|---|---|---|
| DHESI | G.014378 | Asia | 30. 6.70 | Cysts | Formed stool |
| DUCKWORTH | T.54733 | India | 17. 9.70 | Cysts | Formed stool |
| WITHERS | T.54530 | Far East | 21. 9.70 | Cysts | Formed stool |
| GRIMSHAW | T.54996 | Nigeria | 28.10.70 | HT. | Liquid stool |
| RUSSELL | T.58021 | Iraq | 5. 2.71 | Cysts | Formed stool |
| INGRAM pp Dr Neal | UCH | Kenya | 8. 2.72 | Liver aspirate | |
| MOHAMMED | G.071007 | Asia | 16. 8.73. | Liver aspirate | |

Amoebic dysentery patients

| | | | | | |
|---|---|---|---|---|---|
| 106 | Indian child | Saskatchewan Canada | 1965 | Stool | |
| LIGGINS | T.54247 | Nepal | 5. 8.70 | HT. | Rectal scrape |
| ARNELL | T.58020 | Africa | 24. 1.71 | HT. | Rectal scrape |
| L177 | Adult | Saskatchewan Canada | - 3.71 | Stool | |
| ZOCKLING | T.58709 | Malaya | 2. 6.71 | HT. | Bloody stool |
| ASANTE | T.58884 | Ghana | 17. 1.72 | HT. | Rectal scrape |
| PINTO | T.55537 | India | 15. 2.72 | HT. | Rectal scrape |
| MORELLA | T.59048 | India | 27. 4.72 | HT. | Rectal scrape |
| COOPER | T.61615 | Middle East | 15. 2.73. | HT. | Liquid stool |

Mild amoebic colitis patients

| | | | | | |
|---|---|---|---|---|---|
| SWANWICK | T.56208 | Nepal | 31. 3.71 | Cysts | Formed stool |
| DAWSON | T.60697 | India | 6. 9.72 | HT | Formed stool |
| SCOOTE | T.60699 | Middle East | 30. 1.73 | HT | Formed stool |
| O'GRADY | T.63737 | India | -. 6.73 | HT | Rectal scrape |

Amoeboma patient

| | | | | | |
|---|---|---|---|---|---|
| MOOSA | G.062813 | India | 7.10.69 | HT | Liquid stool |

Symptomless carriers

| | | | | | |
|---|---|---|---|---|---|
| BRUNT | T.37257 | Nepal | 30.12.69 | Cysts | Formed stool |
| EVANS pp Dr Walters | | India | 8. 1.70 | Cysts | Formed stool |
| SCOTT | T.55596 | East Africa | 29. 9.71 | Cysts | Formed stool |
| PYO | T.56365 | Burma | 21. 4.71 | Cysts | Unformed stool |

Additional strains

DKB — Derived from original strain isolated by Drbohlav in 1925.

NIH.200 — Axenic strain, originally from Dr. L. S. Diamond, and growing in the medium described by him (Diamond, 1968b).

B. Entamoeba histolytica - atypical

HUFF — Reisolated from stabilate [illegible].12.70.

LAREDO — Reisolated from stabilate [illegible].12.70.

C. Entamoeba hartmanni

| | | | | | |
|---|---|---|---|---|---|
| AHMAD | T.42470 | Pakistan | 1. 1.71 | Cysts | Formed stool |

D. Entamoeba invadens

Strain from Department of Medical Protozoology, London School of Hygiene & Tropical Medicine.

E. Trichomonas hominis

SEGULEH T.45695 Somalia 20.10.70 Trophs. Liquid stool

F. Trichomonas vaginalis

Strain from Department of Medical Protozoology, London School of Hygiene & Tropical Medicine.

Note:

All the strains mentioned were isolated personally with the following exceptions:

The E.histolytica strains DHESI and MOOSA were isolatec by Dr. G. L. Robinson at Greenwich Hospital. Strains 106 and LL77 were isolated by Dr. R. D. P. Eaton who also donated a strain of DKB.

The atypical E.histolytica strains HUFF and LAREDO and the axenic E.histolytica strains NIH.200 were donated by Dr. R. A. Neal.

The strains of E.invadens and T.vaginalis were donated by Professor W. H. R. Lumsden.

APPENDIX 3

FLUORESCENT ANTIBODY TEST FOR E.HISTOLYTICA

Amoebic antigen was prepared from 24 or 48 hour TTY cultures. Evans Blue was used as counter stain.

Method

Decant culture medium and replace with chilled phosphate buffered saline (PBS). Wash twice in PBS. Adjust volume of suspension to suitable concentration (see below). Place one drop of suspension in each well of a teflon coated slide (12 wells per slide).

Dry at 37°C for 1 hour.

Fix in methanol for 5 minutes at room temperature.

Wash for 15 minutes in PBS using magnetic stirrer. Two changes.

Add one drop of test serum (at appropriate dilution) to each well.

Incubate in humid chamber for 40 minutes.

Wash for 15 minutes in PBS.

To each well add one drop of fluorescein conjugate: 0.1 ml fluorescein tagged antiglobulin (Wellcome Reagents) + 0.1 ml Evans Blue (1%) + 0.8 ml PBS.

Incubate in humid chamber for 30 minutes.

Wash for 15 minutes in PBS.

Blot on filter paper.

Add glycerine-saline (90% glycerine in saline) and view.

N.B. For proper reading at least 20 amoebae should be present in each well at the end of the procedure.

Fluorescence was scored thus:

++ Uniformly green trophozoites

\+ Thick green peripheral staining

± Thin green peripheral staining

\- Uniformly red or red with very thin peripheral staining.

The end point was taken to be between + and ±.

APPENDIX 4

COUNTING METHOD FOR TROPHOZOITES

An improved bright line Neubauer haemocytometer chamber with a thick cover glass was used. Amoebae were counted, at x 80 or x 320 magnification, in the 4 large corner squares (1 mm x 1 mm) of the ruled areas above and below the central moat. Thus, at each filling of the chamber 8 squares were counted, each with a volume 0.1 $mm^3$ (since the chamber was 0.1 mm deep). The count per millilitre of suspension is therefore the mean number counted per square multiplied by $10^4$.

Using suspensions of amoebae from either TTY or Robinson's cultures it was demonstrated that the mean and variance of the counts per square were nearly equal, indicating a random Poisson distribution. The standard deviation of the random error inherent in this counting system is therefore the square root of the total number of organisms counted.

For example:

| Number counted | Confidence range* | | |
|---|---|---|---|
| | 80% | 90% | 95% |
| 50 | ± 9.1 = 18.1% | ± 11.7 = 23.3% | ± 13.7 = 27.7% |
| 100 | ± 12.8 = 12.8% | ± 16.5 = 16.5% | ± 19.6 = 19.6% |
| 200 | ± 18.1 = 9.1% | ± 23.3 = 11.7% | ± 27.7 = 13.9% |
| 400 | ± 25.6 = 6.4% | ± 33.0 = 8.3% | ± 39.2 = 9.8% |

*Standard normal deviate x standard deviation

Whenever possible in this work, in particular for the in vitro studies, at least 100 amoebae were counted and preferably 200 to 400. Sometimes, however, when there were few amoebae or only small volumes of suspension, this was not possible. Suspensions in TTY medium were normally adjusted to give the required numbers in 2 or 3 fillings of the chamber. Suspensions in Robinson medium were counted after thorough mixing of the liquid overlay.

When two counts with Poisson distributions are being compared the significance of the difference is given by:

$$d = \frac{x_1 - x_2}{x_1 + x_2} \quad \text{for one experiment and by} \quad d = \frac{x_1 - x_2}{\frac{x_1}{n} + \frac{x_2}{n}}$$

when the experiment is repeated n times (d is the standard normal deviate). Three examples will illustrate the implication of these expressions:

| Counts | One experiment | Two experiments (n = 2) |
|---|---|---|
| $x_1 = 100$, $x_2 = 125$ (22% difference) | d = 1.67 (p = 0.1) | d = 2.36 (p = 0.02) |
| $x_1 = 200$, $x_2 = 235$ (16.1% difference) | d = 1.67 (p = 0.1) | d = 2.37 (p = 0.02) |
| $x_1 = 200$, $x_2 = 224$ (11.3% difference) | d = 1.16 (p = 0.25) | d = 1.61 (p = 0.1) |

APPENDIX 5

TISSUE CULTURE METHODS

The polyploid cell line, rabbit kidney RK13, and also the HeLa cell line were maintained in medium 199. The basic medium is made up as follows:-

| | |
|---|---|
| Deionised water | 80 ml |
| Foetal bovine serum | 5 ml |
| 199 stock medium (x 10 concentrated) | 10 ml |
| Sodium bicarbonate 5.6% w/v | 1.35 ml |
| Streptomycin, ampicillin mixture (see Appendix IC) | 1 ml |
| Amphotericin B (E.R. Squibb) Diluted to 5 mg ml | 0.2 ml |

Trypsin-versene mixture - 2 ml volumes of trypsin were added to 18 ml of sterile 0.02% versene in phosphate buffered saline.

Cultures were maintained in 50 ml, 100 ml or 200 ml flat medicine bottles and subcultured every 7th day. To subculture, the medium is decanted and replaced by 20 ml Trypsin-versene; after 5 minutes incubation the trypsin-versene is removed and further incubation is continued until cells just begin to peel off the glass, usually about 5 minutes. The trypsin-versene is replaced with 10 ml of medium, and by firm tapping against the hand the monolayer is removed.

Further medium is added and the suspension gently shaken. The final suspension is then dispensed as required.

For experimental work the suspension was dispensed, in 1.5 ml amounts, into 5 ml Carrel flasks or 30 ml flat bottomed plastic bottles (Steralin) with a basal cover glass; in the latter case a further 9 ml of 199 medium was added.

Medium, versene mixture and glassware were warmed to 37° before use.

Note:

The cell lines RK13 and HeLa were kindly donated by Dr. D. Bidwell of the Nuffield Institute, London.

APPENDIX 8

DIETS USED IN PART 14 TO FEED RATS

Percentage composition of diets in grammes and their protein value (NDpCal%):

| | DIET A | DIET B | DIET C | DIET D | |
|---|---|---|---|---|---|
| Rolled oats (Quaker Oats Ltd) | 45 | 50 | 55 | wheat | 30 |
| Casein | 22 | 4 | – | barley | 25 |
| Dripping (beef) | 25 | 25 | 3 | meat meal | 8 |
| Maize starch | 3 | 21 | 30 | fish meal | 7 |
| Salt mixture* | 4 | 4 | 4 | grass meal | 5 |
| Mixture of B vitamins* | 1 | 1 | 1 | dried yeast | 5 |
| Fat soluble vitamins* | + | + | + | molasses | 9 |
| | | | | salt mixture | 1 |
| NDpCal% | 9.6 | 5.8 | 4.5 | 8-9 | |
| Presentation | Powder | Powder | Powder | Pellet | |
| Interpretation of diet | 'Balanced' | Low protein | Low protein high calorie | Standard | |

* See Stewart and Sheppard, 1971.