A FINE STRUCTURAL AND CYTOCHEMICAL INVESTIGATION INTO PATHOGENICITY OF ENTAMOEBA HISTOLYTICA STRAINS USING CELL LINE MONOLAYERS

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VOLUME I

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ABSTRACT

Past literature on the pathogenesis of amoebiasis is reviewed. Cytochemical and electron microscopy techniques are carried out to investigate the potential mechanisms of initial cell damage through the interaction between cultivated pathogenic strains of Entamoeba histolytica and cell line monolayers.

Experiments demonstrated :

1) Contact between the amoeba and cultured cells is essential for pathogenesis to occur.

2) No evidence to suggest that the amoebic enzyme-containing organelles or surface lysosomes are responsible for cell damage.

3) No evidence of any amoebic cytotoxic enzyme involvement in cell damage.

4) A toxin, probably plasmalemma associated, that appears to act on the plasma membrane of the contacted cell, leading to the breakdown of selective permeability. Cell lysis results from osmotic effect. The pathological changes in injured cells leading to cell death, and the engulfment of the injured cell by the amoeba by the process of phagocytosis are shown.

5) That cell lysosomes play no part in the early development of cell injury and there is a delay in changes in the distribution of the lysosomal hydrolases after the addition of trophozoites.

6) That cell death is not the immediate consequence of viral genome transfer into the host cell, though the presence of viral genome in the amoeba may have some connection with pathogenicity.

The usefulness of cell-line monolayers in evaluating the virulence of cultivated strains of Entamoeba histolytica is discussed.

Further work to define the chemical nature of the amoebic plasmalemma is necessary and also to isolate the toxic factor and to determine its role in pathogenicity.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES — VOLUME I

FIGURES — VOLUME II

*a LM Light microscope
EM Electron microscope
SEM Scanning electron microscope

		Page
39, 40	Amoebic surface (SEM)	15
41	Amoebic pseudopodium (SEM)	16
42	Uroid (SEM)	"
43a, b	Filopodia (SEM)	"
44a, b, c	Filopodia (SEM)	17
45	Rhesus Monkey Brain culture cell	18
46	Higher magnification of Fig. 45	"
47a, b	Section through contact zone between E. histolytica and Rhesus Brain cell	19
48	CV-1 culture cell	"
49	CV-1 cell showing the various cell organelles	20
50	Contact zone between E. histolytica and CV-1 cell	"
51a, b	Portion of cytoplasm of an affected CV-1 cell in contact with E. histolytica	21
52, 53	Later stage of interaction between CV-1 cells and E. histolytica	22
54	Initial stage of contact between an amoeba and CV-1 cell	23
55	Later stage of cell injury (CV-1 cell)	"
56-62	Stages of amoebic phagocytosis	24-27
63	Uroid of E. histolytica	27
64a	Trophozoite in cell-denuded areas of a CV-1 cell monolayer	28
64b	Uroid of E. histolytica	"
65	Trophozoite in contact with a CV-1 cell	29
66 68	Sections through healthy BD-VI culture cells	29, 30
69	Portion of cytoplasm of BD-VI cell in contact with E. histolytica	31
70	Initial stage of cell injury (BD-VI cell)	"
71	Later stage of cell injury in BD-VI cell in contact with an amoeba	32
72	Contact zone between an amoeba and BD-VI cell	"
73	Prior to lysis of cell in contact with E. histolytica	33
74	An affected BD-VI cell showing a breakdown in the cytoplasmic membrane	"
75	Section showing two lysed cells (BD-VI) near the amoeba	"
76a, b	An amoeba in contact with two BD-VI cells	34
76c-76e	Stages of amoebic phagocytosis	35, 36
76b	An affected BD-VI cell	37
78a	Phagocytotic bulb	38
79	A section of affected and non-affected BD-VI cells in a lesion	"
80	RF13 culture cells. Dorsal view (SEM)	39
81	Cell surface of a RF13 cell (SEM)	"
82, 83	Amoeba in contact with RF13 cells (SEM)	"
84a, b	Later-l view of amoeba in contact with the RF13 cells (SEM)	40
85	Later stage of cellular injury (SEM)	"
86a, b	Affected RF13-cells (SEM)	40, 41
87a, b	Burrowing activity of E. histolytica trophozoite (SEM)	42
88a	Amoebic emerald threads (SEM)	"

B. INTRODUCTION

B.1 AMOEBIASIS IN GENERAL

In 1875 Fedor Alekseandrovich Lösch, working in St. Petersburg, identified in the stool of a fellow countryman suffering from dysentery a protozoon, an amoeba, and demonstrated its pathogenic properties by feeding dogs with the patient's faeces. It has since been shown that at least 6 species of amoebae are found in human faeces but one, the Entamoeba histolytica. (Schaudinn, 1903) is the pathogen responsible for most cases of non-bacillary dysentery in man.

The disease amoebiasis caused by E. histolytica is cosmopolitan in distribution and it is estimated that about 10% of the world's population is infected by E. histolytica (WHO, 1969). The incidence is maximal in tropical regions but the determining factors are socioeconomic and sanitary rather than climatic. (Miller, 1974). The distribution of the disease in the United States of America, for example, has been shown to be in those large rural areas where unsatisfactory sanitary conditions exist (Juniper, 1971) and for the same reason another investigation of an Indian reserve in sub-arctic Saskatchewan found that 38% of the population harboured the parasite, 8% suffering from amoebiasis (Knight et al., 1973).

The parasite is basically a gut lumen dweller, the amoebae inhabiting the lumen of the colon following ingestion of infective cysts. The amoebae within the cysts excyst and become trophozoites which, feeding on cell debris and possibly on bacteria, multiply within the caecum and produce further cysts. These serve to propagate infection in new hosts and are able to survive for considerable periods outside the body. Infestation may last for

several years without damage to the wall of the gut and in such cases the amoebae simply live as a commensal without producing dysenteric symptoms (Dobell, 1928; 1931). Under certain conditions, however, the lumen-dwelling amoebae become tissue invaders and attack the intestinal wall feeding on erythryocytes and tissue elements giving rise to mucosal ulceration which may be characterised clinically by abdominal pain, diarrhoea and the passing of blood streaked mucus in the stool. Severe disease with superimposed bacterial infection of the ulcerated areas results in loss in weight, low grade fever, and leucocytosis (Juniper, 1971) while anaemia is a concomitant both of chronic infection and of blood loss.

Once the colonic mucosal barrier has been transgressed, trophozoites may gain access to the portal circulation and may be carried to the liver where they multiply, causing tissue necrosis resulting in the formation of hepatic abscess. Some such abscesses become very large and potentially dangerous (Knight, 1974) as they have been known to rupture into pericardium, lung with the formation of broncho-hepatic fistula, or lung abscess, gut and peritoneal cavity all occur.

Amoebiasis is therefore a disease due to a parasite with a relatively simple life cycle which depends on its human host for survival of its species. The biochemical basis of pathogenicity of this relatively simple protozoon has remained unexplained despite nearly 100 years of research but what is certain, from the host's point of view, is that potential morbidity can be expected from the moment E. histolytica invades the colonic mucosa. This thesis records a histochemical and fine structural study of cellular invasion by the parasite.

B.2 PATHOGENESIS OF AMOEBIASIS

B.2.1. Commensal - pathogen argument

Since Lösch's discovery of Entamoeba, numerous studies have been made to explain the unusual behaviour of the parasite, E. histolytica. The concept of living as a gut commensal or as a pathogen was first put forward by Kuenen and Swellengrebel (1913), who also stated that in the majority of hosts harbouring this parasite, the infection was symptomless and the host a carrier. This dual nature of the parasitic way of life did not initially gain general acceptance, and it was believed that asymptomatic carriers did not exist. E. histolytica was regarded as a totally pathogenic organism and it was thought that lesions, both small and large were present in all carriers. Dobell, the principal critic of the commensal mode of life did not believe that trophozoites could live commensally feeding on bacteria. He suggested that the presence of bacteria within the amoebae was the result of invasion of microorganisms into degenerating or dead protozoa (Dobell, 1919). Dobell however, in 1928, changed his mind when, in dealing with macaque monkey which may be naturally infected, he found that the trophozoites neither invaded nor fed upon the tissue of the large intestine. In a review of the evidence for and against a commensal phase in the life cycle, Hoare (1952) stressed that a case could be made for recognising a commensal phase. His conclusion was based on the observations made on the food habits of E. histolytica in chronic cases and asymptomatic carriers. Contrary views were also expressed notably by Elmassian (1909) who put forward the idea that the amoeba lives as a pure commensal in the intestine of the host without invading and causing disease. His conclusion was supported by Brumpt (1925),

who suggested that the amoeba found as a commensal belonged to a new species, E. dispar. The whole issue was made more confused when it was postulated that there were races of E. histolytica. Wenyon and O'Connor (1917) stated that the species, E. histolytica consisted of different "races" according to the cyst-size. The division of E. histolytica into "races" was also supported by Dobell (1919), who concluded that there were more than 5 demonstrable races. Brumpt (1925, 1949) added to the confusion by sub-grouping amoebae producing 4 nuclei-cysts into 2 morphologically indistinguishable species, E. dysenteriae and E. dispar on the basis of pathogenesis and geographical distribution. He indicated that E. dispar, a commensal, lived in the lumen of the gut without ever producing lesions, and had a world-wide distribution while E. dysenteriae was pathogenic but it could also live as a commensal in asymptomatic carriers.

It was accepted that in E. histolytica infections, there was a great variation in the size of the trophozoites, especially between those which ingest erythrocytes and the non-invasive luminal dwellers. The former measured 20-40 μm and were regarded as magna forms, whereas the latter were 10-15 μm and were considered minuta forms. Cysts from the stools of dysenteric patients, usually measured 11-16 μm in diameter. Cysts of a smaller size, 6-8 μm in diameter were, however, described by Von Prowasek (1912), who named the species E. hartmanni, but its morphology was strikingly similar to that of E. histolytica. The discrepancy in cyst-size was also noticed by other authors, especially Sapero et al. (1942), who pointed out that the trophozoites from the smaller cysts had not been observed to ingest red blood cells and thus questioned pathogenicity. Whether

size-variation was either an environmental or a genetic feature was investigated thoroughly by Freedman and Eladon-Dew (1959). By growing the 2 strains together in the same environment, in the same culture, they showed a size-distribution curve with 2 peaks. The results implied genetic differences in the two strains. Burrows (1959) in a morphological study of amoebae, reached the same conclusion namely that the small race trophozoites, although morphologically similar, were different from the large race amoebae on the basis of size-distribution of both cyst and of trophozoite. Both Burrows, and, Freedman and Eladon-Dew suggested that the small amoebae should be regarded as a separate species, E. hartmanni. The problem was complicated further by the description of amoebic strains - Laredo, Huff, 401, JA A 40 - with quadrinucleate cysts that resembled E. histolytica morphologically and appeared to be extremely adaptable to room temperature as well as to body temperature, and to changes in osmotic pressure (Richards et al., 1966). These E. histolytica-like amoebae have been shown to differ from the true E. histolytica group both biochemically and biologically (Goldman, 1969). Neal and Johnson (1968) working with the same 5 E. histolytica-like strains, all of which were brought to their laboratory by Goldman, tested for virulence by inoculation into the caecum of young rats. The results revealed that all strains failed to produce caecal ulceration indicating that they were not pathogenic. Such tests for virulence were also used to answer questions such as whether totally non-pathogenic strains of E. histolytica exist in asymptomatic carriers: or are all strains of E. histolytica pathogenic, capable of invading the host under certain conditions. Neal (1957) showed that there was a difference in virulence between amoebae

isolated from dysenteric patients, and those isolated from carriers. He concluded that E. histolytica was divided into 2 groups, one of which was avirulent and a commensal, while the other was virulent and pathogenic. Later Neal (1971), changing his mind, no longer believed in division into 2 groups on epidemiological grounds and also from laboratory evidence indicating that E. histolytica could change from a virulent to an avirulent state and vice versa. Bos and Hage also tested the virulence of strains both from dysenteric patients and carriers by experimentally infecting the liver of hamsters and strongly supported the concept that all strains of E. histolytica have pathogenic potential (Bos, 1973; Bos and Hage, 1975). They concluded that strictly commensal isolates of E. histolytica do not exist.

B.2.2. Host-parasite equilibrium - Some influencing factors

The complicated nature of amoeba-man relationship has been described succinctly by Elsdon-Dew (1964) as "from the parasite point of view, it and its host must survive together and it is rarely of value to a parasite to destroy its host. It is the environment which selects the equilibrated host-parasite relationship and a change of environment may well upset the equilibrium". As E. histolytica is totally dependent on a human host for the survival of its species, an avirulent phase must be regarded as being normal, an intact host increasing the chance of amoeba survival. As a corollary to Elsdon-Dew's concept environmental change leading to amoebic invasion of the intestinal mucosa will disrupt the perfect host-parasite relationship. Neal (1971) reaffirmed that amoebae are normally avirulent in the intestinal lumen, and that in response to environmental change, they are transformed into virulent organisms.

Many factors have been claimed from time to time to affect the parasite's intestinal environment. They include pH, oxidation-reduction potential, steroids, cholesterol, bacterial flora, other parasites and the condition of the intestinal wall (Bos, 1973). Diet, genetic factors involving the host and psychosomatic illness (Bird, 1961) have also been considered. Attention has been given to the study of bacteria, diet, oxidation-reduction potential and the part played by the parasite itself.

B.2.2.a Bacteria

To determine the effect of the combined administration of E. histolytica and bacteria, bacteria-free cultivated trophozoites have been inoculated into experimental animals together with bacteria. Germ-free animals were used reared under germ-free conditions (Phillips et al., 1955). Germ-free and conventional guinea pigs were inoculated intracaecally with E. histolytica cultures. The results showed that intestinal amoebiasis was not producible in the germ-free guinea pigs but only in those with a normal bacteria flora. Germ-free animals monocontaminated with either Escherichia coli or Aerobacter aerogenes developed acute amoebic ulcers. When the caecal epithelium was traumatized by scraping the mucosa or by making numerous puncture wounds with a hypodermic needle only 4 of 12 germ-free animals developed small amoebic lesions (Phillips et al., 1958; Wright et al., 1958).

In a review on experiments conducted on germ-free animals over 6 years, Phillips and Wolfe (1959) concluded that bacteria contributed most to the development of amoebic disease by providing factors that are essential in establishing amoebic infection in the intestinal lumen.

Whether different species of bacteria have any effect on

the pathology of amoebiasis was investigated by Phillips and Gorstein (1966) using monocontaminated germ-free guinea pigs. Amoebic lesions occurred in degrees varying with different species of bacteria showing that the type of bacteria affects directly the severity and pathology of amoebiasis. The work of Phillips and Gorstein was criticized by Wittner and Rosenbaum (1970), who stressed that Phillips and Gorstein were dealing with a primary bacterial enteritis, as the bacteria which they selected might have been pathogens. Wittner and Rosenbaum using non-pathogenic bacteria reached, however, the same conclusion but they went further in attempting to isolate the bacterial factor that might be responsible for enhancing the virulence of the amoeba. They suggested that as the amoebae will only exert their virulence after direct contact with living bacteria, an episomic-like factor, which is bacterial cell-bound, enters the amoeba possibly by phagocytosis. Although there is strong evidence that E. histolytica is only able to cause pathology and produce disease when it is associated with bacteria, the opposing view has been put forward (Neal and Vincent, 1956). Their experiments showed that a loss of virulence in strains of E. histolytica could be restored by passage through the liver, which is bacteriologically sterile. Abscesses were produced in the liver. The authors concluded that the bacteria played only a modifying role in parasitic virulence.

B.2.2.b The effect of diet

The importance of diet as an inducing factor in amoebic penetration was stressed by Taylor et al. (1952), who tested the effect of 2 different compounded diets on experimental E. histolytica infection in 2 hosts, the rat and the guinea pig. When the guinea pigs were fed on the modified guinea pig diet, almost

all the animals died of amoebiasis. When the diet was changed to rat breeder diet, the mortality rate was lowered by 20%.

Histological alterations in the caecal mucosa due to diet changes have been observed by Lynch (1957). He noticed that when guinea pigs were fed on a synthetic diet, a "severe, fulminating type of infection" developed whereas guinea pigs fed on a normal diet did not produce the same degree of infection. He ascribed these differences as being due to alterations in the caecal mucosa. The alterations consisted of a thinning of mucosa and dilatation of the glandular area with vacuoles indicating secretory retention. He postulated that such conditioning of the caecal wall is sufficient to permit invasion by the "symbiotic amoebae and bacteria".

Excessive mucus production could also enhance susceptibility to infection was shown by Thompson (1971). He demonstrated that a salmon diet enhances the severity of intestinal amoebiasis in dogs by stimulating mucus production.

B.2.2.c Oxidation-reduction (O-R) potential

E. histolytica trophozoites are known to be influenced by changes in the oxidation-reduction potential especially in the presence of bacteria. Jahn (1934) found that in order to achieve good growth of intestinal protozoa in sterile peptone broth, the medium should contain bacteria to lower the potential from +200 mv to -200 mv or less; the O-R value of the rat caecum. The presence of bacteria as an important factor in lowering the O-R potential to promote good growth of trophozoites was also verified by Jacobs (1941). Furthermore, Jacobs correlated the longevity of amoeba cultures with the maintenance of reducing potentials. Recently Bos (1973) observed that in monoxenic cultures, which are maintained at high redox potential, the amoebae multiply at a higher rate than

amoebae in bacteria-associated cultures with lower redox potential. Phillips et al. (1958) working on germ-free guinea pigs observed that there is a difference, which was quite substantial, in the O-R potential of the caecum between the germ-free (-90.1 mv) and the conventional guinea pigs (-376.2 mv). Since amoebic infection failed to develop in the germ-free animals, the authors investigated the hypothesis that reduction in O-R potential is a contributory factor in altering the environment of the intestinal mucosa. The potential was lowered by applying chemical reducing agents, Sodium thioglycollate, and L-cysteine hydrochloride, and in such cases local amoebic lesions developed only at sites of inoculation and failed to spread within the gut wall.

Eaton and Meerovitch (1973), following a number of in vivo and in vitro experiments, suggested that E. histolytica was closely dependent on the precise O-R potential of its environment. In vivo experiments, using caecally infected rats illustrated that E. histolytica failed to invade the caecum when there was a rise in the inspired air pO_2. The authors believed that the degree of invasiveness of different strains of E. histolytica depended on the adaptation of these strains to an existence at varying O-R potential.

2.2.2.4 Steroid (Cholesterol)

Virulence was found to increase by adding cholesterol to cultures of E. histolytica or by feeding rats and guinea pigs on this steroid, but the literature on the effect of cholesterol is conflicting, as stressed by Knight et al. (1973).

Singh (1959) tested the effect of cholesterol on strains of E. histolytica, which were initially non-invasive to rats; the strains being isolated from carriers. Ulceration, which was not

produced in the rats' caeca in the absence of cholesterol, developed. Neal and Vincent (1960) repeated the experiments of Singh (1959) using the same methods and strains of E. histolytica. The results showed no rise in infectivity or in invasiveness and no increase in growth rate or size of amoebae in vitro was observed. Recently Singh, Srivastava and Dutta (1971) published details of their work on the virulence of three non-invasive strains of E. histolytica. They found that the acquired virulence of these strains could be maintained by feeding amoebae with cholesterol.

B.2.2.c Paragitic virulence

Several authors have pointed out that the amoeba itself has a part to play in host-parasite equilibrium (Phillips and Bartgis, 1954; Phillips, 1973; Dee, 1973; Dee and Hagre, 1975; Thompson, 1971; Wittner and Rosenbaum, 1970; Phillips et al., 1972; Tanimoto, 1971; Diamond et al., 1973). They demonstrated that continued cultivation, monoxenically and axenically can lead to a loss of virulence and infectivity in laboratory animals. That virulence may be restored by reassociation with bacteria or by passage through the hamster liver or intestine has also been demonstrated (Thompson, 1971; Wittner and Rosenbaum, 1970).

The loss of virulence of amoebae was not observed until strains were grown and maintained in bacterial-free cultures. Some workers noticed a loss of virulence when the amoebae were transferred from a bacteria-associated culture to a monoxenic culture with T. cruzi as an associate (Lattermoser and Phillips, 1952). Phillips and Bartgis (1954) reported that the virulence was restored by returning the amoebae to a culture with selected bacteria, thus again indicating the possible role of bacteria in providing a stimulus for pathogenicity.

Amoeba grown in monoxenic cultures with Crithidia sp.

as an associate and axenic cultures behave in the same way (Bos, 1973; Wittner and Rosenbaum, 1970). Their virulence is restored on direct contact with bacteria. A contrary finding has been reported when a total loss of virulence occurred in axenic strains of E. histolytica (Bos and Hage, 1975; Phillips, 1973).

Some strains have been tested for virulence with and without concomitant bacteria (i.e. HK-9 and 200 : NIH) by more than one author (Table 1). The results must be interpreted bearing in mind 2 factors, firstly, selection of laboratory animals and secondly the number of amoebae in an inoculum and the volume of the suspension used.

1) As far as selection of laboratory animals is concerned, the preferred model would seem to depend upon the objectives of the experiment. Thompson (1971) has investigated the problem of animal selection and concluded that animals particularly useful for sensitive assay of virulence are kittens infected intracaecally or young hamsters infected intrahepatically. Young animals are preferred to old as the former are more susceptible to infection than the latter. The authors mentioned in Table 1, however, have not all followed Thompson's recommendation. Host species strains have also some relevance as far as amoebic virulence is concerned. The Wistar strain of rats produced more consistent results than the Sharman strain (Healy and Gleason, 1966).

2) Two routes of inoculation are commonly used; either direct inoculation of amoebae into the liver or into the caecum. Many workers prefer the former route so that the barrier of the intestinal wall and other intestinal factors are avoided (Bos, 1973). Phillips et al (1972) could not produce caecal abscesses in either germ-free rats or guinea pigs with 2.5×10^5 - 4×10^6 amoebae

TABLE I ENTAMOEBA HISTOLYTICA AXENIC STRAINS TESTED FOR VIRULENCE

Authors*1	Strains of amoebae	Animals as host	Site of inoculation	Number of amoebae in inoculum	Volume of inoculum	Production of lesions (without bacterial associates)	Production of lesions (with bacterial associates)
Vittner and Rosenbaum (1970)	200:NIH	weanling hamsters guinea pigs	liver : intrahepatic or via portal system	$5x10^4$ - $2.5x10^5$	0.1ml	-	+
Tandeta et al.,*2 (1971)	HK-9	hamster	liver : intrahepatic	$1.2x10^7$	?	+	ND
Phillips et al., (1972)	a) 200:NIH b) HK-9	Hartley strain guinea pigs Wistar strain rats	Caecum	$2.5x10^5$ - $4x10^6$	0.5-0.75ml	a) - b) -	ND ND
Phillips (1973)	200:NIH	Hartley strain guinea pigs	Caecum	$3-4x10^5$	1.0ml	(Refer to Phillips et al., 1972)	-
Diamond et al., (1973)	HK-9	Syrian golden hamster Jirds	liver : intrahepatic	$1.6x10^5$ - $1.5x10^7$	0.2-0.25ml	+	ND
Rex and Zaga (1975)	HK-9	Hamster	liver : subcapsular injection	10^7	0.05ml	-	-

*1 Authors have also quoted other strains being used in the experiments, but in this table only HK-9 and 200:NIH strains are mentioned

*2 The results were compared to the lesions produced in hamsters inoculated with monoxenic-bacteria cultures. The lesions produced by axenized amoebae were slower to develop and more circumscribed

ND = not done + = lesion developed - = no lesion

with an HK-9 axenic strain of amoebae, but in their later experiments, amoebic lesions were produced by a dose increase to 4×10^6 to 1.5×10^7, and by inoculating the amoebae into the liver intrahepatically rather than into the caecum (Diamond et al., 1973). Such findings were contradicted by Bos and Hage (1975). They demonstrated a total loss of virulence in the same strain, HK-9 by inoculating the same number of amoebae as Diamond et al., (1973) by the hepatic route. No necrotic abscesses could be found. They emphasized, however, that they used a much smaller inoculum (0.05 ml instead of 0.2 - 0.25 ml) and did not damage the liver parenchyma during inoculation (subcapsular instead of intrahepatic injection).

It would appear that informed opinion lacks unanimity in this matter but as pointed out by Neal (1971), virulence is an unstable characteristic of E. histolytica and prolonged cultivation in vitro of virulent strains of the organism results sooner or later in a gradual loss of invasiveness and infectivity both in rats and guinea pigs. This progressive loss of virulence has been studied extensively by Diamond et al (1974), working on 9 strains of axenically grown E. histolytica. Virulence was found by inoculating the amoebae into hamster's livers, to vary from zero to 100% invasiveness. The variation was not related to length of individual strain culture time and strains cultured for the same length of time did not prove to be equally virulent. The same workers also reported a strain grown for a year, which was avirulent whereas another cultivated for 12 years produced lesions in 45% of inoculated animals.

Many explanations have been offered to explain why cultures lose their ability to produce amoebiasis. It has been

suggested that loss of virulence is related to the loss of the organism's ability to encyst (Phillips and Bartgis, 1974; Phillips, 1973). These authors regarded the encystment of the amoebae as an essential part of the life cycle. They tried to induce encystment by transferring the amoebae from axenic cultures to bacterial-associated media. This resulted in vigorous growth of amoebae but cysts did not appear. Bos and Hage (1975) regarded Phillips and Bartgis' evidence as being inconclusive. They used Crithidia sp. to maintain strains of amoebae over a period of years without the development of cysts, and the virulence of his strains did not diminish with time. Wittner and Rosenbaum (1970) postulated the existence of an episomal-like factor associated with living bacteria which was transferred to the amoebae through phagocytosis. The factor once acquired by the amoeba could then be gradually lost during subsequent transfer through bacterial-free subcultures.

Another hypothesis (Bos and Hage, 1975) suggests that restoration of virulence by reassociation with bacteria or by passage through the liver or by direct intestinal passage can be explained by the existence of both virulent and nonvirulent individuals within the population of one strain. The environment would then play a part in influencing the selection of individuals. In a bacteria-free environment, selection might result in a very low incidence of virulent individuals, while passage through the liver and the intestine or bacterial cultures might favour the selection of virulent forms. Individual differences within a population were also noticed by Diamond et al., (1974) working with Marino. They, investigating the virulence of cloned cultures derived from 3 axenic strains, found that cloned cultures of the

some age varied in virulence. Some clones were completely avirulent.

Perhaps, the most promising hypothesis is that an episomal-like material exists which acts as a virulence factor (Wittner and Rosenbaum, 1970). Whether it is a viral associate or not the authors do not specify. The carrying of a viral agent within the amoebae has been postulated in 1961, when Bird in his thesis on "Studies on Amoebiasis including the morphology and behaviour of certain Parasitic amoebae of man and Animals" suggested that "the carrying of virus material by the amoebae or the co-existence of a localised mucosal reaction to the concurrent viral infection, could account for all the observations connected with the virulence of amoebae and does therefore require investigation." To examine such a concept may be difficult, but Khadem-Dav (1964) quoted an adequate parallel in C. diptheriae where a viral infection of the bacterium induces a genetic change affecting the virulence of the organism. Similarly a strain of Trichomonas gallinae, that had become avirulent after a long period of axenic cultivation, became virulent after being treated with a cell-free homogenate of a virulent strain (Honigberg and Read, 1960). The authors suggested that virulence is a genetically controlled character of strains of T. gallinae, since the addition of RNase to the homogenate cell mixture blocked the transformation.

Virus-like particles within Entamoeba histolytica were first described by Miller and Swartzwelder (1960) who observed a small array of particles about 40 nm in diameter within the peri-nuclear cytoplasm of a trophozoite of E. histolytica cultivated in the presence of bacteria. Bird (1961) described 2 distinct types of particle in trophozoites invading a biopsy specimen from an

established case of amoebic dysentery. One type was a corrugated cylindrical profile approximately 150 nm long and 50 nm in diameter. Further investigation revealed a resemblance between such particles and rhabdoviruses isolated from plants, arthropods and vertebrates including man (Bird, McCaul and Knight, 1974; Bird and McCaul, 1976). The role of amoeba-virus relationship in pathogenicity has interested several workers (Diamond et al., 1972, 1974; Hruska et al., 1972, 1973a, 1973b, 1974; Mattern et al., 1972, 1974; Mattern and Diamond, 1976). Two morphologically different viruses, one filamentous developing in the nucleus and the other polyhedral developing in the cytoplasm in 9 axenically cultivated strains of Entamoeba histolytica have been identified. The former has been found in all strains examined, the latter in only one strain (Diamond et al., 1972; Mattern et al., 1972; Hruska et al., 1973a, 1973b). Such viruses are of interest since it was found that each amoebal strain is resistant to its indigenous virus, and also the viruses from each strain will infect and lyse amoebae of some other strains (Hruska et al., 1972). The same authors also observed that HK-9 amoebae infected with HB-301 polyhedral virus showed a 3 to 5-fold post-infection increase in DNA synthesis (Hruska et al., 1973a, b; 1974). Using DNA hybridization studies, they were able to demonstrate that such newly synthesized DNA in the HK-9 amoebae and the DNA from the infected amoebae were primarily viral in nature. It was also shown by studying with the metabolic inhibitors of DNA synthesis that both viral types are composed of DNA and their DNA is double stranded. According to Mattern et al., (1974), the icosahedral or polyhedral amoebal virus presents an arrangement of its nucleic acid which is unique among viruses and clearly distinguishes this amoebal virus from the bacterial and

algal viruses which it otherwise closely resembles. The possible relationship of filamentous and icosahedral viruses to virulence of Entamoeba histolytica, however, is uncertain as attempts to produce cytopathology in chick and mouse embryo cells and human fibroblast cultures with these agents have failed (Diamond et al., 1972; Mruska et al., 1972). The same workers also failed to induce pathology in suckling and adult mice inoculated with filamentous and polyhedral viruses using intracerebral, intraperitoneal and subcutaneous routes. It would seem also that these viruses are avirulent for mice and there is as yet no evidence of immunological responses in man, either in those infected with E. histolytica or in laboratory workers potentially exposed to such viruses (Mattern and Diamond, 1976). It is also uncertain whether the rhabdoviruses of E. histolytica play a role in pathogenesis of amoebiasis as such viruses are not pathogenic to suckling mice and have not as yet been induced to replicate in Xenopus and BHK-21 cell lines (Bird and McCaul, 1976).

B.2.3 Mechanism of invasion

It will have been noted that in order to become pathogenic the amoeba must first invade the tissues of its host. The mechanism of such invasion must now be considered. Many theories have been put forward to explain such a process. One suggestion is that the amoebae secrete a powerful cytolytic enzyme (Dobell, 1919; Dobell and O'Connor, 1921). The theory that a lytic toxin was produced by the amoebae (Councilman and Lafleur, 1891) was firmly rejected by Westphal (1939) who considered that E. histolytica was capable of both intra- and extra-cellular digestion and its pathogenic mechanism was based on the production of proteolytic enzymes. Westphal's theory was further extended by Villarejas (1962) who proposed that

the mechanism was the result of decomposition of amoebae within the tissues. This hypothesis stated that amoebae disintegrate as a result of exhaustion of food supply. In this case, healthy cells within the host's tissue are acted upon by the endocellular enzymes released by the amoebae during penetration. It is suggested that since amoebae are not able to feed on intact cells, death will occur leading to their disintegration, the products of which lyse the surrounding host's cells.

As amoebae can be successfully grown in various culture media, attempts have been made to gain understanding of pathogenicity by directing interest towards the study of morphology and the biochemistry of trophozoites of E. histolytica in isolation from its host (Kagan, 1974). The biochemical components within the amoebae have been investigated to determine whether lytic enzymes are responsible for cell destruction (Neal, 1960; Jarumilinta and Maegraith, 1969; DaLamater et al., 1954). Neal (1960) failed to detect hyaluronidase, collagenase and lecithinase. It was found impossible to distinguish clearly between pathogenic and non-pathogenic strains of E. histolytica by comparing proteolytic enzyme patterns as in each case trypsin, pepsin, gelatinase, caseinase, exopeptidases and dipeptidases and glutaminase are present. Carboxypeptidase, however, is found only in avirulent strains (Jarumilinta and Maegraith, 1969). Recently, Meerovitch (1975) compared the enzyme pattern of acid phosphohydrolase in the plasma membrane fractions of axenic E. histolytica and axenic E. invadens with other eukaryotes, including protozoa. The plasma membrane fraction in the case of E. invadens was 50 to 60 times and of E. histolytica 100 times as high as that from mammalian liver cell plasma membranes. It is still unclear whether such

results have any significance in the context of pathogenicity.

The ultrastructure of E. histolytica grown monoxenically with a bacterial, or crithidial associate (Rosenbaum and Wittner, 1970; Feria-Velasco and Trevino, 1972) grown polyxenically with a mixed bacterial flora (Lowe and Maegraith, 1970c), grown axenically (Lowe and Maegraith, 1970a; Rosenbaum and Wittner, 1970; Feria-Velasco and Trevino, 1972) or obtained direct from patients with amoebic colitis (El-Hashimi and Pittman, 1970), or hamsters with liver abscess (Feria-Velasco and Trevino, 1972; Lowe and Maegraith 1970b) has been compared and found to be similar in most respects. Exceptionally, trophozoites obtained from the human colon were found to have a "fuzzy" coat and this was not seen in those grown in vitro (El-Hashimi and Pittman, 1970). Again the significance of such a finding is unclear.

It will be seen that morphological and biochemical studies in isolation have so far failed to elucidate the mechanism of amoebic invasion. This was succinctly expressed by Jarumilinta and Maegraith (1969) who concluded that "a study of the parasite alone would not be adequate to distinguish pathogenicity, and for the present, the pathogenicity must continue to be established only in terms of the host-parasite relationship". Studies on such a relationship using experimentally produced amoebiasis in laboratory animals and also examination of samples of human colonic exudate containing trophozoites of E. histolytica have been non-contributory as far as elucidating the penetration problem. Fletcher et al. (1962) working on the fine structure of E. histolytica, postulated that lytic enzymes are activated in or at the surface of the amoebic vacuoles under certain environmental conditions and that the secretion of such enzymes across the plasmalemma might account for

the tissue invasiveness of E. histolytica. El-Hashimi and Pittman (1970), working on biopsy specimens obtained from a patient with acute amoebic colitis, failed to reveal a mechanism by which extracellular secretion of enzyme might take place but they supported on scanty evidence Villarejos' hypothesis (1962) that endoenzymes released following the death of amoebae might account for pathogenic activity.

As E. histolytica is harmful to tissue cells in vivo, will the trophozoites extend their toxic effects on cells in vitro? Jarumilinta and Kradolfer (1964) tested this theory, and detected a definite cytotoxic in-vitro effect with various strains of E. histolytica on polymorphonuclear leucocytes from man, sheep, rabbits, chicken, guinea pigs, hamsters, rats and mice. This effect was not observed with Entamoeba coli and Acanthamoeba species. Although unable to provide definite evidence, Jarumilinta and Kradolfer postulated that, as the leucocytes are damaged in response to contact with the amoebae, the "noxa" of E. histolytica disrupt the lysosomes in the leucocytes and such disruption causes the release of the lysosomal enzymes which cause digestion of other organelles resulting in cell damage and death. Further proof that contact between the amoeba and host is necessary for cytotoxic effect to take place was furnished by Eaton et al. (1969, 1970) who studied the lytic effect of E. histolytica on monolayers of mammalian cells, and by Knight, Bird and McCaul (1975) working on the in-vitro morphological changes following the addition of E. histolytica to a rabbit-kidney culture cell-line. Eaton et al. (1969, 1970) suggested that lysis was not due to the release of any soluble enzymic or toxic product by the amoeba, but depended on contact with surface-active lysosomes. They suggested that these lysosomes are

equipped with simple or compound tubular triggers, which can be easily released on contact with another organism. Surface-active lysosomes were also demonstrated in E. histolytica obtained from colonic biopsy specimens (Proctor and Gregory, 1972b) but their illustrations are not convincing. El-Hashimi and Pittman (1970) and Griffin (1971), also using colonic material, were unable to confirm this work. Surface lysosomes were however observed in trophozoites of non-pathogenic Entamoeba species - E. coli and E. moshkovskii (Scandalli et al., 1974a, c). Knight et al. (1975) suggested that the so-called surface lysosomes might be digestive vacuoles or other vacuolar structures sectioned near the amoebal surface. In recent years, the part played by the multiplicity of host factors, physico-chemical and biological, in obscuring the elucidation of the amoebic penetration problem has gained acceptance. Neal (1971), cognisant of the difficulties, considered whether "we do not yet have a convenient simple model for the determination of invasiveness". It is suggested that a simple model, unaffected by the complexity of host factors, is an in-vitro cell culture system. This work is based on such a system.

B.1 INVESTIGATION OF AMOEBIC PATHOGENESIS

In this study of the pathogenicity of E. histolytica the following investigations were undertaken :

I. Histochemical

a) Study of lysosomal enzymes at light microscopical level in trophozoites of E. histolytica (Texas strain) and in cell cultures both separately and in combination to determine whether cell injury is the result of disruption of lysosomes as suggested by Jaramillo and Kradolfer (1964) (See Table IIa).

b) Study of two lysosomal enzymes, acid sulphatase and

acid phosphatase, at electron microscopical level, both for the reason stated in (a) and to investigate the existence of the "surface lysosome" concept of Eaton et al (1969, 1970).

c) Study of other enzymes of known cellular distribution in cell culture before and after interaction with E. histolytica (Table IIb).

II Normal transmission electron microscopic techniques used in :

a) The examination of the morphology of the various strains of E. histolytica grown monoxenically and axenically.

b) The investigation of pathological changes taking place during the interaction between E. histolytica and cell cultures.

c) Testing the effect of an antihistamine on the interaction between amoebae and cell monolayers.

d) Examining the effect of a homogenate of a strain of E. histolytica upon a monolayer in order to explore Villarejos' hypothesis (1962) that the pathogenic mechanism of invasion is the result of decomposition of amoebae.

e) The investigation of Rhabdovirus particles in Entamoeba spp. (refer to Bird and DeCaul, 1976).

III Scanning electron microscopic techniques to demonstrate the lytic effect of E. histolytica on cell-culture systems.

IV Chromium release experiments to demonstrate the lytic effect of E. histolytica and E. invadens on cell-culture systems.

TABLE II

IIa Localisation of lysosomal hydrolases :

Non-specific esterase
Aryl sulphatase
Acid phosphatase
β-galactosidase
β-glucuronidase
N-Acetyl-β-D-glucosaminidase

IIb

Localisation of enzyme "markers"	Cellular site
Thiamine pyrophosphatase	Golgi apparatus
Inosine diphosphatase	Endoplasmic reticulum
Catalase	Peroxisomes
Mitochondrial ATPase	Mitochondria
Na^{+}/K^{+} - Nitrophenyl phosphatase	
Mg^{2+} dependent ATPase	Plasma membrane
Ca^{2+} " "	
Alkaline phosphatase	At various sites, mostly on plasma membrane
Leucine aminopeptidase	

C MATERIALS AND METHODS

C.1 CELL-LINE MONOLAYERS

C.1.1 Cell-line types

a) CV-1, an established epithelial cell line from African Green Monkey Kidney was obtained from Professor K.E. Rose of the Department of Biochemical Pathology, University College Hospital Medical School, London.

b) BF-VI, an established epithelial cell-line from rat (BD strain) liver, was also supplied by Professor Rose.

c) Tertiary cultures of adult rhesus monkey brain consisting of glial cells, choroid epithelial cells and fibroblasts were obtained from Dr K. N. Taiquaye, Department of Microbiology, London School of Hygiene and Tropical Medicine.

d) RK13, a rabbit kidney epithelial cell-line was obtained from the American Type Culture Collection (BCL-725).

C.1.2 Propagation

All cultures were incubated as monolayers at 37°C. CV-1 and BD cell-lines were maintained in 10 oz. rolling bottles and 8 oz. pyrex babies' feeding bottles. Adult Monkey Brain cell line was grown in polystyrene tissue culture flasks (Falcon Plastics Company). RK13 was cultivated in 100 ml medicine bottles. When required for experimental purposes the monolayers were grown on glass coverslips in Leighton tubes. Only the RK13 cell-line was subcultured continuously. Stock cultures of RK13 were maintained by seeding 100 ml medicine bottles containing 30 ml growth medium and laying flat for 7 days. A confluent monolayer was trypsinised for 2-5 minutes in Versene solution (1:5000) containing 0.05% trypsin. Before the cells started to dislodge from the glass, the chelating agent was decanted, leaving a residual

2ml. The cells were first suspended by shaking and knocking the bottle against the palm of the hand, then resuspended in 20 ml of culture medium, which was then dispersed into new bottles. The subculture ratio was maintained at 1:4 to 1:5. The medium was renewed every 3 to 4 days. The subculturing procedure was performed once weekly in a positive flow cabinet to obviate bacterial contamination.

C.1.3 Culture media

The requirements of the various cell-lines are shown in Table III. The media for the HK13 cell-line were obtained from Wellcome Reagents Limited, and were supplemented by 100 units/ml penicillin, 50 ug/ml streptomycin sulphate and 2.5 ug/ml Amphotericin B.

C.2 E. HISTOLYTICA STRAINS

C.2.1 Monozenic strains

The eleven strains, listed in Table IV, were obtained from Dr B. Knight (Liverpool School of Tropical Medicine) They were cultured in TYI medium with a Crithidial associate (Diamond, 1968a).

All 11 strains tested had a cytopathic effect upon HK13 cell monolayer (Knight, personal communication). Only the Evans strain was maintained continuously.

C.2.2. Axenic strains

Two strains HM-1:IMSS and 200:NIH were kindly supplied by Dr L.S. Diamond and were brought to England by Dr B.G. Bird in TP-S-1 medium (Diamond, 1968b). They were kept for 24 hours in an incubator at 36.5°C before being inoculated onto a HK13 cell-line. An attempt was made to culture the two strains axenically, but no growth was obtained.

TABLE III

CELL-LINE	MEDIUM	SERUM
CV-1	Eagle's B.E.M. with non-essential amino-acids and vitamins.	10% Foetal calf serum
BS-VI	Williams's Medium E	"
RK13	Medium 199	"
Rhesus Monkey Brain (Toiquaye, 1975)	HA16-1	"

TABLE IV

MONOXENIC STRAINS

STRAIN	PLACE OF ORIGIN	PATIENT N°.	CONDITION OF PATIENT	TIME OF ISOLATION
LIGGINS	Nepal	T.54247	Dysentery patient	5.8.1970
106 *a	Saskatchewan	(Canada)	" "	1965
ARNELL	Africa	T.58020	" "	24.1.1971
ASANTE	Ghana	T.56884	" "	17.1.1972
IL77 *a	Saskatchewan	(Canada)	" "	March 1971
SWANWICK	Nepal	T.56208	Mild colitis	31.3.1971
DAWSON	India	T.60697	" "	6.9.1972
RUSSELL	Iraq	T.56021	Liver abscess; strain from cysts in stools	5.2.1971
SCOTT	East Africa	T.55596	Symptomless carrier	29.9.1971
EVANS	India	-	"	8.1.1970
DKB	(derived from strain isolated by Drbohlav in 1925)			

*a IL77 and 106 strains were isolated by Dr E.D.P. Eaton; the remainder by Dr R. Knight at the Hospital for Tropical Diseases, London

Axenis '200' was supplied by Dr R. Neal (Wellcome Research Laboratories, Beckenham).

C.2.3. Culture media

C.2.3.a The formula used for the monophasic TYI medium for monoxenic growth of E. histolytica strain, Evans, and stock cultures of Crithidia sp. was a modification of Diamond's original medium (Diamond, 1968a)

Tryptose (Difco)	8.0 g
Trypticase (BBL)	8.0 g
Yeast extract (Difco)	8.0 g
D-glucose (Analar BDH)	4.0 g
L-cysteine hydrochloride	0.8 g
L. ascorbic acid	0.32g
Sodium chloride	4.0 g
Dipotassium hydrogen orthophosphate	1.28g
Potassium dihydrogen orthophosphate	1.28g
Distilled water to make	1000 ml
pH adjusted with 1.0N NaOH to 6.8	

The tryptose was first dissolved in water with the aid of heat (50°C), then the remaining ingredients were added and dissolved one by one in the order given. After adjusting the pH with 1N NaOH, the medium was dispensed in 13 ml amounts in Flow-Lab. tissue-culture tubes (125 mm. long and 16 mm. diameter), with plastic screw caps. The tubes with the broth were sterilised by autoclaving at 121°C for 10 mins., and then kept at 4°C for up to 6 weeks, when the colour of the medium turned from light yellow to yellowish brown. When required the following supplements were added :

Defibrinated human blood	1-2 drops
Horse Serum (Wellcome H.I. inactivated)	0.5 ml
Streptomycin sulphate	1700 ug/ml of medium
Benzyl penicillin	1700 units/ml of medium

Propagation of E. histolytica monoxenic culture :

The Evans strain was maintained at 37.0°C and subcultured at alternate intervals of 72 and 96 hours. Before transferring, 1 to 3 million Crithidia/ml of culture medium from stock cultures were added to the TYI medium with added supplements.

To transfer the amoebae, the medium from old cultures was decanted aseptically and replaced with fresh TYI medium without added supplements. The tubes were then chilled in ice-water for 10 minutes and inverted several times to loosen amoebae attached to the wall of the tubes. Centrifugation at 1500 rpm for 3 minutes was sufficient to pellet the amoebae. Supernatant fluid was decanted except for the last 1 ml. The pellet was then used for inoculating new cultures, which were inclined at 15° from the horizontal. Subculturing was performed aseptically in a positive flow cabinet.

Elimination of bacteria :

Tubal bacterial contamination necessitated additional antibiotics. The offending organism was a Pseudomonas sp. Sensitivity testing** suggested that Carbenicillin sodium at a dosage of 200 ug/ml was indicated from the anti-bacterial point of view but it was found at this concentration the antibiotic had an adverse effect on amoebic growth rate. Accordingly ampicillin sodium (1000 ug/ml) with Gentamicin sulphate 30 ug/ml of medium and benzyl penicillin (1700 units/ml of medium) were substituted. C.2.3.b The formula used for monophasic TP-S-1 medium (Diamond, 1968b) for axenic growth of E. histolytica strains, 200:HK and HM-1:IMSS was as follows :

** Sensitivity tests were carried out by Dr J. Orange in the Department of Microbiology, Middlesex Hospital, London and by Mr Moody in the Department of Pathology, Hospital for Tropical Diseases, London

Trypticase (BBL)	10.0g
Panmede Liver digest (P & B)	200.0g
Glucose	
L-cysteine hydrochloride	1.0g
Ascorbic acid	0.2g
Sodium chloride	5.0g
Dipotassium hydrogen orthophosphate	1.0g
Potassium dihydrogen orthophosphate	0.6g
Distilled water to make	875 ml

pH was adjusted with 1.0N NaOH to 7.0

The nutrient broth was passed through Whatman Filter paper No.1, and autoclaved in 200 ml medicine bottles for 10 mins. at 121°C. After autoclaving and cooling to room temperature, the following supplements were added :

	HM-1:IMSS strain	200:NIH strain
a) Calf Serum	15.0ml	10.0ml
b) Vitamin 107 mixture	2.5ml	2.5ml
c) Nutrient broth	82.5ml	87.5ml

Difficulty was experienced in maintaining both strains axenically. In retrospect the importance of the relative proportion of L-cysteine hydrochloride and ascorbic acid was not fully appreciated**a.

**a Singh, Das and Dutta (1973) demonstrated that the success of axenic cultivation depends on the oxidation-reduction (O-R) shift, which is controlled by the addition of reducing agents, L-cysteine hydrochloride and ascorbic acid. The authors noticed that an improvement in the growth and multiplication of amoebae takes place in a medium with a low negative O-R potential. At a higher negative O-R potential the amoebae tend to die. The authors pointed out that ascorbic acid can interact with cysteine causing a shift in the O-R potential to the positive side, and the amoebae then die within 3 to 5 days without multiplication. If the amoebae are maintained in an axenic medium containing only cysteine, they multiply rapidly due to the lowering of the O-R potential.

C.3 INOCULATION OF E. HISTOLYTICA ONTO CELL-LINES

Initially cell-lines were grown on a round coverslip, or thick glass disc (1.0 mm thick and 32 mm diameter) placed in an airtight flat-bottomed sterile plastic container of 30 ml capacity as described by Knight et al (1975). Later in the present study, a revised technique was used. A smaller thin coverslip (9 x 35 mm) or a thick rectangular glass (7.5 to 8.0 mm x 38 mm) adapted for Leighton cell-culturing tubes was found to be more efficient and economical.

In all experiments, the cell-lines were allowed to reach confluence. This took between 3 and 5 days. The subconfluent layers were never used.

C.3.1 Inoculation of whole amoebae

Only 3 strains of E. histolytica were inoculated on cell-lines; Evans strain (monoxenic) and HM-1:IMSS and 200:NIH (axenic). The amoebic medium in 48 to 72 hour cultures was replaced with fresh-chilled medium and the tubes placed in ice-water for 10 minutes and then inverted several times to detach the amoebae from the glass wall of the tubes. Three minutes centrifugation at 1500 rpm loosely pelleted the organisms. The supernatant medium was removed and the amoebae suspended in the last few drops of the medium by gently shaking the tube. The amoebae were then counted using a haemocytometer. The inoculum varied from 10,000 to 40,000 trophozoites per culture/Leighton tube. Before adding the amoebic suspension to the monolayer, the cell-line culture medium was rinsed off using a further fresh sterile culture medium. The fluid was again removed and replaced by fresh culture medium containing 5% serum. The cell-lines were covered with enough liquid (1.5 to 2.0 ml) to avoid rapid shift

in oxidation-reduction potential due to contact with air. Spillage down the neck of the Leighton tube bringing with it the suspended amoebae was avoided by incubating the tubes in a slightly slanted position (5° - 10° to the horizontal). The inoculation procedure was performed aseptically. A 1 ml syringe with a (40/9, 20G) 1 1/2 ins. needle was found to be satisfactory.

The preparations were all incubated at 37°C and were fixed periodically. (For preparation of specimens for light and electron microscopy see section C.4).

C.3.2 Inoculation of whole amoebae with an addition of promethazine hydrochloride, an antihistamine, onto a cell-line

In view of Judah's findings (1962) that an antihistamine must be present during infection of a cell-cultured system if the cells are to be protected, promethazine hydrochloride was added with the inoculum of E. histolytica trophozoites. Promethazine hydrochloride is readily available commercially as 'Phenergan' (25 mg/ml), a sterile solution in water, free from dissolved air and with suitable stabilising agents.

The inoculation procedure used was similar to that in section C.3.1., except that the cell-line monolayers were rinsed twice with PBS, and then incubated for 15 mins. at 37°C with fresh PBS. The saline was replaced with sterile TPY medium containing 10^{-4} - 10^{-5} M Promethazine hydrochloride and 5% Horse Serum. The amoebae were inoculated onto the monolayers. The preparations were incubated at 37°C for 2 hours, after which they were fixed with appropriate fixatives (see section C.4.2). Controls were prepared in the same way except that antihistamine was omitted.

C.3.3. Inoculation of homogenate of E. histolytica trophozoites onto a cell-line

Homogenization of amoebae

4 Flow-Lab. tubes, each containing 100,000 to 250,000 trophozoites were used. The fluid medium in 48 hour cultures was replaced with chilled sterile PBS, and the tubes placed in ice-water for 10 minutes and then inverted several times to detach the amoebae from the glass wall. At the same time, a small sterile glass tissue blender was placed in a bath containing crushed ice. 3 minutes centrifugation at 1500 rpm was found sufficient to pellet the organisms. The supernatant medium was decanted and replaced by 1 ml/tube PBS (0-4°C), pH 7.2. The suspension was thus transferred to the blender and homogenized. Homogenization was performed in the crushed ice-bath to ensure minimal enzymatic decomposition. The whole operation was carried out aseptically in a positive flow cabinet. Extraction of cell debris from the homogenate was carried out using a Millipore filter (0.22 um) thus avoiding the use of a high speed centrifuge, a long process in which the activity of enzymatic components may deteriorate. The homogenate was then added onto the cell-lines, covered with TTY medium and horse serum. Following inoculation of the homogenate, 2 experiments were carried out :

i) The homogenate was left on RK13 monolayer for 2 hours before fixing with glutaraldehyde (see section C.4.2);

ii) The homogenate was left on the cell-line for 2 hours. The cell-line was then washed thoroughly with PBS twice before adding fresh 199 Medium containing 10.0% Foetal Calf Serum. The preparation was left for a further 48 hours in an incubator at 37°C before fixing with an aldehyde (see section C.4.2).

C.4 SPECIMEN PREPARATION

C.4.1 Specimen preparation for light microscopy

Both cell-line cultures and amoebae prepared for light microscopy (histochemical demonstrations) were cultivated only on thin coverslips. Storage was in Leighton tubes. Fixation is described in section C.6.1.

C.4.2 Specimen preparation for electron microscopy

Special methods in electron microscopy, such as those employed in localisation of enzymes and visualisation under scanning microscopy are detailed in sections C.5 and C.6.

C.4.2.a Fixation

The fixatives used in this work were glutaraldehyde and osmium tetroxide. Glutaraldehyde, introduced by Sabatini et al. (1963), has been found to be a most efficient cross-linking agent for protein, and is superior to the other aldehydes, such as glyoxal, hydroxyadipaldehyde, crotonaldehyde, pyruvic aldehyde, acetaldehyde, acrolein, methacrolein and formaldehyde. Since the aldehyde does not stabilise unsaturated lipids and phospholipids, post-fixation was carried out in osmium tetroxide so that the materials were not dissolved in the dehydration liquids and lost.

Since it is necessary to add a buffer of roughly physiological pH to all fixatives to prevent wide ranges in pH in fixatives and a possible acidic wave of injury as the fixative penetrates the cell, sodium cacodylate, the most popular buffer used in electron microscopy, was used.

Although most animal tissues are fixed near the optimum physiological pH values of 7.2 to 7.5, tissue cultures show great variation in the physiological pH, ranging from 6.8 to 7.4 for cell-lines and 6.1 to 7.0 for those of *E. histolytica*. Buffers

and fixatives were therefore maintained at an average physiological pH, 6.8.

C.4.2.b Washing and dehydration

Embedding media used are not miscible with water, and the washing and dehydration are therefore conventionally carried out in two stages. The organic solvent, ethanol miscible with the embedding media is often used but still if the tissues are improperly washed after osmium tetroxide fixation, osmium will react with ethanol forming fine colloidal particles visible at high magnification(Dawes, 1971; Mercer and Birbeck, 1972). Methanol, which does not react with osmium, was therefore used as a substitute when feasible (see below).

C.4.2.c Embedding

For tissue embedding prior to sectioning a number of commercial types of epoxy resin, which when polymerized become very strong, is available. The resins used, Spurr, Araldite and Epon, require a transitional solvent because of nonuniform impregnation by the plastic when alcohol is used. Propylene oxide, recommended by Luft (1961), is highly volatile and toxic, and toluene was therefore preferred.

C.4.2.d Stains

Uranyl acetate staining was done during dehydration, and 0.25% solution of the salt in 30% methanol was used. Lead staining was done after sectioning (Reynolds, 1963a; Venable and Coggeshall, 1965).

Detailed formulae for the fixatives, buffers and embedding media employed in this study are detailed in Section C.4.2.h.

One of two methods of processing specimens for electron

microscopy was used, depending on the nature of the material being handled:

a) 'In situ' fixing and embedding of cell-line monolayers and E. histolytica strains growing on thick glass coverslips in Leighton tubes.

b) Fixing and embedding of pellets of E. histolytica monoxenic strains.

C.4.2.a 'In situ' fixing and embedding

Cells were continuously grown on thick glass coverslips**.
The tubes containing cell-line monolayers with or without amoebae, and E. histolytica were carefully taken out of the incubator. The medium was pipetted off quickly but carefully and 3% glutaraldehyde in 0.066M cacodylate buffer (pH 6.8) warmed to 37°C was gently added by pipetting slowly onto the side of the Leighton tubes. Usually this reagent is used at 4°C but high temperature fixation enhances the speed of the chemical reaction between fixative and cell components. In doing so the chance of shrinkage of the amoebae was minimised and external structural features were preserved.

Fixative was first maintained at 37°C for 15 minutes,

**The main difficulty in the 'in-situ' fixation and embedding of in-vitro cultured cells on glass coverslips for electron microscopy is the separation of cells after embedding and polymerisation of the resin. Various authors have attempted to replace the glass coverslip by alternative materials e.g. polystyrene (Richters and Valentin, 1973; Nelson and Flaxman, 1972; Brinkley et al., 1967); Teflon (Zwijh and Hoeli, 1975a); Teflon-FEP film (Zwijh and Hoeli, 1975b). Others have attempted to precoat the glass coverslip with a chemical to allow easy separation of the resin after polymerisation e.g. Formvar (Buckley and Porter, 1975); silicone (Rosen, 1962); carbon (Robbins and Gonatas, 1964) or collagen gel (Hayner, 1963). In this study, due to the nature of the experiments, such precoated glass coverslips and alternative substrates were avoided. The reason for this is that these techniques invariably give atypical growth patterns, as stressed by Moore (1975).

and was then completed at room temperature (22-25°C) for 30 minutes. Following fixation, the slides were removed from the tubes, and one end of the glass marked with a diamond pen. They were then placed individually into flat-bottomed 2 ml plastic tubes, with plug-caps, filled with buffer solution (0-4°C), 0.066M cacodylate buffer pH 6.8. From this washing stage to the dehydration stage, the tubes with the samples were kept cold in a crushed-ice bath. Rinsing with cacodylate buffer to remove excess gluteraldehyde was repeated twice; each step took 15 minutes. The slides were then left in buffer for a further two hours. Specimens were further fixed with 1% osmium tetroxide in 0.066M cacodylate buffer at 0°C for 30 to 45 minutes. The material was post-fixed at 0°C to prevent osmium removing intracellular material which happens at room temperature. Following post-fixation, the slides were thoroughly rinsed with buffer several times to remove excess osmium. The specimens were then left overnight in cacodylate buffer at 4°C. The following day, they were taken out of the tubes and placed into a small container containing 30% methanol. After 5 minutes, the solvent was removed with fresh 30% methanol. After a further 5 minutes, the alcohol was pipetted off and replaced with 0.25% uranyl acetate dissolved in 30% methanol. The slides were thus stained for 30 minutes, after which the specimens were dehydrated further through serial dilutions of methanol; 60%, 70%, 90%, 100% and 100% - each step taking 5 minutes.

The next stage, that of embedding, varied according to the embedding mixture used.

Araldite embedding :

a) Slides were passed through toluene twice (5 minutes each).

b) Volume replaced by araldite and the specimens left for 30 minutes at room temperature.

c) The slides were then left in fresh araldite for a further 30 minutes at room temperature.

d) This step repeated but slides left overnight at room temperature.

e) The dish containing the glass slides and the embedding mixture was put into an incubator ($60^{\circ}C$) for 10 minutes to warm up the embedding medium in order to facilitate easy handling. Each glass strip was drained carefully and placed onto a light microscopy glass slide, (76 mm x 25 mm). A fresh plastic mixture was then pipetted onto the top of the cultures. Care was taken to avoid spillage over the edge of coverslips onto the glass slide (3 to 5 drops were found to be adequate).

Epon embedding :

a) Infiltration with propylene oxide was carried out twice (5 minutes each step).

b) The Epon mixture was added and kept overnight at room temperature in a dessicator. This stage is considered to be important as Epon is slightly hydrophilic, and if the mixture is left overnight on the bench, poor polymerization results.

c) As for Araldite embedding (e).

Spurr embedding :

a) The slides were passed through a 1:3 solution of absolute methanol and Spurr embedding mixture for 30 minutes at room temperature.

b) Passage through 1:2 solution of methanol and Spurr for 30 minutes at room temperature followed.

c) This step was then repeated with a 1:1 solution.

d) The slides were then left in a fresh Spurr mixture overnight at room temperature.

e) As for Araldite embedding (e).

The removal of the resin from the glass was achieved by using a modified version of the tool originally designed by Ellis (1971). This modified borer is designed to fit onto the revolving substage of the microscope. The details of the borer are to be described elsewhere (Bird and Chapman, pers. communication). The advantage of such a tool is that the microscope-slide containing the Leighton tube coverslip need not be removed from the specimen stage until the boring procedure is finished. The cell-culture areas were visualized under light microscopy using phase contrast. The pre-selected areas were marked with a specially designed diamond pen which is attached to the revolving stage of the microscope. Without moving the slide from the specimen stage, the marked areas were bored down to the glass by the boring attachment. The bored areas were cracked off from the glass by rapidly cooling them with cardice-ice. (For in-situ embedding, araldite was found to be preferable to both Epon and Spurr since it was more easily removed after rapid cooling with dry-ice). Each plug containing the bored area was mounted on the conical end of a 1.5 cm. length of perspex rod for section cutting.

Hydrofluoric acid, which can be used to remove embedded cell-cultures from glass coverslips (Moore, 1975) was avoided because of its corrosive potential and also because of its possible effect on fine structure under high resolution investigation.

C.4.2.f Fixing and embedding of pellets of E. histolytica monoxenic strains

The fluid medium in 48 - 72 hour cultures was replaced with fresh-chilled TTY medium, and the tubes placed in an ice-bath

for 5 minutes. They were then inverted several times to detach the amoebae from the glass. Three minutes centrifugation at 1500 rpm followed loosely pelleting the amoebae, which were then fixed with 3% glutaraldehyde in 0.066M cacodylate buffer (pH 6.8) for 45 minutes at 4°C. Excess glutaraldehyde was removed by repeated washings with the cacodylate buffer. As the trophozoites were not easily agglutinated by the fixative, it was necessary to pre-embed the amoebae in 2% Difco Bacto Agar (Gowans, 1971). The agar blocks were post-fixed for 1 hour with 1% osmium tetroxide with 0.066M cacodylate buffer.

Steps for subsequent washing, dehydration and embedding were performed in the same way as for 'in situ' embedding except for the final stage for which a Beem Capsule was used.

C.4.2.g Sectioning and observation

Ultrathin sections were cut on a Reichart OMU2 microtome and collected on uncoated Smethurst Mew 200 copper grids (3.0 - 3.05 mm diameter) and coated 150 grids (Graticules Ltd.). Sections were further stained with lead citrate (Reynolds, 1963; Venable and Coggeshall, 1965) and examined using EM 801 (AEI) and EM 9A3 (Zeiss). Ilford film, EF332, an Electron Microscope film with a polyester base was used for photographic recording.

C.4.2.h Formulae

Buffers :

a) Phosphate buffer :

Stock solutions A : 0.2M - $NaH_2PO_4 . H_2O$

19.6g/500 ml of distilled water (DW) stable for a week or more if kept in refrigerator

B : 0.2M - $Na\ HPO_4 . 12H_2O$

35.8g/500 ml of DW
(or 0.2M Na_2HPO_4 - 14.2g/500 ml of DW)

0.1M Phosphate buffer :	Solution A	Solution B	DW
pH 7.4	19.0 ml	81.0 ml	100 ml
pH 7.0	39.0	61.0	100
pH 6.8	51.0	49.0	100

b) Cacodylate buffer : (Sabatini et al., 1963)

Stock solution A : 0.2M cacodylate buffer
21.4g sodium cacodylate/500 ml DW

0.066M cacodylate buffer : Solution A 165 ml
DW 335
Adjust pH with either 1N HCl or 1N NaOH

Fixatives : Aldehyde fixatives are unstable and should be made up fresh. Glutaraldehyde is available as 25% stock solution, which is stored at 4°C to prevent decomposition.

a) 3% glutaraldehyde (GA) in 0.066M cacodylate buffer :

0.2M cacodylate buffer (Stock solution A) 33.0 ml
25% GA 12.0 ml
Adjust pH with 1N HCl, then dilute to 100 mls with DW

b) 3% glutaraldehyde in 0.1M phosphate buffer :

Add 12 ml of 25% GA to every 50 ml of solutions A and B (phosphate buffer, 0.2M). Adjust pH with either 1N HCl or 1N NaOH, then dilute to 100 ml with DW.

c) 4% formaldehyde in 0.1M phosphate buffer :

Add 10 ml of 40% formaldehyde to every 50 ml of solutions A and B (phosphate buffers 0.2M). Adjust pH with either 1N HCl or 1N NaOH, then dilute to 100 ml with DW.

d) 4% [illegible] in 0.066M cacodylate buffer :

40% formaldehyde 10.0 ml
0.2M cacodylate buffer (stock solution A) 33.0 ml
Adjust pH, then dilute to 100 ml with DW.

Paraformaldehyde is prepared for formaldehyde fixation since the commercial 40% formaldehyde solution (formalin) contains methanol as a preservative, which is detrimental to fixation :

i) Dissolve 10g of paraformaldehyde powder in 25 ml distilled water (40% formaldehyde) by heating to 60°-70°C and stirring.

ii) Add 1-3 drops of 1M NaOH. Stir until solution clears.

iii) When cool add buffer and adjust pH with 1M NaOH or 1M HCl, and then dilute with water to the required concentration.

iv) The solution is filtered before use.

e) Osmium fixatives :

Since osmium tetroxide dissolves very slowly, osmium fixatives are made up a day before use. Osmium tetroxide is supplied as crystals in ampoules (0.1g/ampoule). As osmium tetroxide fumes are harmful to both eyes and respiratory tract, the solution is made up in the fume cupboard.

1% osmium in 0.066M cacodylate buffer :

0.1g osmium is dissolved in 6.7 ml of distilled water before adding 3.3 ml Solution A (0.2 M cacodylate buffer).

Osmolarity :

The osmolarity of both fixatives and buffers was determined by the freeze point depression method using an electronic semi-micro osmometer (Knauer).

The following measurements were obtained :

	Milliosmol/kg
3% glutaraldehyde in 0.066M cacodylate buffer (pH 6.8-7.4)	450-480
3% glutaraldehyde in 0.1M phosphate buffer (pH 6.8-7.4)	460-490
4% formaldehyde in 0.066M cacodylate buffer (pH 6.8-7.4)	>1600*a
4% formaldehyde in 0.1M phosphate buffer (pH 6.8-7.4)	>1600*a
0.066M cacodylate buffer (pH 6.8-7.4)	130-150
0.1M phosphate buffer (pH 6.8-7.4)	150-170

*a Maximum value on osmometer scale

Embedding media :

a) **Araldite** (Durcupan ACM - Fluka)

Epoxy resin	CY212	40 ml
Hardener	964 (Dodecyl succinic anhydride)	50
Plasticier	(Dibutyl phthalate)	1.0
Accelerator	(Tridimethyl aminomethyl phenol)	2.0

Resin, hardener and plasticier are mixed in a plastic beaker. As the medium is very viscous, a mechanical stirrer is used. After 30 minutes the accelerator is added and mixed. The final mixture is then degassed to remove air bubbles, and dispensed in small containers (_ 20 ml). Store in a deep freeze (-20°C).

b) **Spurr** (Spurr 1969)

Epoxy resin	ERL 4206 (Vinyl cyclohexene dioxide)	10.0g
Hardener	NSA (Noneyl succinic anhydride)	26.0g
Plasticier	DER 736 (Diglycidyl ether of propylene glycol)	6.0g
Accelerator	S1 (Dimethylaminoethanol)	0.4g

The resin, hardener and plasticier are mixed very thoroughly with a glass rod. The mixture is then degassed to remove all air bubbles. The accelerator is then added and the mixture stirred. The resin is degassed again and dispensed in small containers and stored in a deep freeze (-20°C).

c) **Epon**

Epoxy resin	812		50 ml
Hardener	i)	DDSA (Dodecenyl succinic anhydride)	80
	ii)	MNA (Methyl nadic anhydride)	10
Plasticier	-		
Accelerator	BDMA (Benzyl idimethyl amine)		0.2 ml*

*per 10 ml of resin and hardeners or 13 drops of pasteur pipette/10 ml

Resin and hardeners are mixed well and dispensed in 10 ml quantities in small containers. Store in deep freeze (-20°C). Before use, add the accelerator. Mix well and then degas.

C.5 SPECIMEN PREPARATION FOR SCANNING ELECTRON MICROSCOPY

For stereoscan observations, the HM13 cell-line and trophozoites were grown on thin glass coverslips and cut down to (9 x 10 to 13 mm) in Leighton tubes. The stereoscan specimen stub will only hold materials under 13 mm diameter. The Entamoeba histolytica strain was seeded onto the cell-line in the usual manner. The cells were then rinsed with PBS, pH 7.2 at 37°C to get rid of cell debris which might hinder stereoscan observations. The samples were fixed with 3% glutaraldehyde in 0.06M cacodylate buffer at pH 6.8. In the ensuing 30 minutes they were rinsed twice with 0.06M cacodylate buffer pH 6.8, and then postfixed in 1% osmium in 0.06M cacodylate buffer for 30 minutes. Two rinses in buffer solution, each for 30 minutes were followed by dehydration with acetone of increasing concentration from 30% through 60, 70, 90 and 100%. Each stage took 10 minutes. The samples were then transferred into liquid CO_2 in a Polaron critical-point apparatus. After freeze-drying, the coverslips were mounted onto specimen stubs by a silver conducting cement, and coated with gold (400-450 Å thick) using a gold diode splutter coater (Polaron Equipment Ltd.). The specimens were examined in the Cambridge Instruments Co. Stereoscan microscope operating at 20 kv. The tilting stage was usually maintained at 45°.

Initially cell cultures grown on glass coverslips showed disproportionate cracks along cell-junctions in some areas of the monolayers. As such appearance was not seen in TEM preparations, these cracks were therefore not due to the processing during

fixation and dehydration but occurred during critical point drying. An alternative substrate which appeared less rigid than glass was tried in an attempt to allow cell shrinkage without altering surface morphology. Previously the RK13 cell-line culture had been grown on solvent-resistant millipore filters for E. histolytica interaction (Knight et al. 1975). Millipore, however, would give an irregular background under stereoscan observations. Instead, Nucleopore filters (13 mm diameter, solvent resistant, 0.6 um diameter pore size) were suggested (Cowper, personal communication) for cell-line culture.

Newell and Roath (1975) have devised a container based on the 'Beem' embedding capsule for supporting the Nucleopore filter. Such a container is not suitable for handling filters with monolayers of cultured cells, since the capsule of the container bends the filter as it is snapped onto the rim portion of the lid. The design of this container also involved undue handling of the filter and damage to the cell-face. A container was therefore devised based on a polythene 'snap-on' cap for 10 or 20 ml disposable specimen tubes (Emscope Ltd.) (see fig. I). The filter is placed in the cap with the cells side up. A metal or plastic disc (14 mm in diameter with an internal hole of about 10 mm in diameter) is placed over it. The disc is held in place by a clip, which is maintained in position by the curved lip of the inner rim of the 'snap-on' cap. The advantage of this system is that there is a gap between the filter and the disc. This gap allows for shrinkage of the nucleopore during critical point drying. Adequate drainage of the solvent is obtained by punching holes in the bottom of the cap.

After critical point drying, the filter is removed and

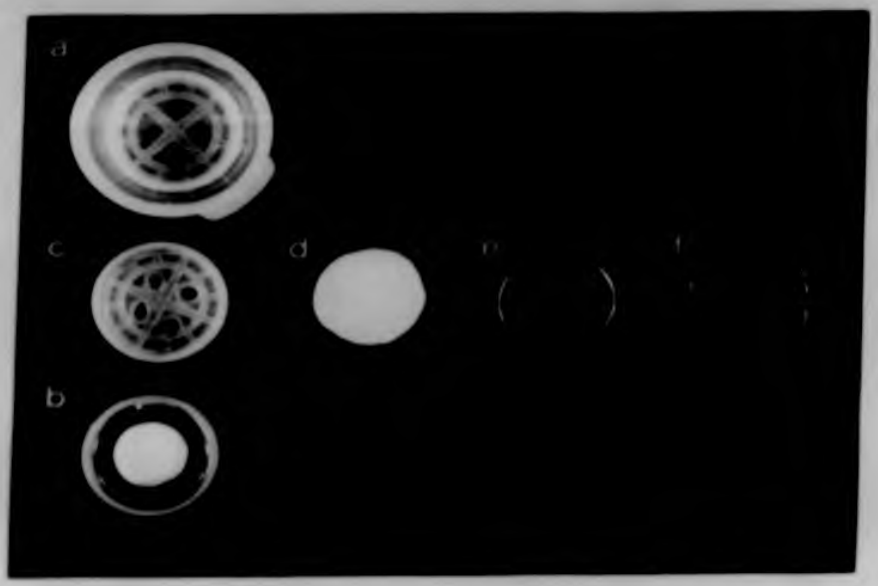

Fig. I Unmodified and modified capsules for critical point drying of samples cultivated on Nucleopore membrane filter

a - Polythene 'Snap-on' cap

b - Modified capsule. Such a capsule can be supported in the boat of the critical point drying apparatus (Polaron).

c - 'Snap-on' cap with rim removed, and holes punched in the cap to aid adequate drainage of solvent.

d - Nucleopore membrane filter

e - Metal disc (14 mm in diameter with an internal hole of about 10 mm in diameter).

f - Metal clip.

attached to the stub by means of a conducting cement. It is then coated and observed as described previously.

C.6 HISTOCHEMICAL TECHNIQUES

C.6.1 Light microscopy techniques

The fixatives used were glutaraldehyde and formaldehyde. Since the ability of the fixative to reduce enzyme activity depends greatly on the cross-linking ability of the fixative with protein the lower the number of cross-links, the greater the enzyme activity will be preserved. As glutaraldehyde is a powerful cross-linking agent, the enzyme activity will be low. Formaldehyde, on the other hand, has a limited cross-linking ability, and the preservation of enzyme is therefore much greater. Formaldehyde is therefore the fixing agent of choice in histochemistry. The nature of the experiments in the present study, however, requires quick and rapid fixation which would enable cells to remain agglutinated on glass. Preliminary tests showed that this was not always possible with formaldehyde and therefore the use of glutaraldehyde became mandatory. Although glutaraldehyde permits only moderate preservation of enzyme activity in comparison with formaldehyde, the speed of chemical reaction between the glutaraldehyde and the enzyme is lowered by decreasing the fixation temperature to 0-4^{o}C and shortening the fixation time to 1-2 minutes. Formaldehyde was only used when the activity of the enzyme was known to be entirely inhibited by glutaraldehyde fixation.

As histochemical techniques are usually designed for cryostat sections, which can withstand variations in osmotic strength of fixatives and washing solutions, buffers of roughly physiological pH were added to all solutions in studying enzymes in cultured materials.

As in all histochemical investigation, adequate controls were used to remove the possibility of artefact. Omission of substrate from the incubating medium was regarded as a satisfactory control. Any enzyme activity observed in control sections was regarded as a false result. Liver and kidney cryostat sections* with known enzymes provided a further control, as failure to detect reaction product indicated faulty technique. The inclusion of specific enzyme inhibitors can also be used as a control measure. In this study, only ouabain, which inhibits Na^+, and K^+ active transport, has been used.

Strict attention was paid to the following points :

a) The samples were prewashed with PBS, pH 7.2 (37°C) for a few seconds before fixation to remove serum which might give false enzyme localization.

b) Fixation was carried out at 0 to 4°C.

c) The samples were left in buffer after fixation for a minimum period of 1 1/2 to 2 hours before incubating in order to remove free fixative which might inhibit substrate-enzyme reaction.

d) The pH of the incubation medium was corrected.

e) The incubation medium was filtered before use.

f) All samples, unless otherwise stated, after incubation and subsequent washing were counterstained with 2% methyl green (chloroform extracted). Methyl green stained nuclei green.

All the essential details i.e. type of fixative, fixation period, incubation period, temperature of incubation, colour of the reaction product, washing buffer, etc. are recorded in Table V.

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*Cryostat sections from mouse liver and kidney were kindly supplied by Dr Jarrett, Histological Laboratory, University College Hospital, London

TABLE V

LOCALIZATION OF ENZYMES AT LIGHT MICROSCOPY LEVEL

ENZYME	FIXATIVE	FIXATION*a PERIOD (in mins.)	MEDIUM (pH and concentration)	INCUBATION*b PERIOD (in mins.)	COLOUR OF REACTION PRODUCT
ACID PHOSPHATASE					
a) Gomori technique	35 GA*c	3	cacodylate buffer pH = 6.8 0.066M	120	black
b) Azo-dye coupling method	35 GA	5		120 - 180	red
NON-SPECIFIC ESTERASE					
a) α-naphthyl acetate method	35 GA	3		30	brick red
b) Indophenol indoxyl method	"	3		half hour - 12 hrs	blue
c) Indoxylase method	"	3		60	purplish-brown
ARYL SULPHATASE					
a) Dye coupling method	"	3		120	red
b) [illegible] method	"	3		60	black
β-GLUCURONIDASE	45 FA*c	30	phosphate buffer 0.1M pH = 6.8	120 - 150	red
β-GALACTOSIDASE	"	30		15 hrs	purplish-blue
N-ACETYL-β-GLUCOSAMINIDASE	"	30		2 - 12 hrs	red
LEUCYL AMINOPEPTIDASE	45 FA	30		120	purplish-red
ALKALINE PHOSPHATASE					
a) Gomori method	35 GA	3	cacodylate buffer pH = 6.8 0.066M	120	black
b) Dye coupling method	"	3		2 - 15 hrs	red
ATPase; Mg^{2+} ACTIVATED	"	3		60	black
ATPase; Ca^{2+} ACTIVATED	"	3		120	black
[illegible] PHOSPHATASE	45 FA	30		2 - 15 hrs	black
THIAMINE PYROPHOSPHATASE (TPPase)					
a)	35 GA	3	cacodylate buffer pH = 7.0, 0.066M	60 - 90	black
b)	45 FA	30		60 - 90	black
INOSINE DIPHOSPHATASE (IDPase)	"	30		45 - 60	black
MITOCHONDRIAL ATPase	"	30	cacodylate buffer pH = 6.8, 0.066M	30 - 40	black
CATALASE	35 GA	15	cacodylate buffer pH = 7.4, 0.066M	120	brownish-black

*a The pH of all fixatives was maintained at 6.8 except for the following : TPPase and IDPase (7.0) and Catalase (7.4)

*b The temperature of the incubation media was maintained at 37°C

*c FA = formaldehyde
GA = glutaraldehyde

C.6.2. Lysosomal enzymes

Six such enzymes were used in this study :

Acid phosphatase
Non-specific esterase
Aryl sulphatase
β-glucuronidase
β-galactosidase
N-Acetyl-β-D-glucosaminidase

C.6.2.a Acid phosphatase

Two reliable techniques for the demonstration of acid phosphatase were used :

i) Gomori lead phosphate method (Bancroft, 1967, p. 190-191)

This method involves the reaction of phosphate liberated by acid phosphatase from the substrate, with lead salt resulting in the formation of lead phosphate. The deposits are visualized under light microscope by the conversion of lead phosphate to black sulphide following treatment with ammonium sulphide.

Preparation of incubating solution

0.05 Veronal acetate buffer pH 5.0	10.0 ml
Sodium β-glycerophosphate (Sigma No. G-6251)	32 mg
Lead nitrate (pH 5.0)	20 mg

The lead nitrate is dissolved in buffer before adding the substrate.

Incubating method

After incubation, the material is :

a) Washed in several changes of buffer. This step is important as excessive artifact staining with ammonium sulphide will occur if improperly washed.

b) Immersed in 1% ammonium sulphide, made fresh for 2-5 minutes.

c) Washed well in distilled water

d) Counterstained with 2% methyl green (chloroform extracted).

e) Washed in tapwater

f) Mounted in glycerin jelly.

ii) AS-Dr simultaneous coupling method using substituted naphthols

The substituted naphthols esters are hydrolysed rapidly by acid phosphatase yielding extremely insoluble naphthol derivatives, which are then made to react with a diazonium salt to produce an insoluble azo dye at the sites of enzyme activity. Pararosanilin hydrochloride as a diazonium salt recommended by Bancroft (1967) was used as it gives a sharp localisation of enzyme.

Preparation of stock solutions
(Naphthol AS- Phosphate method, (Barka, 1960 as cited by Bancroft, 1967)

1) Substrate solution :
Naphthol AS-BI phosphate (Sodium salt. Sigma. No. N-2250) 50 mg
Dimethyl formamide 5 ml

2) Veronal acetate buffer 0.2M

3) Sodium nitrite 400 mg
Distilled water 10 ml
(It is important that solution 3 is made fresh)

4) Pararosanilin - HCl stock:
Pararosanilin hydrochloride 2 g
2N HCl 50 ml
Heat gently to 60°C, then cool to room temperature and filter

5) Distilled water

Incubating method

Bancroft stressed that:

a) it is necessary for the success of the technique that equal parts of solutions 3 and 4 are mixed together and allowed to stand for two minutes before being added to the incubating medium;

b) after counterstaining and staining, the samples are

dehydrated rapidly through fresh alcohols to xylene and mounted in Canada balsam.

C.6.2.h **Non-specific esterase**

3 methods were used : I) α - naphthyl acetate method (Davis and Ornstein (1959) as cited by Bancroft (1967))

Preparation of stock solutions

1) Substrate solution :

α -naphthyl acetate (Sigma No. N-6750)	50 mg
Acetone	5 ml

2) 0.2M phosphate buffer

3) 4% sodium nitrite

4) Pararosanilin-HCl stock solution (see previous page)

5) Distilled water.

Preparation of incubating solution

Solution 1	0.25 ml	
" 2	7.25 ml	
" 3	0.5 ml	0.4 ml of solutions 3 and 4 are mixed before adding to incubating solution
" 4		
" 5		

pH - 6.9 with 0.2M phosphate buffer (solution 2)

Incubating method

As for Azo-dye coupling method (Acid phosphatase see p. 52)

II) Indoxyl methods : II(a) Indigogenic indoxyl method or metal catalysed oxidation method (Holt, 1954)

The substrate used is 5 - Bromo - 4 - chloro indoxyl acetate, which is hydrolysed by esterase to produce 5 - Bromo - 4 - chloro indoxyl, a soluble product. The indoxyl is oxidised by the potassium ferricyanide to an insoluble indigo dye.

Preparation of incubating solution

5-Bromo-4-chloro indoxyl acetate (Sigma No. B-4977)		1.5 mg
Ethanol		0.1 ml
Tris Buffer (0.2M) pH 7.2		2.0 ml
Potassium ferricyanide		17 mg
Potassium ferrocyanide		21 mg
Calcium chloride		
Distilled water	make up to	10 ml

The substrate is dissolved in the ethanol, and the buffer then added. The remaining chemicals are dissolved in distilled water and the solution mixed.

Incubating method

a) As for Ana-dye coupling method (p. 52)

b) A cacodylate buffer is substituted for Tris Buffer, 0.1M, pH 7.2

c) The samples are counterstained in Mayer's Carmalum for 5 minutes to stain the nuclei red.

II(b) Indoxylase method (Debellis and Fishman, 1965)

Preparation of incubating medium

Solution 1	5-Bromo-4-chloro indoxyl acetate	1.0 mg
	Ethanol	0.1 ml
	Tris-HCl buffer pH 7.4	5.0 ml
	Distilled water	4.75 ml

The substrate is dissolved in ethanol before adding the buffer and water.

Solution 2	Pararosanilin - HCl stock solution	0.125 ml
	4% sodium nitrite	0.125 ml

The two solutions are mixed and allowed to stand for 1 minute before adding to solution 1.

Finally, 22 mg $CaCl_2$ is added and the pH adjusted by adding 0.1M NaOH to 6.1 to detect lysosomal non-specific esterase, although some cytoplasmic activity may be present.

Incubating method

As for Ana-dye coupling method (p.52)

C.6.2.a Aryl sulphatase

Two techniques are used in locating aryl sulphatase :

I Simultaneous coupling method (Bancroft, 1967, p.242-243)

Preparation of stock solutions

1) Substrate solution :

Naphthol AS-BI sulphate (Monopotassium salt, Sigma No. N-2375)	20 mg
Sodium chloride	8.0 ml

2) 0.2M acetate buffer

3) 4% sodium nitrite

4) Pararosanilin-HCl stock solution (see p. 52)

Preparation of incubating solution

Solution 1	8 ml	
" 2	2 ml	
" 3	0.6 ml	0.3 ml of solutions 3 and 4 are mixed and left standing for 1 minute before adding to incubating solution
" 4		

NaCl (260 mg) is then added
pH = 6 to 7.

Incubating method

As for Ano-dye coupling method (see p. 52).

II Lead-nitrocatechol sulphate method (Nassau-Hawa et al., 1972)

Preparation of incubating solution

p-nitrocatechol sulphate (Sigma No. N-7251)	160 mg
Distilled water	4 ml
0.1h acetate buffer, pH 5.5	12 ml
6% lead nitrate	4 ml
pH = 5.5	

The substrate is dissolved in water before adding the buffer. The pH is adjusted before adding lead nitrate, which is added dropwise while stirring continuously.

Incubating method

a) As for Gomori lead technique (see p.51)

b) Before incubation, the slides are thoroughly rinsed in 0.1M acetate buffer, pH 5.5. It has been shown that cacodylate ions inhibit the enzyme (Hopsu-Havu et al., 1967).

C.6.2.4 β-glucuronidase

Simultaneous coupling method (Hayashi, 1964)

Preparation of stock solutions

1) Substrate solution :

Naphthol AS-BI-β-D-glucuronide (Sigma No. N-1875)	7 mg
0.05M sodium bicarbonate (420 mg/100 ml)	0.3 ml
0.1M acetate buffer, pH 5.0	25.0 ml

The substrate is dissolved in sodium bicarbonate before adding buffer

2) 4% sodium nitrite

3) Pararosanilin-HCl stock solution (see p. 52)

4) Distilled water

Preparation of incubating medium

Solution 1 10 ml
" 2 0.6 ml
" 3
" 4
pH = 5.2

0.3 ml of solutions 2 and 3 are mixed and left standing for 1 minute before adding to incubating solution

Incubating method

a) As for Azo-dye coupling method (see p. 52)

b) Formaldehyde is used as a fixative. Others have shown successful localisation of the enzyme in tissue fixed with glutaraldehyde (Bowen, 1971, 1973; Livingston et al., 1969) but it was found that control mouse liver sections fixed with 3% glutaraldehyde revealed the reaction products very faintly after 3 hours of incubation at 37°C. On the other hand, sections fixed in 4% formaldehyde gave a very intense staining. It may be that

β-glucuronidase is significantly inhibited by glutaraldehyde.

e) Phosphate buffer is used throughout as cacodylate buffer inhibits β-glucuronidase activity (Bowen, 1971).

C.6.2.e β-galactosidase

A post-coupling azo-dye method is preferred to a simultaneous coupling technique as diazonium salts completely inhibit enzyme activity (Pearse, 1972).

Preparation of incubating medium

6-Bromo-2-naphthyl-β-D-galactopyranoside (Sigma No. B-7627)	100 mg
Methanol	1.5 ml
Hot distilled water (70°C)	200 ml
Phosphate-citrate buffer pH 4.95	85 ml

The substrate is dissolved in methanol, and hot distilled water is then added to the dissolved substrate. On cooling, phosphate-citrate buffer is added with a further 100 ml of distilled water. (The solution is stable for 6 months at 4°C).

Incubating method

a) Incubated at 37°C for 12-15 hours

b) After incubation, transfer the slides to a freshly prepared solution of Fast Blue B salt (Sigma No. D-3502), 1 mg/ml at 4°C, pH 7.4 - 7.8, with gentle agitation for 3 to 5 minutes.

c) Samples are then washed 3 times in cold water and mounted in glycerin jelly.

C.6.2.f. [illegible]

Simultaneous coupling method (Hayashi, 1965)

Preparation of stock solutions

1) Substrate solutions
   Naphthol AS-BI-N-Acetyl-β-D-glucosaminide (Sigma No. N-3378) 3 ml
   Ethylene glycol monoethyl ether 0.5 ml

2) 0.1M citrate buffer (pH 5.2)

3) Pararosanilin-HCl solution (see p. 52)

4) 4% Sodium nitrite

5) Distilled water

Preparation of incubating solution

Solution 1	0.5 ml	
" 2	5.0 ml	
" 3	0.6 ml	0.3 ml of solutions 3 and 4 are mixed before adding to incubating solution
" 4		
" 5	Final volume of 10 ml	

pH = 5.2

Incubating method

As for Azo-dye coupling method (see p. 52)

C.6.2.c Leucine naphthylamidase (Pearse, 1972)

Preparation of substrate stock solution

L-leucyl-β-naphthylamide HCl (Sigma No. L-0376)	40 mg
Ethanol	0.1 ml
Distilled water	4.9 ml

The substrate is dissolved in ethanol before adding distilled water.

Preparation of incubating medium

Substrate stock solution	2.0 ml
0.1M acetate buffer, pH 6.5	20 ml
0.85% sodium chloride	16 ml
0.02M potassium cyanide (65 mg/50 ml)	2.0 ml
Fast Blue B salt	20 mg

Incubating method

a) Following incubation, a 2 minutes rinse in 0.85% sodium chloride (saline) is followed by immersion in 0.1M copper sulphate for a further 2 minutes. After a further rinse in saline for 2 minutes, counterstaining with methyl green is carried out.

b) Dehydration is followed by mounting in Canada balsam.

C.6.3. Enzyme markers associated with known cellular sites

All the phosphatases outlined in Table IVb (as well as acid phosphatase and aryl sulphatase) were localised using

histochemical techniques involving precipitation of lead salts. The phosphate ions liberated by enzymatic hydrolysis of the substrates were trapped 'in situ' by lead ions present in the medium to form highly insoluble precipitates. The precipitates of lead are colourless, but are seen by exposing the materials to ammonium sulphide, which transforms the precipitate into a highly insoluble black granular deposit.

There are, however, certain drawbacks to the lead salt method, such as insolubility of lead in the medium, the formation of the lead precipitate and non-specific binding of lead to tissue structures occurs (Hugon, 1974; Essner, 1974; Weakley, 1972; Barka and Anderson, 1962). In the present study, the following principles were carefully applied :

a) Phosphate or carbonate buffers were avoided for their specific action on the lead ions present in the media.

b) To avoid the formation of lead carbonate precipitates only fresh distilled water, boiled and cooled immediately before use, was employed in preparing incubation media.

c) Since lead forms a precipitate above pH 8.0 unless some chelating agent is present, Tris-maleate was added.

d) Lead at a high concentration will inhibit the substrate and increase the solubility of the reaction product. In a low lead concentration medium, not all the lead will capture the phosphate ions liberated from reaction sites and such ions will diffuse through the material giving rise to artefactual staining. The lead concentration must therefore be strictly controlled.

e) The preparation of the incubating medium requires special attention. Precautions such as those taken by Hugon et al., (1970) result in cleaner preparation. They recommended that in the

preparation of the solution, the lead nitrate is added dropwise and slowly to the buffer and that the pH is adjusted by carbonate free sodium hydroxide (1.0M). The substrate is then dissolved in distilled water and brought to the required pH before being added slowly to the lead and buffer solution. When any other constituents have been added, the medium is filtered and used immediately.

f) It is important not to overincubate the materials as non-enzymatic hydrolysis of the substrate in the presence of lead in the medium may occur leading to the precipitation of lead phosphate, which may bind non-specifically to tissue structures.

C.6.4 Plasma Membrane Markers

C.6.4.a Alkaline phosphatase

Two techniques were used in the demonstration of alkaline phosphatase : I. Gomori calcium phosphate method (as cited by Bancroft, 1967)

Preparation of incubating solution

2% sodium veronal	2.5 ml
2% calcium nitrate	5.0 ml
1% magnesium chloride	0.25 ml
2% Na-β-glycerophosphate	2.5 ml
Distilled water	1.25 ml

The reagents are added in the order given. The final pH of the medium is adjusted to between 9.0 and 9.4 with either 1N HCl or 1N NaOH.

Incubating method

Slides after incubation and several rinses in buffer and water are a) treated with 2% cobalt nitrate for 3 minutes

b) Washed well in distilled water

c) Immersed in 1% ammonium sulphide for 2 minutes

d) Counterstained, washed and finally mounted in glycerin jelly.

II Naphthol AS-BI method

Preparation of stock solution

Naphthol AS-BI phosphate	2.5 mg
N:N - Dimethyl formamide	1.0 ml
Distilled water	1.0 ml
IM Na_2CO_3	1 drop

The reagents are added in the order given and sufficient IM Na_2CO_3 is added until pH is 8.0.

The following solutions are then added :

Distilled water	30 ml
0.2M Tris buffer pH 8.3	10 ml

Preparation of incubating solution

Stock solution	20 ml
Fast red TR (Sigma No. F-1500)	20 mg

Incubating method

a) After counterstaining, the slides are washed with water before mounting in glycerin jelly.

C.6.4.b Magnesium and calcium activated ATPases
(Jacobsen and Jørgensen, 1959, a modification of the Wachstein and Meisel's medium, 1957)

Preparation of incubating solution		Final concentration
ATP . $2H_2O$ (Sigma No. A-3127)	18.2 mg	3mM
$MgSO_4 \cdot 7H_2O$ (15mM)	2.0 ml	3mM
(For localisation of Ca^{2+} activated ATPase, substitute $MgSO_4$ for $CaCl_2 \cdot 2H_2O$ (15mM))		
Lead nitrate (15mM)	2.0 ml	3mM
Buffer, Tris-maleate 0.2M pH 7.2	4.0 ml	
Distilled water	2.0 ml	

pH = 7.2

Incubating method

a) As for Gomori lead technique (acid phosphatase, see p. 51)

C.6.4.c **Potassium-dependent nitrophenyl phosphatase** (Ernst, 1972a, b)

Preparation of incubation medium		Final concentration
p-nitrophenyl phosphate (Sigma No. 3-6750)	93.0 mg	5mM
$MgCl_2 . 6H_2O$ (100mM)	5.0 ml	10mM
KCl (100mM)	5.0 ml	10mM
Strontium chloride (200mM)	5.0 ml	20mM
Tris-HCl buffer 0.2M, pH 9.0	25.0 ml	
Distilled water	10.0 ml	

In a control medium, ouabain is added with the substrate.

Final concentration of inhibitor = 10mM.

Incubating method

a) The presence of yellow nitrophenol in the medium after several minutes of incubation is indicative of enzymatic hydrolysis of nitrophenyl phosphate.

b) After incubation, the material is rinsed with 3 changes of 0.1M Tris-HCl buffer pH 9.0 with 0.1M sucrose at room temperature. Sugar is added to make the rinsing solution iso-osmotic with the standard incubation medium.

c) Treatment with [illegible] minutes rinses (twice) with 2% lead nitrate at room temperature follows.

d) Free lead is removed with 0.2M sucrose.

e) The slides are rinsed in tap water thoroughly before submerging in 1% ammonium sulphide.

f) After washing and counterstaining, the slides are finally mounted in glycerine jelly.

C.6.5 **Golgi Apparatus Marker**
Thiamine pyrophosphatase (Novikoff and Goldfischer, 1961)

Preparation of incubation solution

Thiamine pyrophosphate (Cocarboxylase - Sigma No. C-8754)	25 mg
Distilled water	7 ml
Tris-maleate buffer pH 7.2, 0.2M	10 ml
1% lead nitrate (0.03M)	3 ml
0.5% Manganese chloride (0.02M)	5 ml

pH = 7.0 - 7.2

Incubating method

a) As for Gomori lead technique (Acid phosphatase, p. 51)

C.6.6. Endoplasmic Reticulum Marker

Inosine diphosphatase (Novikoff and Goldfischer, 1961)

Preparation of incubating solution

As for TPPase; substitute Thiamine pyrophosphate by inosine diphosphatase (Sigma No. I 4375)

C.6.7. Mitochondrion Marker

Mitochondrial ATPase

According to Pearse (1972), the degree of localization obtained by Ogawa and Mayahara (1969) was superior to that obtained by other workers. Ogawa and Mayahara incorporated 2-4-dinitrophenol, which stimulates the activity of ATPase in the mitochondrion, into the medium.

Preparation of incubating solution		Final concentration
0.2M Tris-HCl, pH 8.5	1.4 ml	28mM
ATP	18.7 mg	3mM
Magnesium sulphate (15mM)	2.6 ml	3.9mM
Reynold's lead citrate reagent with pH adjusted to 9.2-9.4 with 0.1M HCl	4.0 ml	2.0mM
Dinitrophenol (1mM)	1.0 ml	0.1mM
Distilled water	1.0 ml	

Incubation method

a) As for Gomori lead technique (Acid phosphatase, p. 51)

C.6.8 Peroxisome Marker

Catalase (Novikoff and Goldfischer, 1969)

The catalase activity can be demonstrated cytochemically by using 3'3 diaminobenzidene tetrahydrochloric acid (DAB) as a substrate. The catalase in the material acts as a catalyst to the hydrogen peroxide which oxidizes the substrate to produce a brown granular deposit. The reaction products are insoluble and are able to withstand dehydration and mounting in Canada balsam.

Preparation of incubating solution

DAB (Koch-Light No. 1543p.)	20 mg
0.05M 2-amino-2-methyl-1,3-propandiol buffer, pH 10.0	9.8 ml
1% H_2O_2	0.2 ml

pH = 9.0

Incubation method

Fixed slides are a) rinsed very thoroughly in cacodylate buffer (pH 7.4);

b) transferred to the incubating solution. After 1 hour the medium is replaced with a freshly-prepared medium to avoid excessive accumulation of the DAB oxide, as a result of auto-oxidation;

c) rinsed in several changes of distilled water;

d) dehydrated through graded alcohols to xylene and mounted in Canada balsam.

C.6.9 Electron Microscopy Techniques

The location of an enzyme activity can only be determined with great accuracy if preservation of cellular organelles is adequate. The best preservation of fine structure is obtained with glutaraldehyde, a powerful cross-linking agent, but the enzyme activity will be either low or absent. The quality of the preservation of the ultrastructure is proportional to the number of cross-links. Thus, it is impossible to obtain a high degree of enzyme activity with good morphological preservation. A reasonable compromise is to use formaldehyde which has a limited cross-linking ability but the morphological preservation may be relatively poor, making observational findings of the reaction sites very difficult. Fortunately most of the enzymes localised in this study are hydrolytic enzymes, which are more resistant to glutaraldehyde than the oxidative enzymes and glutaraldehyde is

therefore used extensively. In order to retain some degree of enzyme activity, the ultrastructural preservation has to be sacrificed by shortening the fixation time and decreasing the fixation temperature from 37°C to 4°C. The usual fixation time for ultrastructural observations on cell-cultured systems is 30 minutes, but for histochemical purposes, the time was therefore cut to 10-15 minutes.

The enzymes investigated ultrastructurally were aryl sulphatase, acid phosphatase, alkaline phosphatase, thiamine pyrophosphatase, Mg^{2+} activated ATPase and catalase.

The ultrastructural localisation of the above enzymes, with the exception of catalase, is based largely on the adaptation of light microscopic lead methods to electron microscopy. Lead precipitates on reaction sites have sufficient density for easy viewing in the electron microscope. The only steps altered are those following incubation and outlined below :

Procedure following incubation

Slides are a) rinsed very thoroughly in cacodylate buffer (15 minutes each step - 3 times), and then left in buffer for 2 hours at 0°C - 4°C;

b) postfixed with 1% osmium tetroxide in 0.066M cacodylate buffer for 30-45 minutes at 0°C - 4°C;

c) rinsed several times in buffer to remove free osmium;

d) dehydrated and embedded as described in section C.4.2e.

Counterstaining with uranyl acetate and lead was not attempted.

C.6.9.a Thiamine pyrophosphatase

Novikoff and Goldfischer's medium (see p. 62) is used. A shorter incubation period (45 minutes) is employed to avoid diffusion artefact.

C.6.9.b Magnesium activated ATPase

The method of Jacobsen and Jørgensen (1969) proved satisfactory (see p. 61).

C.6.9.c Alkaline phosphatase

A different incubation medium from that used for light microscopy is used as Millonig and Millonig (1974) reported that the Gomori reaction with the conversion of calcium phosphate into lead phosphate (see p. 51) is too sensitive for electron microscopy since lead precipitates are produced in the cytoplasm, over the chromatin and the nucleolus.

Preparation of incubating solution (Wachstein and Meisel, 1956)

Tris-maleate buffer 0.2M pH 6.2	1 ml
Sodium-β-glycerophosphate (1.25%)	2 ml
Distilled water	5.7 ml
Lead nitrate	1.3 ml
$MgCl_2$	3 drops

pH = 5.0

Incubating method

a) The lead nitrate is added very carefully into Tris-maleate buffer.

b) Distilled water is added to the substrate, which is then mixed with the lead and buffer.

c) The rest of the reagents is then added.

d) After a few minutes, the pH of the solution is adjusted to 6.2 with 1N NaOH. The medium is warmed for 15 minutes at 37°C, during which precipitates are formed in the solution. It is then kept at room temperature for 1 hour. The solution is filtered and used immediately.

C.6.9.d Acid phosphatase

2 methods are employed in the localisation of acid phosphatase, both of which are modifications of the original

Gomori lead method.

I Barka and Anderson's medium

Preparation of incubating solution

1.25% sodium-β-glycerophosphate (Freshly prepared and adjusted to pH 5.0 with IN HCl)	10 ml
0.2M Tris-maleate buffer	10 ml
0.2% Lead nitrate in distilled water	20 ml
Distilled water	10 ml

II Novikoff's CMP medium (Novikoff, 1963)

Cytidene 5' monophosphate (CMP) is substituted for β-glycerophosphate in the Gomori medium, as the rate of hydrolysis of CMP is superior to that of the latter (Essner, 1974).

Preparation of incubating solution

CMP (Sigma No. C-1131)	25.0 ml
Distilled water	14.5 ml
0.1M Manganese chloride	1.25 ml
0.2M acetate buffer, pH 5.0	6.25 ml
1% lead nitrate	3.0 ml

pH = 5.0

C.6.9.e Aryl sulphatase

Nitrocatechol sulphate method (Hopsu-Havu et al., 1967, p. 55)

Although incubation media utilizing lead as a capturing agent are widely used for visualization of aryl sulphatase, Hopsu-Havu et al. (1967) pointed out the inhibitory effect of lead on the substrate. They recommended that the coupler, lead nitrate, is replaced by barium chloride. The effect of 5% barium chloride on aryl sulphatase activity is negligible in the incubation medium. The reaction product, barium sulphate has sufficient density for viewing in the electron microscope.

Preparation of incubating solution

p-nitrocatechol sulphate	160 mg
Distilled water	4 ml
0.1M acetate buffer, pH 5.5	12 ml
5% barium chloride	4 ml

pH = 5.5

Incubating method

a) Before incubation, the slides are carefully rinsed in 0.1M acetate buffer, pH 5.5. This step is important, as cacodylate ions inhibit the enzyme.

C.6.9.f Catalase

Novikoff and Goldfischer's medium is used (see p.63). Oxidised DAB is readily visualised by both light and electron microscopy and the incubation medium adapted for light microscopy can be used.

Incubating method

a) The materials are rinsed with 0.066M cacodylate buffer, pH 6.8, to remove free DAB and auto-oxidised DAB, before post-fixing with osmium. This step is important as any free auto-oxidised DAB present will form polymeric complexes with osmium (Hanker et al. 1972).

C.6.10 Observation

For materials prepared for electron microscopy see section C.4.2g.

Light microscopy specimens were examined using the Wild light microscope (M 20) with a Nikon Dark Box camera attachment (M-355). Ilford film Pan F 135 (a black and white film) and Agfachrome 50L 135 (a reversal colour film) were used for photographic recording.

C.7 CHROMIUM (^{51}Cr) RELEASING CYTOTOXICITY TESTS

C.7.1 Amoebic Strains

Only 2 axenic strains of amoeba were used in this study: E. histolytica Ax. 200 strain and E. invadens EAR strain. The trophozoites were maintained at 37°C for the Ax. 200 strain and 26°C for the EAR strain in 100 ml medicine bottles containing 90 ml TPS-1 medium, 5% Adult Bovine serum and 2.5% Vitamin 107 mixture (Diamond, 1968b). The supernatant medium in 48 hour cultures was decanted except for the last 20 ml. The bottles were then placed in ice-water for 10 minutes and then inverted several times to detach the amoebae from the glass wall of the bottles. The medium with the suspended amoebae was then transferred to Universal containers. Three minutes centrifugation at 1500 rpm loosely pelleted the organisms, following which the supernatant medium was removed and the amoebae suspended in the last 5 ml of the medium by gently shaking the containers. The amoebae were then counted using a haemocytometer. The supernatant medium was used to test its effect on the labelled cells.

C.7.2. Labelling of Chang liver cells

Confluent 100 ml medicine bottles of Chang human liver cells were washed with Eagle's medium and the cells removed by the addition of versene for 10 minutes at 37°C. The cells were concentrated by centrifugation and washed twice with Eagle's medium containing 5% foetal calf serum. They were then pooled and resuspended in 5 ml Eagle's medium with 5% serum and $Na^{51}Cr$ (total concentration = 50 μCi) was added. The cells were maintained in suspension for 60 minutes at 37°C. The excess of radioactive label was then removed by washing 3 times in about 20 ml of Eagle's solution, after which the cells were centrifuged at 4°C for 5 minutes

at 1500 rpm and resuspended in Eagle's solution. Viability was assessed by the Trypan-blue exclusion test. The concentration was adjusted to be 100 times less than the highest dilution of amoebic suspension (usually 15 - 20 x 10^4 cells/ml).

C.7.3 Cytotoxic tests

The Cr-releasing cytotoxicity tests were used a) to determine the pathogenicity of the amoebic strains using i) intact trophozoites and ii) homogenized amoebae by ultrasonication;

b) to investigate the effect of the supernatant fluid of a TPS-1 culture medium obtained from 48 hour amoebic cultures;

c) to investigate the inhibition effect of both promethazine hydrochloride and Rosenthal's inhibitor or DL-2, 3-distearoyloxypropyl-(dimethyl)-(2-hydroxyethyl)-ammonium acetate on the amoeba-culture cell interaction.

For a)i) 100 µl of Chang cell suspension was added to 100 µl of each dilution of amoebic suspension. The ratios 100, 32, 10, 3.2, 0.32, 0.032, 0.0032 amoebae to 1 labelled Chang cell were employed. The volume was made up to 300 µl by adding TPS-1 axenic medium (Diamond, 1968b). As a control, the labelled cells (100 µl) were added to TPS-1 axenic medium (200 ml). To check for ^{51}Cr background release, Chang cell suspension (100 µl) was added to 200 µl of Eagle's medium.

For a)ii) the amoebae (20-40 x 10^5 in 5 ml TPS-1 medium) were homogenized by ultrasonication (MSE ultrasonicator, 2 minutes at 6mu). 100 µl of Chang cell suspension was added to 100 ul of each dilution of amoebic homogenate. The ratios of approximately 0.32, 0.032, 0.0032 homogenized amoebae to 1 labelled Chang cell were used.

For b) 100 µl of Chang cell suspension was added to

100 µl of the supernatant fluid obtained from amoebic cultures, and 100 µl of TPS-1 axenic medium.

For c) the ratio of 3.2 amoebae to 1 labelled Chang cell was employed for the inhibition experiments. The inhibitors used were promethazine hydrochloride and Rosenthal's inhibitor (Calbiochem) (Rosenthal and Geyer, 1960). 100 µl of Chang cell suspension, and 100 µl of amoebic suspension were added to 100 µl of each dilution of the inhibitors. The initial concentration of Rosenthal's inhibitor was 2.2×10^{-2}M. 3 serial dilutions of Rosenthal's inhibitor were used to obtain different concentrations i.e. 6.9×10^{-3}M, 2.2×10^{-3}M, and 6.9×10^{-4}M. As a control for testing the effect of Rosenthal's inhibitor on Chang cells, 100 µl of the inhibitor (2.2×10^{-2}M, 6.9×10^{-4}M, 6.9×10^{-4}M) was added to the Chang cell suspension (100 µl) and TPS-1 medium (100 µl).

The initial concentration of promethazine hydrochloride was 10^{-2}M, and 3 serial dilutions of the inhibitor were employed to obtain different concentrations i.e. 10^{-3}M, 10^{-4}M, 10^{-5}M. As a control for testing the effect of promethazine hydrochloride on Chang cells, 100 ul of the inhibitor (10^{-2}M, 10^{-3}M, 10^{-4}M, and 10^{-5}M) was added to the Chang cells (100 µl) and TPS-1 medium (100 µl).

The serial dilutions of both inhibitors were done in sterile distilled water, making the resulting medium slightly hypotonic.

The resulting mixtures in flat-bottomed plastic tubes (2 ml) for all experiments were incubated for 4 hours at 37°C when E. histolytica was used, and for 18 hours at room temperature in the case of E. invadens. The reaction was stopped by adding 1.0 ml of cold Eagle's medium to each tube followed by centrifugation at

1500 rpm for 5 minutes. Aliquots of the supernatants (900 µl) were taken for counting the spontaneous released labels (A). The remaining pellet and supernatant medium (400 µl) was also taken in order to count the remaining releasable ^{51}Cr (B).

The released ^{51}Cr was counted in a gamma spectrometer. Raw gamma counter data were punched on paper tape and specific release computations were made automatically with an electronic calculator system. All tests were performed in triplicate.

Cytotoxicity was expressed in terms of percentage of chromium release :

$$\text{Cytotoxicity} = \frac{\frac{1400}{900} \times B}{A + B} \times 100\% \ ^{51}Cr \text{ released}$$

The percentage specific release was calculated as the percentage release in cultures containing the experimental samples minus the percentage release in cultures containing the appropriate controls.

D. RESULTS

The aim of the present investigation was to study the pathogenesis of amoebiasis by using a variety of cell culture systems acted upon by both pathogenic and non-pathogenic strains of E. histolytica.

In so doing information has been gained on certain enzyme action sites in the cultured cells and trophozoites separately and after contact also on the ultrastructural changes related to pathogenicity.

Furthermore by release of Chromium from labelled cultured Chang cells it has been confirmed that contact between amoeba and cells is necessary for the initial cell damage to take place. Blocking of the Cr. release was achieved and evidence is put forward to indicate that a toxin may be the cause of a change in the cell membrane permeability, the first step which leads through secondary intracellular changes to eventual cell death.

D.1 [illegible] TROPHOZOITES IN [illegible] MONOXENIC AND AXENIC STRAINS OF ENTAMOEBA HISTOLYTICA

General morphology :

Fig. 1 illustrates the structure of a trophozoite of Entamoeba histolytica.

D.1.1 Nucleus

The nucleus in trophozoites of monoxenic and axenic strains is oval shaped and the nucleoplasm is more electron dense than the cytoplasm (Fig. 2b). The nuclear membrane shows a double membrane with nuclear pores (40 to 70 nm in diameter) (Fig. 2a). Fig. 3 demonstrates a diaphragm bridging the pore. This diaphragm has no obvious trilaminar structure and is more diffuse in appearance than a typical membrane. Remley et al (1976) by freeze- fracturing

technique revealed pores studded with numerous globules but these are not seen in the present study. One nucleus per trophozoite is usually found. Trophozoites with two nuclei are uncommon. In one monoxenic strain, Liggins, 3 nuclei are detected (Fig. 4). The chromatin material is displaced towards the peripheral part of the nucleus under the nuclear membrane, and forms irregular clumps which are not uniformly laid out (Fig. 2a). The karyosome is irregularly shaped and electron dense. Fig. 2a shows microtubules (18-20 nm in diameter) radiating from a centrally situated karyosome. Also present in the nucleus are various inclusions which vary in size. The non-vesicular inclusions (non-membrane bound) are confined to the euchromatin area (Figs. 2b, 4). Such inclusions contain either osmiophilic rings (Fig. 4) or coarse granules which fill the whole core (Fig. 5). The vesicular-type inclusions (membrane bound) are confined to the peripheral region of the nucleus in the heterochromatin area (Figs. 5,6).

The vesicular-type inclusions vary in shape and size. They may either be haricot bean shaped, spherical or even pear-shaped (Figs. 5,6). They contain either electron-lucent material or have an electron-dense membranous structure which looks the same as that of a nuclear membrane (Fig. 5). Sometimes ribosomal material is seen (Fig. 6).

In some sections, only the vesicular-type bodies are seen to move out of the nucleus. Once having passed through the nuclear membrane, the contents of electron-lucent material are dispersed into the cytoplasm (Figs. 7a, 7b).

Filamentous strands are found in the nucleus, but such a finding is rare (Fig. 8). The individual filament is about 5 nm in diameter. Its length cannot be determined in sectioned

materials. The strands are probably not the filamentous viral particles (10 nm in diameter) first described by Diamond et al. (1972) as there is a size discrepancy.

D.1.2 Cytoplasm

D.1.2a External membrane and the subpellicular bodies

The plasma membrane is typically trilaminar with a thickness of about 100 nm. The middle electron transparent layer is 60-75 nm thick. A fuzzy coat on the outside of the external membrane is not observed in all trophozoites (Fig. 9).

Electron opaque deposits are occasionally seen on the cytoplasmic side of the inner membrane (Figs. 10, 11). These deposits accumulate and resemble the subpellicular dark granules with a lens-shaped profile described by previous workers, (Bird, 1961; Ludvik and Shipstone, [illegible]; [illegible], [illegible]; Proctor and Gregory, [illegible] (Fig. 11). The contents of the sub-pellicular bodies at a late stage of development resemble the non-vesicular type nuclear inclusion, which, in this section (Fig. 12), is found in the cytoplasm of an axenic cultivated trophozoite. The sub-pellicular bodies are common in axenic strains, whereas in monoxenic strains they are found to be few in number or completely absent. Fig. 10 is a micrograph of a section, an amoeba of [illegible] strain, illustrating the subpellicular body. After several years of subculturing, however, such bodies are not in my experience to be found (Figs. 72, 76b, 76c).

D.1.2b Surface lysosomes

Surface-active lysosomes equipped with a 'trigger mechanism', first described by Eaton et al (1969, 1970), are not detected in specimens used in this study. Fig. 13 shows an appearance suggestive of such an entity but on close examination,

the apparent lysosomes are seen to be 2 vacuoles which have collapsed during centrifugation before pellet fixation.

B.1.2e Vacuoles

The most abundant vacuoles are the food vacuoles and within such vacuoles, membranous whorls, concentric rings or myelin-like figures are observed (Fig. 15). These membranous whorls come from digestion of bacteria.

Some of the strains of Entamoeba histolytica were sporadically contaminated with bacteria, mainly of Pseudomonas sp., and the digestion of the ingested bacteria can be readily followed. The bacterial cells are surrounded by a rigid polysaccharide cell wall which protects them (Fig. 14).

Once the bacterium is taken into the vacuole, the cytoplasmic membrane breaks down (Fig. 14). Eventually the outer membrane of the cell wall, containing the lipopolysaccharide component ruptures, and the cytoplasm leaks out. The undigested outer membrane then forms concentric rings or myelin-like figures (Fig. 15).

The reaction product for acid phosphatase is localised at light microscopic level within the amoebic vacuoles. Gomori's modified technique gives a more intense reaction product (Fig. 17) than that of the dye technique which produces a more diffuse one (Fig. 16). No reaction product is present in trophozoites incubated in substrate-free medium. Under the electron microscope, the product for acid phosphatase is present in large cytoplasmic vacuoles (Figs. 18, 19, 20). There is no difference in either the distribution or intensity of the cytochemical product using either

Novikoff's CMP (Figs. 19, 20) or Barka and Anderson's β-glycerophosphate (Fig. 18) methods. The lead reaction product is either restricted to the walls of the vacuoles and their contents or the whole vacuole. The droplets observed in one of the vacuoles of Fig. 19 are perhaps fat bodies released from decomposed cristidia whose remains are visible. No acid phosphatase is seen in the intra-nuclear bodies (Fig. [illegible]). The deposits of lead on the plasma membrane and cytoplasm of vacuole (Figs. 18, 19) are artefact due to non-specific absorption of lead onto the cell.

At light microscopic level, catalase, an enzyme-marker for peroxisomes is localised in vacuolar-like structures (Fig. 21). At electron microscopic level, peroxisomes are definitely not present in the trophozoites, and catalase is strictly confined to food vacuoles, which probably contain digested Crithidia or bacteria (Fig. 22). No catalase is located in either the intra-nuclear bodies or the nucleoplasm (Fig. 23).

The trophozoites used for the localisation of catalase were fixed 'in situ' i.e. directly onto the slides. In situ fixation reveals an extracellular component, the uroid or tail end which is not evident in sections of Entamoeba histolytica trophozoites fixed after centrifugation. The tail is surrounded by clumps of cellular debris and bacteria (Fig. 23). No reaction product is present when the trophozoites were incubated in a substrate-free medium (Fig. 24, see after Fig. 25).

In the trophozoites incubated for thiamine pyrophosphatase

activity (TPPase) the reaction product, lead phosphate, is precipitated in vacuolar-like structures in the trophozoites when observed under the light microscope (Fig. 25). TPPase is only found in specimens fixed with 4% formaldehyde, as glutaraldehyde fixed materials only show the reaction products on the periphery of the nucleus (Fig. 26). Electron microscopy preparations confirm that glutaraldehyde fixed trophozoites show the products deposited randomly along the periphery of the nucleus (Fig. 27). Artefactual nuclear staining is a common phenomenon in lead salt techniques (Barka and Anderson, 1962).

At electron microscope level, the reaction product for TPPase is heavily localised in the amoebic vacuoles (Fig. 28). No electron dense granular deposit is seen in the intranuclear bodies and the rest of the nucleoplasm. At a higher magnification, TPPase fills either the whole of the vacuoles or just the periphery of the vacuoles (Fig. 29).

As TPPase is observed within amoebic vacuoles (Figs. 28, 29), observations were carried out on normal fixed material to identify any specialisation in the structure of the vacuole which could play an important role as a secretory organ in the same way as that of a Golgi apparatus. At ultrastructural level, small invaginations of the limiting membrane of the vacuole are observed (Figs. 30a, b). Later they become detached by pinching to form little vesicles which move into the cytoplasm.

An alternative explanation would be that these invaginations result from a fusion between vacuoles and vesicles. Such vesicles

appear to acquire a fuzzy coat on their cytoplasmic surface. A pinocytotic vesicle with an external fuzzy coat can be seen on the surface of the plasmalemma (Fig. 30b).

D.1.2d Ribonucleoprotein particles and helices

Scattered in the cytoplasm of many amoebae are fragments of polyribosomes showing a helical configuration (Fig. 30b). These short helical fragments resemble the RNP helices of E. invadens described by Siddiqui and Ridzinska (1963) and the RNP particles of E. histolytica, first mentioned by Lowe and Kaegraith (1969).

In some trophozoites, during pre-cystic stage, short ribosomal helices aggregate to form a crystalloid structure or the chromatoid body (Fig. 31).

D.1.2e Rhabdovirus particles

The morphology and the formation of rhabdoviruses are described elsewhere (Bird and McCaul, 1976). In all strains, the rhabdovirus particles are either scattered throughout the cytoplasm or arranged in rosettes around areas of specialized cytoplasm (Fig. 32). The number of rosettes per trophozoite varies. Fig. 32 shows 4 groups of rosettes.

Fully formed virions demonstrate characteristics of a rhabdovirus: a bullet-shaped virion with an outer envelope and two distinguishable helices (Fig. 33).

D.1.2f Other cytoplasmic features

The remainder of the cytoplasm consists of glycogen particles. Other distinguishable features such as endoplasmic reticulum, mitochondrion, and a typical Golgi apparatus with its stacks of flattened sacs could not be identified.

In the cytoplasm of trophozoites of axenic strain, Axenic 200, are numerous granules of varying size (200 nm - 320 nm)

(Fig. 34). These granules form a granular mass unbounded by membranes. They resemble the paramuclear body first described by Proctor and Gregory (1974a).

Parallel arranged bundles of microfilaments are plentiful in Axenic 200 (Fig. 35). In monoxenic strains, however, the filaments are arranged individually (Fig. 36).

B.1.2g Scanning electron microscopy of [redacted] (Bunns strain)

Normally the amoeba appears slug-like, with a single pseudopodium projecting in front of a hump-like main body (Fig. 37). The size of the pseudopodium may vary depending on the actual mobility of the amoeba (Fig. 38). Generally only one pseudopodium is seen but under certain conditions several smaller pseudopodia may flow from different positions (Fig. 38). The surface morphology reveals a smooth surface with slight infolding (Fig. 39), which can sometimes be fairly marked depending on the state of the amoebae (Fig. 40). The appearance of the surface of the pseudopodium and of the main body of the amoeba is identical. At the junction between the pseudopodium and the main body, however, horizontal striations on the surface would suggest that the membrane is stretched (Fig. 41). This is not unexpected as the pseudopodium plays an important role in amoebic movement.

Large craters or depressions described by Eaton et al. (1970) and Proctor (1974) on amoebic surfaces have not been identified in the specimens used in this study.

Occasionally clumps of cellular debris remain attached to the tail end or uroid of the amoeba (Figs. 38, 42). Washing with PBS warmed at 37°C before fixation removes the clumps revealing the true nature of the uroid (Figs. 43a, b). For example, filopodia are seen to spread out from the tail end of the pretences in all

directions (Fig. 43b). In some cells, conspicuous cytoplasmic processes, which appear to be morphologically similar to the uroid filopodia, are extended along the lateral edges of the amoebae (Fig. 44a). Some of the cytoplasmic processes have blebs at the end of the stalks (Fig. 44c), and in others they apply closely to the substrate surface (Fig. 44b). It is difficult to determine the significance of these cytoplasmic strands. It may be that they are involved in anchoring the amoebae to the glass-substrate but this is open to speculation. The processes are never seen on the upper surface of the amoebae and are always confined to areas nearest to the substrate surface.

D.2 ULTRASTRUCTURAL STUDY OF INTERACTION OF MONOXENIC STRAIN E. HISTOLYTICA (EVANS STRAIN) ON CELL-LINE MONOLAYERS

D.2.1 E. histolytica (Evans strain) and Rhesus Monkey Brain cultured cells interaction

Fig. 4f illustrates the structure of the normal cultured brain cells. The mitochondria, with its internal components the cristae and the intramitochondrial granules and the endoplasmic reticulum are normal in appearance (Fig. 46). The cisternae or flattened vesicles of granular endoplasmic reticulum are studded with ribosomes.

Changes in the Rhesus Monkey Brain cultured cells :

After the trophozoite is added to the cell-line monolayer, fine structural changes take place in the cultured cells. The mitochondria and other organelles are swollen and the cell membrane shows signs of breaking down (Fig. 47a). The cell next to the contact cell is immune to the toxic effect of the amoeba. Fig. 47a was taken after 10 minutes of interaction.

Fig. 47b shows clearly swollen mitochondria. The cisternae of granular endoplasmic reticulum have degenerated into small vesicles.

D.2.2 E. histolytica (Evans strain) and CV-1 cell-line interaction

Fig. 48 illustrates the structure of the normal unstained CV-1 monolayer. The mitochondria, nuclei, endoplasmic reticulum, glycogen particles, lysosomes, lipid droplets and fibrils are all present. The chromatin of the nucleus is divided into lighter and darker areas. The dense areas, known as heterochromatin, are scattered mostly along the periphery of the nucleus. The paler areas, known as euchromatin, cover most of the nucleus. Usually up to 2 nucleoli per nucleus are seen in this culture (Fig. 48).

The cisternae of the rough endoplasmic reticulum are relatively scarcer than those seen in the glial cells, and they are not heavily studded with ribosomes (Fig. 49). When the cisternae are cut tangentially, the ribosomes are seen to occur in groups forming rosettes, which are usually described as polyribosomes.

Microtubules, running across the cell, and the golgi complexes are illustrated in Fig. 49.

a) Changes in the CV-1 cells :

Dramatic pathological changes within the cells occur between 0 and 10 minutes after the addition of Entamoeba histolytica trophozoites. Some cells lyse completely within 5 minutes (Fig. ??) whereas others take 30 minutes (Fig. 53). This time difference illustrates the influence of factors such as changes in culture conditions, age of subculture, degree of confluence and intrinsic pathological state, determining susceptibility of cultured cells to infection. It is for this reason that events leading to cell death are here recorded in terms of pathological change but not necessarily in sequence related to time lapse after contact.

On initial contact with the amoebae, the cells appear to be undamaged. The surface configuration is slightly altered as microvilli increase its length (Fig. 50). When contact is prolonged, gross and rapid degeneration is seen to take place in the cell. The mitochondria begin to lose their normal shape. The mitochondrial matrix becomes diluted as evidenced by decreased density. The electron dense material within the matrix migrates to the periphery of the mitochondria. It would seem that the outer mitochondrion chamber lying between the two membranes of the mitochondrial envelope and extending into the space between the cristae suffers less damage than the inner chamber containing the matrix. The

cristae are also displaced to the periphery and show varying degrees of disappearance (Figs. 51a, b).

The cytoplasmic matrix also loses its overall density. The Golgi apparatus enlarges, and such enlargement can usually be related to an increased secretory activity in order to compensate for the loss of protein secondary to cell destruction. The endoplasmic reticulum also undergoes gross changes. The cisternae of the endoplasmic reticulum swell to an enormous degree and as with the mitochondrial matrix, the endoplasmic reticulum matrix becomes less dense. The polyribosomes at this stage are still attached to the cisternae (Fig. 51b). There is no alteration in appearance of the secondary lysosomes. Small vesicles, which may be primary lysosomes, also show no damage.

At a later stage, the mitochondria completely break down, and the contents of the mitochondrial matrix are released into the cytoplasm through breaks in the mitochondrial limiting membranes (Fig. 52). Some of the mitochondrial membranes are seen to begin to dissolve. The cristae still approximate to parts of the mitochondrial membrane (Fig. 52). Cytoplasmic filaments are present and may have arisen from dissolution of microtubules and microvilli (Fig. 52). The endoplasmic reticulum is vesiculated, and some of the ribosomes have left the cisternae indicating degranulation of rough endoplasmic reticulum.

Prolonged contact leads to the breakdown of the cytoplasmic membrane. Very few swollen mitochondria are present as most of the mitochondrial membranes have dissolved (Fig. 53). Filaments are seen to accumulate and most of the polyribosomes have not yet fully disaggregated as a tangential section through the vesiculated endoplasmic reticulum still reveals groups of polyribosomes

attached to the cisternal membrane (Fig. 53).

The nucleus also undergoes gross changes. The first nuclear alteration is the migration of chromatin to the periphery of the nucleus and swelling of the nuclear envelope (Fig. 54). At a later stage of cell injury, when the cell is already lysed, the nucleus assumes a spherical shape and its contents are almost lost. Only the nucleolus is left (Fig. 55). Fig. 55 also shows the disappearance of the cell membrane, but in some cases the damaged organelles which are not grossly affected are retained by the unbroken parts of the plasma membrane.

b) Study of amoeba in contact with host cell:

The contact side of the amoebic surface becomes active as the area along the contact border is filled with an electron opaque substance of varying density. No food vacuoles or large particulate matter are present in this area. Micropseudopodia are formed which indent the cell without breaking the cellular membrane (Fig. 56). The cell itself is injured as the organelles show signs of damage such as swollen mitochondria and endoplasmic reticulum and ruptured secondary lysosomes. The cytoplasmic membrane of the cell, although distorted, is still intact as the membrane can be observed along one of the micropseudopodia (Figs. 56 and 57). No surface lysosome is observed near the site of contact.

Fig. 57 illustrates a later stage of protrusion of micropseudopodia into a cell. A phagocytotic channel is thus formed, and small projections line the channel. The function of these projections or micropseudopodia is probably to maintain an efficient grip on the cell as the contacted cell is drawn into the channel (Fig. 58). The amoebic cytoplasm around the phagocytosed cell is

fine-reticular and devoid of cell organelles, thus resembling ectoplasm. Along the whole area of contact, there are sites of apparent discontinuity of opposing membranes characterised by membrane fuzziness. The phagocytotic channel is further extended, and the end of the channel becomes invaginated to form vesicles or small vacuoles which may bud off from the channel (Fig. 59). The fate of these vesicles remains undetermined but it is assumed that they fuse with the amoebic lysosomes whose acid hydrolases may break down the contents of the vesicles.

There seems to be no limit to the size of the phagocytotic channel (Fig. 60a). The micropseudopodia may expand and encircle the trapped cellular debris (Fig. 61a). At higher magnification, the detached pieces of cell plasma membrane can be observed along the lining of the phagocytotic bulb; an indication that the cell is engulfed by the amoeba with its membrane intact. The cell is therefore not yet lysed when engulfment first takes place (Figs. 60b, 61b).

Within the channel, further degradation of the organelles takes place, leading to the disruption of the membrane-bound organelles containing the myelin-figures (secondary lysosomes). (Fig. 61b). The microtubules dissociate into filaments and the polyribosomes degranulate from the endoplasmic reticulum (Fig. 61b). The mitochondrial membrane is seen to begin to dissolve as electron dense particles are seen to evaporate from the surface of the cristae (Fig. 62).

Cellular debris is not only taken in at the pseudopodium, as in Fig 63, particulate material is also trapped by small cytoplasmic protrusions or filopodia at the uroid end of the amoeba. The margins of such filopodia fuse and the material then moves into

the cytoplasm as a vacuole. The formation of a small vesicle can be observed adjacent to the inner coat of the vacuole. The uroid is surrounded by an irregular clump of electron-dense material including strings of mucoid substance.

Fig. 64a shows an amoeba in such a lesion. The surrounding cells are injured, as indicated by pathological changes such as swelling of the nuclear membrane, clumping of the nuclear chromatin material, swelling of the mitochondrial and endoplasmic reticulum and breakdown of the plasma membrane. The pseudopodium is seen to ingest a membrane-bound structure, probably a secondary lysosome. At the opposite end of the pseudopodium is the uroid or tail end. This uroid is heavily surrounded by clumps of debris which include swollen mitochondria released from lysed cells and strings of mucoid substance (Fig. 64b). Apart from the almost detached cytoplasmic piece of uroid, even smaller bits of ectoplasm are seen to bud off continuously from the surface of the large ectoplasmic piece (Fig. 64b).

The microvilli of the surrounding cells, distinguishable by internal microtubules, have a peculiar attraction for the uroid and they are seen to point towards the amoebae, especially the uroid (Figs. 64b, 65).

D.2.3 E. histolytica (Evans strain) and BD-VI cell-line interaction

The structure of the normal unimaged liver cell-line monolayer is shown in Fig. 66, which illustrates good cell contact between adjacent cells. The most noticeable features of the cell-line are the mitochondria, endoplasmic reticulum and microbodies.

The mitochondria, which are more oval-shaped than those seen in other cell-lines, are numerous. Its cristae, lamellar in shape, are abundant, and they penetrate right through the matrix

from one end of the wall of the inner membrane to the other (Fig. 67). Occasionally fenestrae appear in some of the cristae. Very few intramitochondrial granules are seen (Fig. 67).

The endoplasmic reticulum is mainly granular in pattern and the cisternae with attached ribosomes occur in various shapes. Some cisternae are seen in loosely arranged groups either as closely packed stacks or as wide-spaced stacks (Fig. 67). No smooth endoplasmic reticulum can be identified.

Hepatocyte microbodies are plentiful in this cell-line. These are round or oval organelles bounded by a single membrane, and contain a fine granular matrix. Some microbodies have amorphous nucleoids but others have none (Fig. 68).

Lysosomes are not very abundant, but it is difficult to differentiate between membrane-bound lysosomes which may contain myelin figures and microbodies with partly formed nucleoid, which may also assume a myelin-figure configuration.

The nucleus is almost spherical (Fig. 66). The euchromatin covers most of the nucleus, as heterochromatin, the dense area, is mainly confined to the periphery of the nucleus. Usually up to 2 nucleoli per nucleus are observed in this cell-line.

a) Changes in BD-VI cell-line :

Dramatic pathological changes are seen within the cells between 0 and 15 minutes after the addition of Entamoeba histolytica trophozoites. The most noticeable change is swelling of mitochondria. At the same time, mitochondrial cristae are seen to fragment except along the periphery of the mitochondria. There is a slight rounding up of mitochondrial matrix densities, and the matrix is pale (Fig. 69). The endoplasmic reticulum is not yet vesiculated and the matrix of its cisternae is of normal density.

In some cells, there is a remarkable hypertrophy of Golgi complexes reflecting an increase in secretory synthesis to compensate for protein loss due to amoebic toxic interference (Figs. 69, 70).

Swelling of the mitochondria is not the only phenomenon to take place as a converse change, mitochondrial condensation can occur (Figs. 71, 73, 74). Here, the mitochondria are generally smaller than those found in unaffected liver cells, and there is a definite increase in the density of the matrix. The cristae have also changed configuration as some of the mitochondria show tubular cristae; an indication of transformation from lamellar to tubular forms.

Fig. 71 also shows vesiculation of the cisternae of endoplasmic reticulum. The cisternae are swollen, and the matrix is less dense. The ribosomes are seen leaving the cisternae. Many filaments are present in the cytoplasm, and they are probably part of disintegrating microtubules.

The nucleus also undergoes pathological change. The first noticeable appearance is the condensation of chromatin along the periphery of the nucleus. The nuclear envelope is also swollen (Figs. 71, 72).

Further contact leads to a reduction in density of the cytoplasmic matrix (Fig. 72) as indicated by cytoplasmic materials which have leaked from the cell. Prolonged contact leads to almost complete loss of cytoplasmic matrix. What is remarkable at this stage is that the condensed mitochondria and the vesiculated cisternae of the endoplasmic reticulum still retain their shape (Fig. 73). Such behaviour is not evident in CV-1 culture cells. The chromatin continues to condense along the periphery of the

nucleus, the interior of which is almost bare (Fig. 73).

Eventually the cytoplasmic membrane breaks down, releasing the cellular contents into the surrounding medium (Figs. 74, 75). Some mitochondria are swollen but most are still in a condensed state. The vesiculated cisternae are seen to be swollen. The nuclear contents are almost lost, the nucleolus alone being clearly seen (Fig. 74).

Fig. 75 shows the lysed cells near the amoeba. Once the cisternae of endoplasmic reticulum are released into the surrounding medium, swelling of these cisternae is accelerated. Microbodies are present which do not seem to be affected. Condensed mitochondria have still not, at this stage, changed shape. Although some nuclei assume a spherical shape, the two nuclei seen in Fig. 75 are distorted due to ballooning of the nuclear envelope.

b) Study of amoeba in contact with host cell :

As in a CV-1 monolayer, gross changes within the cell after amoebic contact are rapid (Figs. 76a, b). The microtubules play an important role in maintaining cell shape. Dissociation of the microtubules into filaments leads to a loss of cell rigidity. Under such a case, the plasma membrane becomes more pliable, and is more prone to the pushing action of the amoeba.

Because of both good contact between amoebic plasmalemma and the cell membrane, and flexibility of the host-cell membrane, any movement of the plasmalemma of the amoeba also affects the shape of the cell. An example is shown in Fig. 76a, where due to the active turnover of the amoebic plasmalemma, the cell plasma membrane shows marked infolding. The amoebic cytoplasm along the contact area shows incipient filling with an electron opaque substance and neither good vacuoles nor particulate matter are

present in this area. The contact areas reveal sites of conspicuous discontinuity instanced by membrane fuzziness (Fig. 77a). Eventually, pieces of attached host-cytoplasm are drawn into the interior of the amoeba by the ectoplasm (Fig. 77b). The phagocytotic channel is seen to deepen and most of the liver cytoplasmic materials are drawn into a channel with a bulbous end (Fig 78a). The nucleus is also drawn into the channel. Fig. 78a shows some nuclear components being so dragged with such force that the nuclear membrane is torn apart. The organelles of this affected cell are damaged but to a minor degree. An condensed mitochondria with concentrated cristae and non-swollen vesiculated cisternae of the endoplasmic reticulum are still present (Fig. 78b), it is likely that this cell is less susceptible to amoebic toxic substance.

The phagocytotic channel and its bulb contain numerous ribosomes, small vacuoles, vesiculated endoplasmic reticulum and filaments (Fig. 78c, 78d). Probably, small vesicles, although not seen, form on the surface of the bulb and these bud and fuse with lysosomes. Such fusion would result in the release of acid hydrolases, which subsequently would breakdown the vesicular products.

Fig. 79 illustrates the differences between an infected and non-infected cell. The infected cell contains swollen mitochondria and endoplasmic reticulum. The difference in nuclear structure is apparent as the infected nucleus shows excessive margination of chromatin, which is not seen in unaffected nuclei.

No amoebic surface-lysosome with its trigger mechanism or any other trigger mechanism was observed.

D.2.4 Scanning electron microscopy of the infection of a RK13 cell-line monolayer with E. histolytica (Evans strain)

Scanning electron microscopy reveals good cell contact between individual cells of the RK13 cell-line monolayer. It is possible to differentiate the nucleus and cytoplasm in cells in which the central mass is flattened (Fig. 80). Occasional artefacts such as fractures along the cell-junctions are common. The surface of the cell is studded with microvilli (Fig. 80). The number of microvilli per cell varies from one cell to another. The rounded-up cells bare the largest number (Fig. 81). It is known that the number of microvilli when cells are rounded up before cell division is significantly higher than in that of normal cells (Follett and Goldman, 1970).

After the amoebae have settled on the monolayer, they form pseudopodia in all directions (Fig 82). The microvilli of cells surrounding the amoebae extend and point towards the amoebae (Figs. 82, 84a). At higher magnification, there is no contact between the microvilli and the plasmalemma of amoebae (Figs. 83, 84b). The surface morphology of the trophozoite is not altered when the trophozoites are added to the cultures cells (Fig. 84b). Large depressions or cracks on amoebic surfaces described by Proctor (1974) have not been seen in this study.

The cells surrounding the amoeba are rapidly destroyed (Fig. 85). The surface of the injured cell is now punctured at various places giving an appearance of an eroded surface. The microvilli seem to be disappearing (Figs. 85, 86a). The ballooning of the cell indicates swelling due to an osmotic effect (Figs. 86a, 86b).

The trophozoite would seem to burrow under the cells (Fig. 87a). As the amoeba continues to move over the cultured cells,

clumps of cellular debris and bacteria occasionally become detached from the uroid end and become agglutinated onto any solid substrate e.g. glass (Fig. 87a) or the surface of an affected cell (Fig. 87b).

Some cells lyse and as a result, the amoeba is coated with cellular debris, which are eventually carried to its uroid end (Fig. 86b). Mucoid threads extending from the tail end or uroid can be long and they sometimes remain attached to the amoebic surface and neighbouring cultured cells (Fig. 88a).

The BK13 monolayer, grown on nucleopore filters, shows even more extensive cracking along cell junctions than those seen on glass-cultivated cells (Figs. 88b, 88c). The width of the crack is apparent in Fig. 88c. The nucleopore filters have pores which provide additional anchorage for the cells, which insert cytoplasmic processes from the underside of the cells into the pores (Fig. 88d). Such strong support prevents possible lateral movement of cells during critical point drying. The Nucleopore filter is thus not suitable for observing monolayers of epithelial cell-lines under the scanning electron microscope.

D.3 LIGHT AND ELECTRON CYTOCHEMICAL INVESTIGATIONS OF CELL-CULTURES AND E. HISTOLYTICA TROPHOZOITES

The distribution of enzymes in cells and trophozoites examined by light microscopy is shown in Tables VI and VII . The results of histoenzymological light microscopy observations of the responses of the RPMI to the interaction with the trophozoites of E. histolytica are also tabulated in Tables VI and VII .

D.3.1 Lysosomal enzymes

D.3.1a Acid phosphatase

Light microscopy level : a) Gomori technique : At light microscopic level, distinct granular deposits are seen in the cytoplasm of the RPMI monolayer (Fig. 89). The enzyme staining varies from one cell to another, and it appears to be on an all or none basis. A slight diffuse cytoplasmic and nuclear staining is noticed but this does not obscure the actual reaction site. Staining is absent when the cultured cells are incubated in a medium lacking β-glycerophosphate.

After the addition of trophozoites to RPMI, there is no increase or difference in size of the particles containing acid phosphatase (Fig. 90). Eventually, there is a progressive enlargement of such particles, whose size can be as large as the nucleus of the amoeba (Fig. 91). The reaction product itself is still confined to these particles.

After prolonged contact, the staining in some host cells is generally diffuse, an indication that the lysosomes have disrupted. In others, lysosomes containing the enzyme-product enlarge without disruption (Fig. 92). One cell, with undisrupted lysosomes, is seen being drawn into the phagocytotic channel of the amoeba (Fig. 92).

b) Azo-dye technique : The density of reaction product

TABLE VI

ENZYMES INVESTIGATED BY HISTOCHEMICAL METHODS AT LIGHT MICROSCOPY LEVEL

	Control*a	E. histolytica	RK13*c	E. histolytica/RK interaction (maximum 2 hours) E. h.	RK
LYSOSOMAL ENZYMES					
N-Acetyl-β-D-glucosaminidase	++	++	-	++	-
β-galactosidase	++	+	+	+	+
Acid phosphatase					
a) Dye technique	++	+	++	++	++
b) Lead (Gomori) technique	++	+++	++	+++	+++
Aryl sulphatase					
a) Dye technique	-	-	-	-	-
b) Lead technique	+	-	++	+*b	++
β-glucuronidase	++	-	+	+*b	++
Non-specific esterase					
a) Delellis & Fishman (1965)	+	-	±	Nd	Nd
b) Metal oxidation	++	-	Nd	Nd	Nd
c) Dye technique	+++	-	+++	+*b	+++

Activity : - negative; ± trace; + low; ++ moderate; +++ high; Nd Not done

Control*a - Cryostat sections of mouse kidney or liver obtained from Dr Jarrett, (Dermatology Dept., U.C.H.)

+*b - The presence of enzyme in E. histolytica results from ingestion of cell debris

RK13*c - Cell-line culture of rabbit kidney epithelial cells

TABLE VII

ENZYMES INVESTIGATED BY HISTOCHEMICAL METHODS AT LIGHT MICROSCOPY LEVEL

	Control[*a]	E. histolytica	HK1[*c]	E. histolytica/HK interaction (maximum 2 hours) E. h.	HK
OTHER ENZYMES					
Thiamine pyrophosphatase	++	+	+	+	+
Catalase	++	+++	-	+++	-
Inosine diphosphatase	++	-	+	++b	+
Mitochondrial ATPase	++	-	+++	++b	+++
ATPase (Mg^{2+} activated)	++	-	++	++b	+++
ATPase (Ca^{2+} activated)	++	-	+	+b	+
P-nitrophenyl phosphatase	++	-	±	-	±
Alkaline phosphatase					
a) Gyr technique	++	-	-	-	-
b) Lead (Gomori) technique	++	-	-	-	-
Leucine aminopeptidase	++	-	-	-	-

Activity, Control[*a], ++b, HK1[*c] : See footnote to Table VII

is weaker than when β-glycerophosphate is used as a substrate. The red deposits can just be visualised (Fig. 199), but the staining is generally diffused as a slight orange-red colour is detected in the cytoplasm of RK13 cultured cells. Staining is absent in cells incubated in a substrate-free medium.

Cultured cells infected with trophozoites show a redder colour in the cytoplasm, but lysosomes containing the reaction product are difficult to see. After 1 to 2 hours of interaction round particles containing the reaction product are visible in the cytoplasm of the amoebae (Figs. 93, 200). Obviously these are cytoplasmic pieces of RK13 being digested in amoebic vacuoles. These vacuoles are generally confined to the amoebic body as vacuoles containing acid phosphatase are absent in the pseudopodium (Figs. 93, 94).

Electron microscopy level: At the electron microscopy level, the only organelles of RK13 which are stained for the enzyme are the lysosomes (Figs. 95a, b) where the deposits are confined either to its membranes or its contents. Adequate preservation of the nucleus and the various organelles especially the mitochondria is noticed in both of these figures (Figs. 95a, b). No staining is seen in RK13 cells, apart from a few lead deposits in the cytoplasm, in substrate-free media both in CMP and β-glycerophosphate methods (Fig. 96).

Enzyme staining appears to be on an all or nothing basis as some cells fail to show reaction product in the lysosomes. Non-enzymatic deposition of lead can be seen in the nuclei both of RK13 cells and of amoebae.

It must be emphasised that the ultrathin sections were not counterstained in order to prevent unselective removal of the

reaction product precipitates and so the membranous structures are not readily visible.

After the initial stage of contact, there is no change in the intensity of enzyme activity within the lysosomes of RK13 cells (Fig. 97). At this stage, a slight mitochondrial swelling is seen, and the cristae of the mitochondria are beginning to fragment. As the mitochondria progressively swell, acid phosphatase is released into the cell cytoplasm by the disruption of the limiting lysosomal membranes (Figs. 98a, 98b). There are, however, some lysosomes whose limiting membranes appear intact, and such lysosomes can withstand osmotic changes in the cell (Figs. 99, 98c) Eventually, most of the organelles, including the mitochondria disaggregate except lysosomes (Fig. 100). As shown in Fig. 100, the reaction product completely fills the internal composition of the lysosomes. Finally the membrane of the affected host cell breaks down, and on lysis, the cellular contents are discharged into the surrounding medium, carrying with it the uninterrupted lysosomes (Fig. 101), whose membranes again appear intact. The lysosomes, however, soon unable to cope with the culture medium's osmotic pressure burst (Fig. 102) and the lysosomes are now irregularly shaped.

No changes in the intensity of the reaction product in the amoebae are detected throughout the interaction, although the vacuolar contents, which are probably ingested RK13 components, of the amoebae contain acid phosphatase (Figs. 103, 104).

Surface-active lysosomes bearing the reaction product are never seen in the plasmalemma of the contacted amoebae, and in one preparation, a surface vacuole contains no acid phosphatase. This vacuole is seen to discharge its fluid contents extracellularly (Fig. 105).

D.3.1b Non-specific esterase

Light microscopy : a) α-naphthyl acetate method : Sections of mouse kidney demonstrate sites of enzyme activity appearing in the cytoplasm of convoluted tubules (Fig. 198). The reaction product has slightly diffused into the brush border of the tubules. No reaction is present in the glomeruli.

RF13 cultured cells show a very strong cytoplasmic reaction of non-specific esterase; so strong that its nuclei cannot be seen (Fig. 106). Entamoeba histolytica trophozoites show no reaction.

Non-specific esterase in RF13 cells surrounding the amoebae enhances after prolonged interaction. Figs. 107 and 108 illustrate both the progressive increase in size of the lesions and the enhancement of non-specific esterase in affected cells. Fig. 107 was taken after 15 minutes of interaction and Fig. 108 after 120 minutes. It was not possible to investigate whether such increase in reaction product is due to disruption of lysosomes.

During the initial stage of contact, unlysed cells surrounding the amoebae show an alteration in microvilli length (Fig. 109).

Eventually a lesion is developed. The amoeba initially shows no enzyme activity (Fig. 201). After one hour of interaction definite particle-bound reaction products are present within the amoebae (Fig. 202).

No enzyme reaction in RF13 cells is seen when the substrate is omitted from the incubation medium.

b) Indigogenic indoxyl method : Sections of mouse kidney show enzyme activity in the cytoplasm of convoluted tubules (Fig. 203). No reaction product is found in the glomeruli. This

method was used only on E. histolytica trophozoites and no reaction site of non-specific esterase is detected even after 18 hours of incubation at 37°C.

a) Indoxylase method : A pattern of discrete brown droplets is observed in RPMI cells, and this is interpreted as being sites of lysosomes (Fig. 110). The reaction product is also deposited throughout the cytoplasm of some cells; an indication that diffusion of the final reaction product has taken place. The colour of the cytoplasmic diffused reaction varies from purple to dark brown.

The enzyme staining again appears to be on an all or nothing basis as lysosomes show either appreciable deposits or none at all. Lysosomes of the giant multinucleate cells tend to stain more heavily than those of normal cells (Fig. 111). The trophozoites show no reaction. As lysosomes in RPMI cells bearing the reaction product are few in number, the indoxylase method was not used to investigate the responses in the lysosomal non-specific esterase of host cells to amoebae.

D.3.1c Aryl sulphatase

Light microscopy : a) Lead-nitrocatechol sulphate method : Granular deposits are easily observed in the cytoplasm of the RPMI cultured cells (Fig. 112). Although deposits are seen in lysosomes, slight cytoplasmic and nuclear staining varies from cell to cell and is more prominent in giant multi-nucleate cells (Fig. 113). The lysosomes bearing aryl-sulphatase are generally smaller than those containing acid phosphatase. No staining is observed in the amoebae.

The infected cells initially show no enlargement of lysosomes (Fig. 114). Prolonged contact either leads to a

progressive increase in the lysosomal size (Fig. 115) or causes no further change in the size of the membrane-bound particles.

b) Simultaneous coupling method : The coupling method was used only once. No enzymatic reaction product can be observed either in the HM1J cells or even in the control kidney sections.

Electron microscopy level : The fine granular barium particles are deposited over dense bodies within the lysosomes of unaffected HM1J cells. The distribution of the reaction product varies according to the shape of the dense body; it can be distributed either irregularly (Fig. 116) or evenly (Figs. 117a, 117b). Occasionally, small barium precipitates appear in small vacuolar-like structures, presumably primary lysosomes (Fig. 117b). Preservation of the nucleus and the mitochondria is adequate the distribution of enzyme activity in HM1J cells at electron microscopy level is, however, much lower than that under light microscopy. It is possible that a longer period of glutaraldehyde fixation has an inhibitory effect on enzyme activity.

No staining is detected over lysosomal dense-bodies in HM1J cells incubated in a substrate-free medium (Fig. 118).

No staining is seen in the intranuclear bodies or the rest of the nucleoplasm of the amoebae (Fig. 119). In some cases, reaction product is confined to the contents of the vacuoles which are probably remnants of Crithidia sp. (Fig. 120). No staining is detected on the vacuolar membranes.

Changes in the intensity of the reaction product in the lysosomes of infected cells are not as dramatic as those recorded for acid phosphatase. The reason for this is that there are fewer labelled lysosomes in this preparation. After initial contact, no change in the intensity of the reaction product in both the primary

and secondary lysosomes is found in contacted cells (Fig. 121). In cells with swollen mitochondria, no further spread or release of aryl sulphatase into the cytoplasm is detected (Fig. 122). The contacted amoeba shows no change in the intensity of the reaction product. During prolonged interaction, no staining is even found in the nucleus of the amoebae. Fig. 123 illustrates the pinching action of the amoeba which has penetrated between the cells. The host-cytoplasm is beginning to be drawn into a phagocytotic channel of the amoebic cytoplasm. In this section, no aryl sulphatase reaction product can be visualized.

D.3.1d β-glucuronidase :

Light microscopy : The stained granules are localized to the cytoplasm of convoluted tubules of mouse kidney (Fig. 204). No reaction, as in other lysosomal enzymes investigated, is observed in the glomeruli. Staining is absent when kidney sections are incubated in a medium lacking substrate. The RK13 cells react weakly with the substrate although a slight pink-coloured diffuse reaction product is detected across the monolayer. No visible reaction is detected in trophozoites.

The first stage of infection produces no change in the reaction product in either Entamoeba histolytica or the contacted RK13 cells (Fig. 124). The damaged cells later show an increased activity in β-glucuronidase (Figs. 125, 205). In this preparation, the nuclei are deeply counterstained with methyl green and consequently the shape and density of the nucleus can be traced. The nuclei of the infected cells condense, characterised by an increase in the density of the nucleus as well as its being more spherical. Prominent deposits are also seen in the vacuoles of E. histolytica trophozoites after one hour of contact (Fig. 206).

D.3.1e β-galactosidase :

Light microscopy : Purplish-blue granular deposits are localized in the cytoplasm of the convoluted tubules of mouse kidney. No reaction is detected in the glomerulus (Fig. 126). Both the cultured cells and the amoebae contain β-galactosidase (Fig. 127). This method, however, is not sensitive enough to record differences in the responses of the enzyme in RK13 cells after interaction with the amoebae, as the coloured reaction product varies greatly between one preparation and another.

D.3.1f VI N-Acetyl-β-D-glucosaminidase :

Only glucosaminidase is detected in Entamoeba histolytica. No reaction product is found to spread from amoeba to cells throughout the interaction period (Fig. 207).

D.3.2. Enzymes localized on plasma membrane

D.3.2a Alkaline phosphatase :

a) Gomori technique : A fine granular deposit of the enzyme reaction is seen around the intima of blood vessels in mouse kidney sections (Fig. 128a). At light microscopic level, no deposit is visualised in both the RK13 cells and the trophozoites. No alkaline phosphatase is detected in either the contacted amoebae or the infected cells throughout the interaction period. At electron microscopic level, the alkaline phosphatase staining along the plasma membrane of the RK13 cells is extremely weak (Fig. 129).

b) Azo-dye technique : Fine red deposits are present around the intima of blood vessels in mouse kidney sections (Fig. 128b). Staining is absent in both RK13 cells and amoebae.

D.3.2b Magnesium activated ATPase :

Light microscopy : Sections of mouse kidney demonstrate sites of enzyme activity in the glomeruli, brush border and

basement membrane of the tubules (Fig. 130). No reaction is present in the cytoplasm of the convoluted tubules.

The plasma membrane of RK13 cells is actively stained with the reaction product (Fig. 131). The amoebae demonstrate no enzyme activity (Fig. 132). After initial contact between the RK13 and the amoebae, no difference in plasma staining is detected. Progressive infection leads to a more diffuse spread of reaction product among the cells surrounding the amoebae (Fig. 133). The mucoid threads of the amoebae are heavily studded with reaction product (Fig. 134). After prolonged contact, plasma membrane staining is very pronounced in areas where cells are affected (Fig. 135). The plasma membranes of the amoebae eventually show up the reaction product (Fig. 135).

Electron microscopy : The plasma membrane of the RK13 cells shows much reaction product (Figs. 136a, b). Slight staining is present in the mitochondria, which also have ATPase. Mitochondria ATPase, however, is not fully revealed as glutaraldehyde fixation has an inhibitory effect on mitochondrial ATPase activity.

Very slight plasma membrane staining is detected in cells incubated in a substrate-free medium (Fig. 137). This presumably indicates a slight non-specific binding of lead. Initially, there is no change in the intensity of the reaction product on the plasma membrane of RK13 cells surrounding the amoebae. Not all cells are stained with granular deposit. In Fig. 138 (after 10 minutes of interaction) no ATPase reaction product is detected along the plasma membrane of the infected cells. Prolonged contact leads to a significant increase in Mg^{2+} ATPase activity along the borders of the infected cells (Fig. 139). During the initial stage of infection, no reaction product is detected on the plasmalemma of the amoeba

(Fig. 140). The mucoid threads are very prominent in this preparation and they extend from the uroid end (Fig. 140). No ATPase is detected in the uroid, which is an active region where particulate matter is ingested (Fig. 141).

As cells surrounding the amoeba are destroyed, the mucoid threads in the lesion become studded with reaction products detached from the injured cells' plasma membrane (Fig. 143). These threads, however, are not apparent after normal staining. In Fig. 143 the amoeba is actively taking in fluid droplets through the uroid by pinocytosis.

Due to the dynamic nature of the plasmalemma of the amoeba, any debris, such as in this instance pieces of cell plasma membrane containing the ATPase reaction granules, are swept to the uroid end as the amoeba moves forward, eventually leading to a large accumulation of cell debris (Figs. 142, 144b). These deposits are then taken into the amoebic cytoplasm either by phagocytosis (Fig. 144a) or by pinocytosis leading to a small vesicular-like bulb (Figs. 145a, b). Later the deposits are seen in amoebic cytoplasmic vacuoles (Fig. 146).

Fig. 147 reveals numerous projections along the base of the amoeba in contact with the glass-substrate. These projections are also seen in scanning electron microscopy preparations (Fig. 44a).

D.3.2c Calcium activated ATPase :

Light microscopy : As in the case of magnesium-activated ATPase, mouse kidney sections show sites of reaction product actively appearing in the glomeruli, the brush border and basement membranes of the tubules (Fig. 148).

The enzyme pattern in the RF13 cells is the same as that

of magnesium-activated ATPase, although the overall staining is relatively weaker. The plasma membrane staining is more pronounced in areas where cells are in contact with amoebae (Fig. 149). In such areas, a faint cytoplasmic staining is detected. Eventually ingested HELJ debris containing the enzyme activity is taken into the vacuoles of the amoeba (Fig. 150).

D.3.2d Nitrophenyl phosphatase :

Light microscopy : The reaction product in the mouse kidney sections is distributed densely in the glomeruli, the basement membrane and the brush border of the tubules (Fig. 151). Non-enzymatic deposition of lead is visible in the nuclei of convoluted tubules (Fig. 151). The demonstrable K^{+}-NPPase activity in the stained areas is not inhibited by ouabain (Fig. 152) although staining intensity is slightly reduced.

Very little enzyme activity is present on the plasma membrane of HELJ cultured cells (Fig. 153). After 18 hours of incubation, very few reaction products are localised on the plasma membrane, although a slight yellowness is detected in the incubation medium, which indicates specific enzymatic hydrolysis of the substrate, *p*-nitrophenyl phosphate. No NPPase reaction product is found on the plasma membrane of trophozoites.

D.3.3. Enzymes of endoplasmic reticulum and Golgi apparatus

D.3.3a Thiamine pyrophosphatase (TPPase) :

Under light microscope, the Golgi apparatus staining is only readily appreciated in some areas of the formaldehyde-fixed HELJ monolayer (Fig. 154). The plasma membrane is extensively stained with reaction product (Fig. 155). In vacuolated cells, TPPase staining is very pronounced in the vacuoles (Fig. 156). This technique for the localisation of TPPase was repeated twice

and the results were consistent. The distribution of enzyme activity seen under the electron microscope shows good correlation with that seen using light microscopy (Fig. 157). Plasma membrane and vacuolar staining is absent in cultured cells incubated in a substrate-omitted medium (Fig. 158).

During the initial stage of contact, the microvilli, which are visualised by TPPase staining of the cells surrounding the amoeba, alter in length and extend towards the amoeba (Fig. 159). After prolonged contact, no alteration in enzyme activity is detected either in the RPMI cells or in the amoebae.

B.3.3b Inosine diphosphatase (IDPase) :

As for TPTase, the plasma membrane and vacuolar staining is seen with inosine diphosphatase staining (Fig. 160). Endoplasmic reticulum staining was difficult to visualise in this study. No staining is detected in cultured cells incubated in a medium without substrate (Fig. 161). No reaction product is detected in trophozoites except during prolonged interaction where granular deposits are visible in the amoebic cytoplasmic vacuoles (Fig. 162).

B.3.4 Peroxisome enzyme

Catalase : Catalase is only present in trophozoites (see p. 77). No enzyme is detected in the RPMI cells. There is no spread of the reaction product from the amoebae when they are added to the cultured cells (Fig. 163).

B.3.5 Mitochondrial enzyme

Mitochondrial ATPase : Mitochondria are easily seen in RPMI cells; its corresponding ATPase staining is very intense (Fig. 164). The cultured cells display various sizes of mitochondria. Rod-like shapes, elongated, short and stumpy forms are seen (Fig. 165). The reaction product is absent in the trophozoites.

After contact, the mitochondria, indicated by appropriate enzyme staining, in contacted cells are dramatically altered from an elongated to a rounded shape (Fig. 166). Cytoplasmic staining is very pronounced in such cells, resulting from breakdown of the mitochondria membrane releasing ATPase into the cytoplasm (Fig. 166). Further contact leads to complete breakdown of the mitochondria (Fig. 167). Here, the amoeba has ingested the mitochondria.

D.3.6 Leucine aminopeptidase

A diffuse enzyme reaction product is seen in the cytoplasm of the tubules of the mouse kidney (Fig. 172). No reaction is present in either the BHK cells or the amoebae. No reaction is detected in either the interacted amoebae or culture cells after prolonged contact.

D.4 ELECTRON MICROSCOPIC EXAMINATION OF THE EFFECT OF A HOMOGENATE OF E. HISTOLYTICA (EVANS STRAIN) ON A CELL-LINE MONOLAYER

D.4.1 The structure of the normal unaffected RF13 cultured cells is shown in Figs. 168 and 169. The mitochondria, known for their pleomorphism, show variation in shape, and the cristae are not as abundant as those of liver cultured cells. The extent of the penetration of the cristae through the matrix is also variable (Fig. 169), as some of the cristae reach from one end of the inner chamber to the other.

The cytoplasmic matrix mostly consists of polyribosomes and filaments (Fig. 168). The inclusion bodies are probably secondary lysosomes. The cytoplasmic matrix is occupied by a rough endoplasmic reticulum, which is studded with ribosomes. The cisternae of the rough endoplasmic reticulum are short and do not always occur in loosely arranged groups of closely packed parallel stacks.

At places along the junction between the cells, interdigitating folds are seen, and they play an important role in cell-to-cell adhesion (Fig. 168). Firmer cell-to-cell attachment is accomplished by modifying the cell surface to form 'tight junctions' which are recognised in Fig. 168 by denser lines at certain places along the junction.

D.4.2 Addition of homogenate

The addition of homogenate or extract of Entamoeba histolytica trophozoites grown for 48 hours has no effect on the monolayer. The cells have not detached themselves from the glass. The cells still retain contact with neighbouring cells (Fig. 170). Under higher magnification, the interdigitating folds and tight junctions are not affected, an indication that the cells have retained their rigidity (Fig. 171).

The mitochondria continue to show pleomorphism and the cristae are not fragmented (Fig. 171). No alteration in density of both the mitochondrial and endoplasmic reticulum matrix is seen. The endoplasmic reticulum has not lost its morphology (Fig. 171).

The nucleus is unaffected as chromatin margination is not seen. Ballooning of the nuclear envelope is absent (Fig. 173).

Figs. 171, 172 and 173 were taken from samples which were subjected to the amoebic homogenate for 48 hours.

D.5 ULTRASTRUCTURAL STUDY OF INTERACTION OF AXENIC STRAINS 200:NIH AND HM-1:IMSS ON RK13 CELL-LINE MONOLAYER

D.5.1 HM-1:IMSS and RK13

The observations recorded below were made on materials which were fixed after 2 hours of HM-1:IMSS and RK13 cell monolayer interaction. The structure of the undamaged RK13 cultured cells is shown in Fig. 174. The trophozoites, intersoting with RK13, contain most of the organelles which have been described in other axenic strains (Lowe and Musgraith, 1970a; Proctor and Gregory, 1972a, El-Sashimi and Pittman, 1970; Faria-Velasco and Trevino, 1972). The intranuclear bodies, electron-dense fibrillar structures, ribosonal helices within double-membraned vacuoles, glycogen particles and vacuoles are all present in HM-1:IMSS trophozoites (Fig. 175). Fig. 175 also shows the presence of short, smooth-walled vesicles referred to by the same authors as part of smooth endoplasmic reticulum. Sub-pellicular bodies are abundant in this axenic strain (Fig. 177) and they lie at irregular intervals along the plasmalemma.

After prolonged contact, the cells are damaged in the same way as that described for BD-VI and CV-1 cultured cells. Only cells in contact with amoebae are damaged, and they rapidly lyse, releasing cytoplasmic contents into the external medium (Fig. 176). Cells next to the lysed cells show incipient damage as swollen mitochondria can be seen. Some of the ruptured contents remains attached at the uroid end of the amoeba (Fig. 176).

At higher magnification, the mitochondria have almost lost their cristae, and the matrix becomes paler. The outer membrane of some of the mitochondria has ruptured. The endoplasmic reticulum is forming vesicles and these too are swollen (Fig. 178). Mitochondrial swelling, however, does not occur in every contacted cell,

as Fig. 179 shows an injured cell with condensed mitochondria.

Along the area of contact, there is an increase in activity on the amoebic side. Here, the membrane becomes undulated forming micropseudopodia (Fig. 180). Some of the cytoplasm of the injured cell is drawn into the phagocytotic channel.

The amoeba can probe any intracellular cavity between cultured cells (Fig. 181). Part of the amoebic pseudopodium, as shown in Fig. 181 has already penetrated the cavity and the rest of the body will follow, widening the gap even more.

It appears that virulent and avirulent individuals can exist within a population of a cultivated strain, as one intercellular amoeba is seen and is attempting to phagocytose normal cells (Fig. 182a). Such cells are not affected as the organelles show no change (Fig. 182b). The cell cytoplasmic matrix, however, is denser than that of neighbouring cells, probably due to the amoeba compressing the contacted cell against its neighbours.

No surface-active lysosomes is seen in this preparation. There is, however, as shown in Fig. 178, a surface vacuole which gives the impression that should this vacuole collapse it would give the appearance of a surface-active lysosome.

5.5.2 200:NIH and NIH

As a relatively small number of samples was studied it was not possible to identify all the organelles described by Proctor and Gregory (1973b) in their study on the ultrastructure of axenically cultivated trophozoites of E. histolytica strain NIH:200. It is noticeable, however, that the number of intranuclear bodies per nucleus is high (Fig. 183). In one section, also, a crystalline structure resembling an intranuclear body is seen in the cytoplasm (Fig. 184).

After 2 hours of interaction, the cells surrounding the amoebae are found to be undamaged (Figs. 185a, b; 186). The organelles of these cells such as mitochondria, and endoplasmic reticulum show an absence of swelling. One section, however, shows a piece of cell being ingested (Fig. 187), and the density of its matrix is the same as that of normal untouched cells, indicating that the trophozoites of NIH:200 axenic strain have lost their virulence on host-cells.

D.6 ELECTRON MICROSCOPY OBSERVATIONS ON THE EFFECT OF AN ANTIHISTAMINE, PROMETHAZINE HYDROCHLORIDE, ON THE INTERACTION BETWEEN E. HISTOLYTICA AND A CELL-LINE MONOLAYER

The BHK3 cells were infected with E. histolytica trophozoites of Kwama monoxenic strain. In one preparation, promethazine hydrochloride (10^{-4}M) was added together with amoebae to the monolayer. In the other, only the amoebae were added. Both preparations were left for 2 hours, and the findings then compared.

D.6.1 Without the addition of antihistamine

The cells surrounding the amoebae are rapidly destroyed (Fig. 188). Fig. 189 also shows an unbonded amoeba as the external surface appears ragged, and its nucleus is not spherical.

The organelles within the contacted cells are greatly affected as mitochondrial swelling, degranulation of endoplasmic reticulum and swelling of the cisternae of the endoplasmic reticulum are observed as recorded in section D.2. At this stage, chromatin migrates towards the periphery of the nucleus (Fig. 189). When the contact is prolonged, lysosomes finally rupture (Fig. 190). The cytoplasmic matrix becomes diluted as evidenced by a decrease in density. The surface of the contact cell opposite to the amoeba has lost its normal relationship to neighbouring cells, as the interdigitating folds and tight junctions have disappeared (Fig. 190). Nuclear margination is evident in Fig. 190 in which also the chromatin is disappearing from the nucleus.

As the surface membrane of the contacted cell loses its rigidity, it easily falls prey to the approaching amoeba, which finally grips a piece of the cell cytoplasm (Fig. 191).

When the cells lyse, the cellular organelles are released into the extracellular fluid. The organelles then adhere to the

amoebic plasmalemma. As the amoeba moves forward, the organelle debris is swept backwards towards the uroid (Fig. 192).

D.6.2 With the addition of promethazine hydrochloride

The cells surrounding the amoeba are unaffected (Figs. 193, 194 and 195). All 3 figures show that the antihistamine effectively prevents the swelling of susceptible organelles. As the cell plasma membrane is not grossly affected, the cell retains its rigidity. Penetration of micropseudopodia into the cell is thus impeded (Fig. 195). Such activity, however, does not prevent phagocytosis occurring as the whole cell seems to be drawn into the cytoplasm of the amoeba (Fig. 196a). This apparently uninjured cell is in good contact with its neighbour, and its mitochondria and nucleus appear perfectly healthy. At the end of the phagocytotic channel, the cell cytoplasm is fragmented and has been rendered into small vesicles, which may bud from the channel and fuse with lysosomes (Fig. 196b).

Promethazine at a concentration of 10^{-4}M does not interfere with the structure of amoebic plasmalemma as filopodia are continually formed at that part of the amoeba (Fig. 197) in contact with the glass slide.

B.7 CHROMIUM (^{51}Cr) RELEASING CYTOTOXICITY TESTS

B.7.1 Cytotoxicity induced by trophozoites of E. histolytica and E. invadens

Results from an experiment in which the trophozoites were added to cultures at various dilutions are depicted in Table VIII and Fig. II. The greater the ratio between the amoebae and the labelled Chang cells, the higher the chromium ^{51}Cr release. The shape of the curves obtained is similar in both the E. histolytica and E. invadens tests, but the slopes of the curves differ in that the slope is steeper when E. histolytica is used, indicating a higher cytotoxic effect of this strain than that of the E. invadens trophozoites. At higher amoebic concentrations (1 amoeba to 0.32 Chang cell), the chromium specific release varied especially when E. invadens trophozoites were used. Such a variation may be due to the competition among an amoebic population. As a result of the above studies, further experiments on cytotoxicity were carried out by using ratios of 1 amoeba to between 1 and 3.2 labelled Chang cells.

B.7.2 Cytotoxicity induced by homogenates of trophozoites of E. histolytica and E. invadens

The results of an experiment in which the homogenate was added to the labelled cultures at various dilutions are depicted in Table IX. The ultrasonicated homogenate was found to have no effect on the Chang cells, since the level of % specific cytotoxicity for all dilutions of homogenate was never more than 1.1%, a very low figure.

B.7.3 Cytotoxicity induced by supernatant medium from 48-hour amoebic cultures

The results from such an experiment are shown in TableIX. The supernatant medium was found to have no effect on Chang cells; an indication that cytotoxic substances is not secreted extracellularly

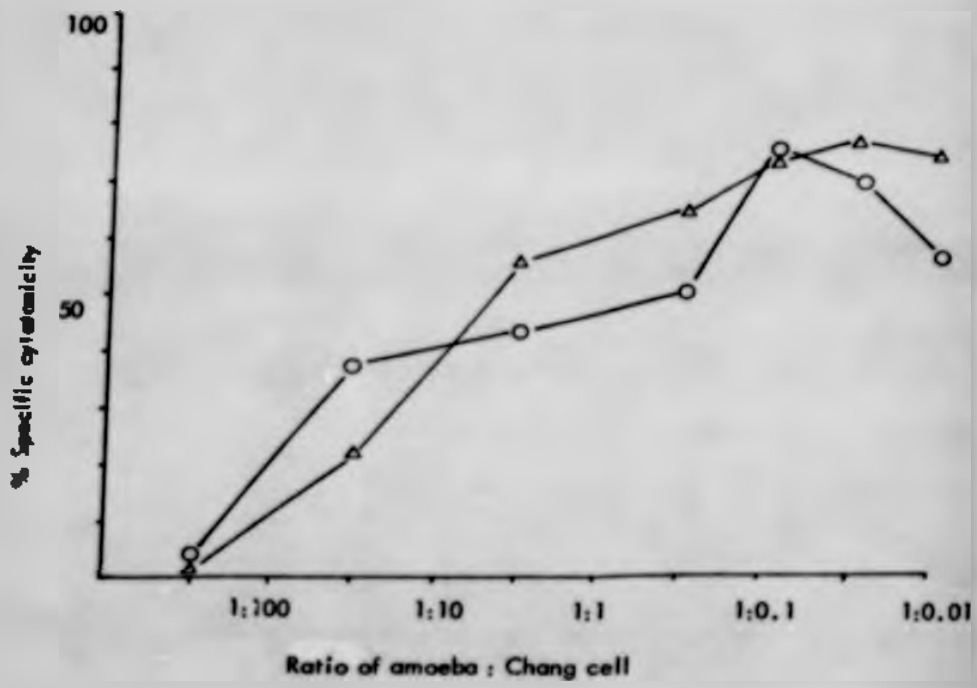

Fig. II Specific release of ^{51}Cr from labelled Chang cells exposed to trophozoites of E. histolytica and E. invadens as a function of log. ratio of amoeba : Chang cell. Symbols : (△) E. histolytica and (○) E. invadens.

TABLE VIII

CYTOLYTIC ACTIVITY OF *E. HISTOLYTICA* AND *E. INVADENS*

Ratio of trophozoites to labelled Chang cells	^{51}Cr release[a]	% Specific cytotoxicity
Ax. 200 (*E. histolytica*)		74.3
100 : 1	83.6 ± 0.6	74.3
32 : 1	86.8 ± 4.7	77.5
10 : 1	81.9 ± 0.4	72.6
3.2 : 1	73.1 ± 3.1	64.1
0.32 : 1	65.3 ± 1.5	56.3
0.032 : 1	29.4 ± 8.3	20.4
0.0032 : 1	8.3 ± 0.3	-0.7
BAR. (*E. invadens*)		
100 : 1	69.1 ± 1.9	54.5
32 : 1	80.9 ± 0.4	70.3
10 : 1	87.9 ± 0.5	77.3
3.2 : 1	55.2 ± 1.5	48.4
0.32 : 1	49.5 ± 0.2	42.7
0.032 : 1	45.5 ± 1.5	38.7
0.0032 : 1	10.9 ± 0.6	4.1
Controls : [b]		
Eagle's medium	10.4 ± 0.4 11.9 ± 0.4	-
TPS-1 (amoebic medium)	9.0 ± 2.0 9.3 ± 0.5	-

[a] The results are expressed as the means ± s.d. of three experiments

[b] Controls were used in the absence of trophozoites to assess background release of ^{51}Cr

TABLE IX

INFLUENCE OF THE HOMOGENATE AND SUPERNATANT FROM 48 hr ANOXIC CULTURE ON THE RELEASE OF ^{51}Cr FROM CHANG CELLS

Ratio of amoebae to labelled cells	% ^{51}Cr release*a	% Specific cytotoxicity
Homogenate		
Ax. 200 :		
0.32 : 1	7.4 ± 0.3	-1.8
0.032 : 1	5.9 ± 0.6	-3.1
0.0032 : 1	6.1 ± 0.2	-2.9
HAH :		
0.32 : 1	6.7 ± 0.5	-0.1
0.032 : 1	7.9 ± 1.7	1.1
0.0032 : 1	6.8 ± 0.7	0.0
Supernatant		
Ax. 200	9.2 ± 0.3	-0.1
HAH	11.4 ± 2.0	2.1

*a The results are expressed as the means ± s.d. of three experiments

into the environment.

D.7.4 Influence of different substances on the cytotoxic action of E. histolytica and E. invadens

D.7.4a Promethazine hydrochloride

The results in which the inhibitor was added to the mixture of amoebae and Chang cells are shown in Table X . Promethazine hydrochloride (10^{-4}M - 10^{-5}M) has been shown to block the cytotoxic action of E. histolytica on RK13 cells (section D.6) by stabilizing the cellular membrane integrity of the cells. The addition of the antihistamine ($3x10^{-3}$M to $3x10^{-5}$M) with the amoebae to Chang cells had no inhibitory effect on the efflux of ^{51}Cr from Chang cells in contact with either E. histolytica or E. invadens trophozoites. When the cells however were suspended in promethazine hydrochloride alone at the same concentrations, the levels of the percentage of specific cytotoxicity were found to be remarkably high (between 51% to 68%). As the Chang cells were found to be sensitive to the inhibitor at the concentration required to protect the cells, it was not possible to elucidate fully the action of the inhibitor on the release of ^{51}Cr. Although the inhibitor had *no effect on the Chang cells at lower concentrations* (between $3x10^{-5}$ to $3x10^{-6}$M), the cytotoxic effect due to E. histolytica was found to be uninhibited at such concentrations. In the case of E. invadens the inhibitory effect of promethazine hydrochloride was found to be very slight; a 7% inhibition is noted at $3x10^{-6}$M, and 14% at $3x10^{-5}$M.

D.7.4b Rosenthal's inhibitor

The results in which the inhibitor was added to the mixture of amoebae and labelled Chang cells are tabulated in Table XI . Figs. III and IV show the dose-response effect of this analogue on the cytotoxic reaction. At higher concentrations of the

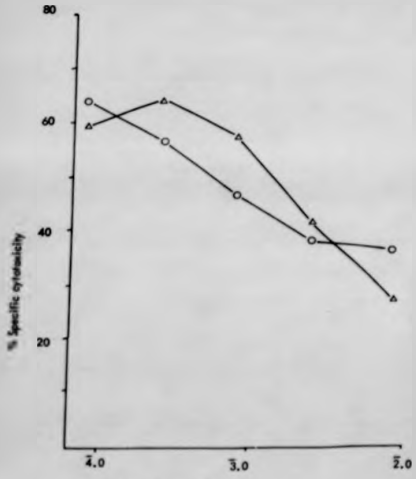

Fig. III Inhibitory effect of Rosenthal's inhibitor (RH) on specific release of ^{51}Cr from labelled Chang cells exposed to trophozoites of E. histolytica and E. invadens as a function of log. concentration of the inhibitor. Symbols : (O) E. histolytica and (Δ) E. invadens.

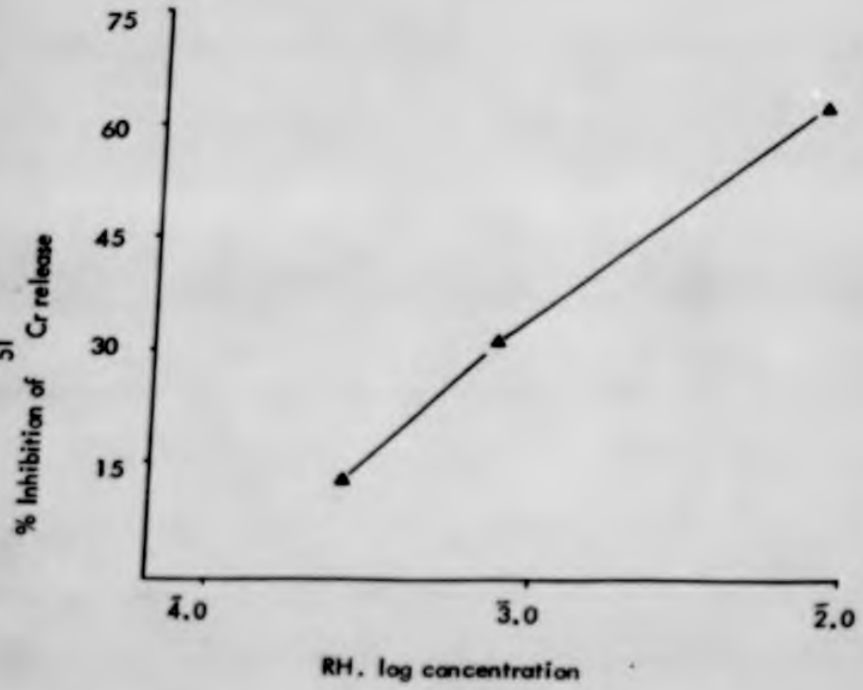

Fig.IV Inhibition of specific release of ^{51}Cr from labelled cells exposed to trophozoites of E. invadens by Rosenthal's inhibitor (RH).

TABLE X

EFFECT OF PROMETHAZINE HYDROCHLORIDE ON CELL LYSIS

A. Concentration of inhibitor in M	% ^{51}Cr release	% Specific cytotoxicity	
Ax. 200 strain[a]		I	II
3×10^{-3}	76.7 ± 3.8	69.6	[d]
3×10^{-4}	69.3 ± 3.4	61.8	[d]
3×10^{-5}	62.3 ± 1.4	54.8	60.5
3×10^{-6}	71.2 ± 1.1	63.7	70.3
BAH strain			
3×10^{-3}	75.7 ± 0.5	68.9	[d]
3×10^{-4}	69.2 ± 0.9	62.4	[d]
3×10^{-5}	52.2 ± 4.8	45.4	50.6
3×10^{-6}	55.5 ± 1.0	48.7	54.4
B. Controls			
a) without inhibitor :			
Ax. 200	73.1 ± 3.1	64.1	
BAH	65.3 ± 0.7	58.5	
b) with inhibitor and without amoebae :			
3×10^{-3} M incubation at 37°C	74.6 ± 0.9	67.8	
" 26°C	73.1 ± 0.2	65.6	
3×10^{-4} M 37°C	58.7 ± 4.0	51.9	
" 26°C	61.5 ± 2.4	54.0	
3×10^{-5} M 37°C	8.6 ± 1.0	1.8	
" 26°C	9.1 ± 0.6	1.6	
3×10^{-6} M 37°C	7.6 ± 0.6	0.8	
" 26°C	8.4 ± 0.8	0.9	

[a] Ratio of amoebae to labelled cells was maintained at 3.2 : 1

[b] Specific cytotoxicity was assessed by the total percentage of ^{51}Cr released from the cells subtracted by the percentage of the label released in the absence of amoebae.

[c] As for [b], except that inhibitor at varying concentrations was added

[d] The controls were unsatisfactory

TABLE XI

EFFECT OF ROSENTHAL'S INHIBITOR ON CELL LYSIS

A. Concentration of inhibitor in M	% ^{51}Cr release	% Specific cytotoxicity		% inhibition of cytotoxicity
Ax. 200 strain*a		I*b	II*a	
7.3×10^{-3}	45.3 ± 0.7	36.3	6.4	90
2.3×10^{-3}	46.5 ± 5.0	37.5	-3.2	-
7.3×10^{-4}	55.8 ± 1.6	46.8	-	-
2.3×10^{-4}	65.3 ± 1.5	56.3	54.0	15
BAN strain				
7.3×10^{-3}	33.6 ± 0.6	26.6	11.7	80
2.3×10^{-3}	48.3 ± 0.1	41.5	30.3	48
7.3×10^{-4}	64.4 ± 0.7	57.6	-	-
2.3×10^{-4}	69.9 ± 1.1	63.1	62.0	0
B. Controls				
a) without inhibitor :				
Ax. 200	73.1 ± 3.1	64.1		
BAN	65.3 ± 0.7	58.5		
b) with inhibitor and without amoebae :				
7.3×10^{-3} M at incubation 37°C	38.9 ± 6.5	29.9		
" 26°C	21.9 ± 0.9	15.1		
2.3×10^{-3} M 37°C	48.2 ± 0.9	40.7		
" 26°C	18.0 ± 0.8	11.2		
2.3×10^{-4} M 37°C	9.8 ± 0.4	2.3		
" 26°C	7.9 ± 0.8	1.1		

*a, b, c See Table I

inhibitor, the release of ^{51}Cr was markedly reduced. The specific cytotoxicity of E. histolytica at 7.3×10^{-3}M concentration was found to be reduced from 64% to 36%; a 90% inhibition (See Table XI). Similarly, the specific cytotoxicity of E. invadens at 7.3×10^{-3}M was found to be reduced from 58% to 26.8%; a 80% inhibition (see Table XI). When labelled Chang cells were incubated at 37°C in the presence of higher concentrations of inhibitor (7.3×10^{-3}M and 2.3×10^{-3}M) the % Cr release was slightly raised (see Table XI). When the controls, however, were incubated at room temperature, the amount of ^{51}Cr release was lowered; an indication of the preference of Chang cells for incubation at room temperature in the presence of the inhibitor.

5. DISCUSSION

5.1 ULTRASTRUCTURAL AND CYTOCHEMICAL STUDIES OF TROPHOZOITES OF E. HISTOLYTICA

5.1.1 Scanning electron microscopy

Scanning micrographs taken during this study show clearly that the surface of E. histolytica trophozoites is rough with irregular infoldings. Large depressions or trigger-like organelles (Eaton et al., 1969, 1970), craters or pores of different sizes (Proctor and Gregory, 1973b; Proctor, 1974) which have lysosomal functions are not identified in the present study.

I agree with Nagmudda et al.(1970) that the two scanning electron micrographs published by Eaton et al. do not demonstrate "trigger-shaped organelles" but can be interpreted as showing tiny protuberances which project into the cavities of craters. Eaton et al. (1969), Nagmudda et al. (1970), Proctor and Gregory (1973b) and Proctor (1974) all have presented scanning micrographs which I believe to be showing degenerated amoebae. These protozoa are rounded and show surface depressions. Their pseudopodia are poorly developed and no uroid is observed. These authors used a technique necessitating centrifugation and suspension during fixation. Such a step is likely to alter the surface morphology leading to artefact. Eaton et al. (1969) although stating that the emphasis was always on obtaining material with the least possible disturbance to the amoebic surface, added suspension of amoebae to the fixative. In their case, 'in-situ' fixation was not attempted. Nagmudda et al. (1970) centrifuged their material before fixation. Proctor and Gregory (1973b) and Proctor (1974) did not specify the method used. In this study, the amoebae were fixed 'in-situ' to maintain the relationship between amoebae and substrate and to preserve extracellular structures such as the uroid, pseudopodia and surface

vacuoles. Martinez-Palomo et al. (1974) agree that in-situ fixation, as proposed by Bird (1961) offers certain advantages over standard fixation methods which involve physical treatment. There is a further feature which has not been noted in previous microscopic studies of the amoeba, namely filopodia*. These extend at places along the base of the amoebae (Fig. 44a). Micrographs of thin sections of fixed specimens also illustrate their presence (Figs. 23, 197). As swellings are sometimes present at the tips of such protrusions, the filopodia may assist in attachment to the substrate. The structural significance of such a finding remains open to speculation. It is likely, however, that the structural and functional role of these protrusions reflects a heightened activity of the amoebic surface membrane.

E.1.2 Amoebic vacuoles

As suggested by Ludvik and Shipetowa (1970), the most morphologically varied organelles in the cytoplasm are the vacuoles. The most abundant are the food vacuoles and within such vacuoles membranous whorls, concentric rings or myelin-like figures are observed, as described by previous workers (Ludvik and Shipetowa, 1970; Lewa and Neagraith, 1970a; Procter and Gregory, 1972a). These membranous whorls come from digestion of bacteria as suggested by Schuster (1963). The bacterial cell wall polysaccharide consists of a disaccharide attached to a tetrapeptide and the whole unit, a murepeptide, is made up of N-Acetyl-β-glucosamine and N-Acetyl-muramic acid joined by a β-linkage (Bronk, 1973).

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*Filopodia is a term used to cover situations where long, slender cell processes or long extensions of the cell cytoplasm covered by cell membrane are observed (Thadially, 1975). Such filopodia have been noted in diploid WI-38 culture cells that are in the process of attachment onto a glass surface or substratum after trypsinization (Rajaraman et al., 1974). These authors suggested that the spherical tips of filopodia may play a direct role as specialised structure of attachment to the substratum.

The unit is linked in two dimensions to form a net-like structure. The disaccharide units join to form polysaccharide chains and these are cross-linked by their respective peptide side chains.

The polysaccharide portion of the peptidoglycan framework has been located ultrastructurally and biochemically on the cytoplasmic membrane in the cell envelope (Costerton et al., 1974). Cytochemical testing (see p. 103) has shown that N-Acetyl-β-D-glucosaminidase is localized to the amoebic vacuole. Once the bacterium is taken into the vacuole, this enzyme breaks down the peptidoglycan framework of the cytoplasmic membrane (Fig. 15). Eventually the outer membrane of the cell wall ruptures, and the cytoplasm leaks out. The undigested outer membrane then forms concentric rings or myelin-like figures.

Although such figures, which represent digestive components of bacteria are seen in the trophozoites studied, Lowe and Maegraith (1970a) noted that such figures are also present in Axenic strains believed to be free from bacterial contamination. They stressed that the myelin forms are therefore not just bacterial in origin, but a means of increasing area for enzymic activity on foodstuffs obtained from material engulfed. It is more probable that such myelin-figures described by Lowe and Maegraith are artefacts due to fixation. Myelin-forms are readily produced in tissue fixed in glutaraldehyde. Since lipids are not fixed by this fixative, some will leach out of the cytomembranes. A combination of such liberated lipid and an aqueous environment produces membranous formations which are fixed by the osmium used as a post-fixative. These then present as myelin-figures.

Some vacuoles are lysosomal in origin, as acid phosphatase is detected in them. Rosenbaum and Wittner (1970) reported that this enzyme is restricted to the inner surface of the vacuolar

membrane and the contents of the digestive vacuoles. In this study, the enzyme is not restricted as suggested but is distributed over the whole vacuole. A lysosome, which is newly synthesized and has not yet been involved in digestive activities is generally regarded as a primary lysosome (Pitt, 1975) and this may be the case here. Lowe and Maegraith (1970a) noted very small "roughly and randomly electron dense bodies" scattered throughout the cytoplasm. These the authors regarded as lysosomes. In this study, as tests for acid phosphatase failed to detect the enzyme, it is questionable if such bodies can be so regarded.

The cytochemical study shows that TPPase is located in some of the vacuoles. It is difficult to identify the actual nature of these vacuoles as the cellular structure beneath the reaction product is poorly preserved. It is necessary to stress at this point that some enzymes are sensitive to glutaraldehyde and thiamine pyrophosphatase is one. Glutaraldehyde permits the exact location of an enzyme with reference to the preserved cellular structure. As glutaraldehyde is not used for TPPase localisation, perfection in the preservation of the ultrastructure has to be sacrificed to allow some localisation of the enzyme.

This is probably the first report of TPPase localisation in an amoebic vacuole. Such a finding indicates that the vacuoles are able to perform the secretory activities of a golgi complex. The correlation between golgi complex and the lytic enzymes has been studied by Locke and Sykes (1975) working on the origin of the membranes and the lytic enzymes which are involved in autophagy in the metamorphosing insect fat body. They demonstrated that the golgi complex gives rise to lytic enzymes. The appearance of fuzzy vesicles on the limiting membrane of the amoebic vacuoles would

suggest that they too may exhibit a secretory role in producing primary lysosomes. The findings of Rosenbaum and Wittner (1970) however would not support the hypothesis that the vacuoles may play such a secretory role. They showed in several micrographs the proximity of RNP helices to digestive vacuoles and suggested that the ribosomes of the RNP helices are active in synthesis and digestive enzymes are transported from the chromatoid bodies to food vacuoles by the helices. The same authors believed that the helical polyribosome of Entamoeba histolytica is analogous to a primary lysosome in man and other animals. Griffin (1971) failed to find any association between the helix and the vacuole and suggested that the chromatoid bodies are best explained as stored aggregates of ribosomal helices available for use after encystment. The presence of a ribonucleoprotein helix in an aerobic vacuole (Fig. 1c), however, would support the hypothesis of Rosenbaum and Wittner (1970) but such a finding is rare. I have only seen it once.

Although unspecific phosphatases are localised within the cytoplasmic membrane of bacterial envelopes (Costerton et al., 1974) and specific phosphatases such as alkaline phosphatase, hexose monophosphatase and cyclic phosphodiesterase reaction products are found in the periplasmic space and at the cell surface of Escherichia coli (Wetzel et al., 1970), it is unlikely that TPPase in an amoebic vacuole is bacterial in origin, as such products are confined to the walls of the vacuoles.

E.1.] Endoplasmic reticulum and Golgi apparatus

Whether reaction products for TPPase are localised in either Golgi apparatus-like, or endoplasmic reticulum-like vacuoles is not easy to discern, as Goldfischer et al., (1971)

have demonstrated that in liver hepatocytes, TPPase and inosine diphosphatase, which is commonly used as an endoplasmic reticulum marker, differ not only in intracellular distribution but also in pH optima. TPPase is most active in the endoplasmic reticulum at pH 5.0, whereas at pH 7.0 the enzyme is most active in the Golgi-apparatus. These authors considered that TPPase is a useful marker for the Golgi apparatus if the pH is kept between 6.5 and 7.0. As the pH of the incubation medium used in the present study was 7.0, the structure bearing the reaction products would therefore appear to be a Golgi apparatus-like vacuole. Because of the poor preservation of the trophozoites localized for TPPase, it is not possible to correlate the functional role of the enzyme with its structure. It is also not possible to demonstrate whether vacuoles with micropinocytotic vesicles (or fuzzy vesicles) are related to the enzymatic activity of TPPase. Only presumptive evidence therefore can be presented. Wivelinski et al. (1974) found a positive TPPase reaction in vacuoles of tissue cells of the frog myocardium and correlated the presence of TPPase production with the elaboration of glycocalyx material or glycoprotein. Ghadially (1975) stated that micropinocytotic vesicles are known to form when protein is being transported into the cytoplasm. Should this be true, then amoebic vacuoles do play an important role in protein transport.

Previous authors have not shown unanimity of opinion regarding the nature of amoebic endoplasmic reticulum and the Golgi apparatus. Love and Wagenaith (1970a, c); Ludvik and Shipetowa (1970); Bird (1961); Miller et al. (1961); and El-Hashimi and Pittman (1970) all failed to find a Golgi apparatus as defined for metazoan cells. A special arrangement of small vesicles and smooth

surface elongated profiles resembling the Golgi apparatus was seen in E. histolytica induced by emetine hydrochloride and metronidazole, both of which are amoebicidal drugs (Trevino et al., 1972b). Proctor and Gregory (1972a) claimed to observe for the first time a Golgi-like complex. They noticed a stack of elongated smooth-walled vesicles, swollen at the distal ends to form sacs. It is difficult to deny that such vesicles were artefacts due to fixation. An endoplasmic reticulum without prominent ribosomes was reported by Lowe and Maegraith (1970c). Osada (1959) and Bird (1961), however, failed to find endoplasmic reticulum. Ludvik and Shipstone (1970) regarded endoplasmic reticulum as consisting of fine tubules and small longitudinal lacunae surrounded by a number of small ribosomes. Numerous small 'hair clip-like strands' of smooth endoplasmic reticulum have been observed in an axenic strain of E. histolytica (NIH:200 strain) (Proctor and Gregory, 1973b). El-Hashimi and Pittman (1970) reported endoplasmic reticulum consisting of a number of small tubules and vesicles within the cytoplasm. In this study, no endoplasmic reticulum was found.

E.1.4 Nucleus and intranuclear bodies

The intranuclear bodies seen are the same as those recorded by several workers (Feria-Velasco and Trevino, 1972; Lowe and Maegraith, 1970c; Ludvik and Shipstone, 1970; Rondanelli et al., 1974b; Zaman, 1973). Ludvik and Shipstone (1970) described intranuclear bodies as 'button-like structures'. Lowe and Maegraith (1970c) regarded such structures as 'vesicles of various size, some empty, some containing irregular dense granules or membrane rings which were sometimes found in the peripheral region of the nucleus'. To describe the morphological differences in the

intranuclear bodies, the terminology of Rondanelli et al (1974b) in their study of intranuclear bodies of Entamoeba coli is useful. Those authors separated the intranuclear bodies into 3 morphological types depending on size.

The first type I): small inclusions often crowded into clumps of numerous elements lying at the periphery of the nucleus in the heterochromatin border.

The second type II): larger inclusions about 0.2-0.5 um in diameter morphologically similar to Ludvik and Shipatone's 'button-like structures', randomly distributed in the nucleoplasm. Both types I and II are non-membrane bound and Rondanelli et al (1947b) regarded them as non-vesicular type nuclear inclusions. In this study, although variation in size of such non-vesicular inclusions is noticed, these inclusions are only confined to the euchromatin area and none is seen in the heterochromatin border (Figs. 2b, 4). The second type inclusion body contains many osmiophilic rings (Fig. 4). Only in the first type body are the osmiophilic rings replaced by coarse granules filling the whole core (Fig. 5).

The 3rd type of nuclear inclusion was described by Rondanelli et al (1974b) as vesicular-type inclusions (membrane-bound) similar to the vesicles described by Lowe and Maegraith (1970c). The present observations show that these bodies are confined to the peripheral region of the nucleus in the heterochromatin area (Figs. 5, 6).

An interesting cytochemical study by Faria-Velasco and Trevino (1972) revealed reaction products for acid phosphatase in the nuclear bodies and the authors suggested that they might be part of the lysosomal system of the parasite. In this study, using

2 different substrates: cytidine 5-monophosphate and sodium β-glycerophosphate, no acid phosphatase was found in the nucleus. Gonzalez-Angulo et al (1973) using cytochemical stains involving enzymatic treatments found that DNase digested DNA in the central core of the non-vesicular inclusions. Such findings indicate that these circular dark bodies may involve replication and interaction of genome during division of the protozoon. Such a finding may explain the functional significance of acid phosphatase in the bodies.

The presence of electron-dense membranous structure and ribosomal material in the vesicular type nuclear inclusions probably indicates that these vesicles have the property to digest portions of their own nucleoplasm. These bodies thus have an autophagic function. This would not be unreasonable as cellular autophagy is a characteristic of a cell and may play a key role in the economy of the cell by participating in turnover of cell constituents (Pitt, 1970).

Zaman (1973) stated that these intranuclear bodies are not essential for survival of the amoebae as occasionally the nuclei are observed to be almost depleted of them. This view is confirmed in Figs. 2a and 7). Zaman also observed these inclusions in Entamoeba coli and believed that they cannot be regarded as an aggressive mechanism.

Similar intranuclear laminated inclusions, comprising alternating concentric rings of electron-dense and electron-lucent material, have also been seen in cells of various types, which are known to be capable of developing a mucoid secretory product (Chadally, 1975). All these inclusions have a single loose-fitting organized membrane. Such evidence would strongly suggest

that the circular dark bodies of the E. histolytica nucleus participate in the formation of mucopolysaccharide. It is known that trophozoites secrete copious amounts of this substance and threads of it extend from the uroid (Bird, 1956; Zaman, 1961, 1972).

The nature of the nuclear pore is clear. A large number of nuclear pores are surrounded by a rim which indents the nucleoplasm. No further detail can be elucidated except that in some sections, diaphragms are seen across the nuclear pores. Martinez-Palomo et al. (1976), and Henley et al. (1976), using a freeze-fracturing technique, revealed a large number of nuclear pores along the fractured faces of amoebic nuclear membranes. Henley et al. also noticed that these pores were regularly spaced, about 35 pores per square micron, and were covered with 'studded globules'. Such globules were not seen in this study using a thin-sectioning technique.

E.1.5 Sub-pellicular bodies

Sub-pellicular bodies or lens-shaped electron dense bodies on the cytoplasmic surface of the inner membrane of plasmalemma have been observed in trophozoites of E. histolytica cultivated axenically or monoxenically with either bacteria or crithidia (Bird, 1961; Ludvik and Shipetone, 1970; Lowe and Macgraith, 1970a, c; Proctor and Gregory, 1972a). Their function however is uncertain. Rondanelli (1967) submitted a hypothesis that they may be concerned with the hydrolytic enzyme activity of pathogenic amoebae. Knight et al.(1975) extended this hypothesis by suggesting that these particles may well be small liposomes (lipid droplets containing enzymes) which originate in the nucleus and move across the cytoplasm coming to lie beneath the internal lamina of the membrane. On contact with the host cell, they appear

to be discharged into the cell or surrounding medium. The Wanna strain (monoxenic culture) used by Knight et al. (1975) initially carried these particles, but after 2 years of subculturing, they disappeared. Their disappearance did not affect the lytic effect of the amoebae on the cultured cells (Figs. 72, 76b, 76c), evidence that these bodies do not necessarily carry hydrolytic enzymes. Love and Musgraith (1970a) also questioned the significance of these bodies as they occur in axenised Laredo strain. Similar bodies have been seen on cytoplasmic faces of the plasma membrane of cloned hybrid cells, in intestine, squid axon, muscle and slime mould and within synaptic vesicles (Larsen, 1975). Such deposits are usually widely separated and contain Ca^{2+} salt. Larsen put forward 3 possible explanations for deposit formation :

a) a high intracellular concentration of calcium inhibits the process of movement of ions across the membrane;

b) Ca^{2+} entering the cell acts as a trapping agent, precipitating anions produced by the hydrolysis of endogenous substrates such as ATP;

c) Ca^{2+} may precipitate with pyrophosphate produced from endogenous ATP by adenylate cyclase.

E.2 [illegible] OF E. HISTOLYTICA IN CONTACT WITH CULTURE CELLS

E.2.1 Existence of amoebic surface lysosomes

Eaton et al. (1969, 1970) put forward an exciting hypothesis that "surface-lysosomes" might be an aggressive mechanism. They

were studying the lytic effect of E. histolytica on monolayers and suggested that the effect was not due to the liberation of toxins or enzymes but was contact-dependent. They published micrographs revealing "surface-lysosomes" which were equipped with simple or compound tubular "triggers" under the amoebic plasmalemma. The authors suggested that on contact with an object, the trigger released lysosomal or cytotoxic enzymes into the cell. These lysosomes were thought to contain acid phosphatase shown by the Gomori staining technique. This present study does not support this theory as no ultrastructural component which might be construed as a surface-lysosome could be found. If surface-lysosomes do exist, cytochemical techniques should reveal a marked and localised reaction product on the interaction area between Entamoeba histolytica and the host-cells. However, in this study, the intensity of reaction product (acid phosphatase dye-technique) revealed only a weak reaction in Entamoeba histolytica trophozoites and even after the addition of amoebae to a monolayer, there was little difference in the intensity of staining, and the reaction product was never seen to be confined to the site of attachment. The reaction product, however, is restricted to food vacuoles (Fig. 200).

Only a few lysosomal enzymes such as N-Acetyl-β-D-glucosaminidase, acid phosphatase and β-galactosidase are present in an amoeba. Other acid hydrolases, non-specific esterase, β-glucuronidase and aryl sulphatase are only present in food vacuoles after a prolonged period of interaction. N-Acetyl-β-D-glucosaminidase is only confined to the amoebic cytoplasm and there is no enhancement of the enzyme when the amoebae are added to the cells. If the surface-lysosome concept was operative a spread of

this enzyme into the cell should occur but none was detected.

Rae (1973) was unable to demonstrate from both acid phosphatase staining and studies of electron micrographs that surface-active lysosomes with triggers are present at the site of contact. De Chor et al. (1973) revealed a strong reaction of acid phosphatase in the pseudopodia and suggested that this enzyme, in keeping with Katen's findings, plays an important role in E. histolytica invasiveness. Surface lysosomes were observed by Proctor and Gregory (1972b) but their photographic evidence is not convincing. Trevino-Garcia Passo et al. (1971a) also claimed to have seen these organelles, but the only localised acid phosphatase on the surface of the plasmalemma beneath which lay "the organelle-like lysosome". In their photograph, this "organelle-like lysosome" shows absence of acid phosphatase.

On the other hand, others (Maghadda et al., 1970; El-Hashimi and Pittman, 1970; Griffin, 1972; Knight et al., 1975) were not able to confirm the presence of surface-like lysosomes. Such authors regarded these organelles as artefacts in preparation or fixation. In reply to critics, Proctor and Gregory (1973a) reaffirmed the surface active lysosome hypothesis illustrating with micrographs progressive stages in the development of trigger mechanisms. These authors, however, conceded that such specialised lysosomes were only revealed in one pathogenic strain (HM:200) and not in the other one (HK-9). In Katen et al.'s hypothesis, the acid hydrolases are released from surface lysosomes by depolarising the membrane of the cell. Lushbaugh and Miller (1974) working on the glycocalyx of E. histolytica could not detect any special charging relationship in association with a surface lysosome.

Although the cytochemical studies in the present work

gave negative results for non-specific esterase. β-glucuronidase and aryl sulphatase, such results do not indicate that these enzymes are wholly absent in E. histolytica trophozoites. The levels of enzyme activity may well be too low for histochemical methods to reveal their presence. At the same time, the inhibitory effect of fixatives may abolish activity. Mayat (1974) stressed, however, that a recovery of 12% of acid phosphatase is sufficient for light and electron microscopy observations. Non-specific esterase has been localised cytochemically at light microscopy level in round or rod-like granules evenly distributed in the cytoplasm of bacteria-free amoebae cultivated with crithidine (Michel and Westphal, 1970). Perhaps such rod-like granules could however be derived from ingested crithidine, whose lysosomes carry non-specific esterases.

The existence of surface lysosomes is most definitely open to doubt.

B. 2.2. Extracellular digestion

Alkaline phosphatase and leucine aminopeptidase are virtually absent in both HK13 and E. histolytica although under the electron microscope, a trace of electron-dense precipitates for alkaline phosphatase is deposited along cell-junctions in the HK13 monolayer.

Alkaline phosphatase, esterase, and leucine aminopeptidase have been found in the holdfast, a specialised organ of attachment, of strigeids (avian parasitic trematodes) (Lee, 1962; Erasmus and Ohman, 1963; Ohman, 1964) and play an important role in both attachment of the holdfast to the host-gut wall, and digestion of neighbouring cells. In vitro tests have demonstrated that alkaline phosphatase and esterases are secreted to the exterior

of the strigeid, Cyathocotyle bushiensis, and onto the surface of the duck caecum (Erasmus and Ohman, 1963). Such secretion had a marked effect on the mucosa of the duck caecum, where the cells ultimately become reduced to a granular mush. The adhesive organ of C. bushiensis thus serves not only for attachment but also for extracorporeal or extracellular digestion in which host tissues were reduced to a granular form in which they could be ingested (Erasmus and Ohman, 1963). Similarly in another strigeid Diplostomum spathini esterases, which are secreted extracellularly by the pseudosuckers in the holdfast, have a histolytic action on the attaching cells thus making ingestion of the tissues easier (Lee, 1962). Several different alkaline [illegible] have also been located at the surface of the Schistosoma tegument and their presence has been correlated with the metabolic uptake of glucose by the tegument from the surrounding medium (Hockley, 1973). Strong leucine aminopeptidase activity is also located in the tegument of Schistosoma mansoni and it has been suggested that this enzyme hydrolysed peptides which have a free α-amino group on a terminal leucine or other related amino acid (Hockley, 1973). The serum proteins are also broken down by leucine aminopeptidase and free amino acids are absorbed through the tegument. Such experimental findings especially those on avian trematodes, strigeids, illustrate extracellular digestion taking place among parasitic organisms where the enzymes synthesised by the cell are passed into the extracellular medium and hence act at some distance from the secreting cell.

It has been suggested that the F. hepatica trephocytes are capable of extra-cellular digestion (Westphal, 1938) on the assumption that proteolytic enzymes are secreted extracellularly

where tissue destruction takes place as a result of the action of such proteolytic enzymes. Evidence to support such a hypothesis is lacking as Neal (1960) stressed that proteolytic enzymes of E. histolytica were not secreted extracellularly in vitro. Furthermore, Jarumilinta and Maegraith (1969) found it was impossible to distinguish clearly between the so-called "pathogenic" and "non-pathogenic" strains of E. histolytica by determining the total pattern of proteolytic enzyme activity and that of certain other enzymes. Eaton et al (1969, 1970)'s concept of 'surface-lysosome' as an aggressive mechanism in pathogenesis would also suggest that extracellular digestion may take place. It will have been seen, however, that evidence against the 'surface-lysosome' hypothesis is considerable.

In this study, the absence of leucine aminopeptidase, alkaline phosphatase and esterase in the amoebae would also suggest that extracellular digestion plays no part in cellular digestion, and cytotoxic enzymes are probably not secreted through the plasmalemma of the amoeba. Histochemical testing shows that N-Acetyl-β-glucosaminidase is only present in the cytoplasm of amoebae, and throughout the interaction period, no spread of the enzyme to neighbouring RK13 cultured cells can be detected; an indication that extra-cellular digestion has not taken place.

Materials, both solid and fluid are probably just taken into the amoeba where they undergo further hydrolysis. It would seem that intracellular digestion takes place in digestive vacuoles, which are formed during phagocytosis and pinocytosis. Non-specific esterases, β-glucuronidase and aryl sulphatase are found to be present in food vacuoles after a prolonged period of interaction; an indication that intracellular digestion is taking place using

host-cell acid hydrolases to break down the ingested host-cell components.

II.2.j. Mechanism of amoebic phagocytosis

The process of uptake of particulate material into a cell cytoplasm designed for intracellular digestion is known as phagocytosis. Such a phenomenon has been shown in trophozoites ingesting particles such as erythrocytes, starch granules, leucocytes and Crithidias. (Bird, 1961; Chewes, 1972; Westphal et al., 1972; Trevino et al., 1972; Proctor, 1974; Zamam, 1962, 1970). Westphal suggested that the trophozoites of E. histolytica are incapable of ingesting tissue cells with intact plasma membranes (Westphal, 1939). Recently, Knight et al. (1975) reported ingestion of cell debris by trophozoites after a complete disintegration of the cell cytoplasm. In this study, the amoebae are seen to be capable of phagocytosing tissue culture cells, whose plasma membranes are intact, although the cell organelles show gross pathological changes.

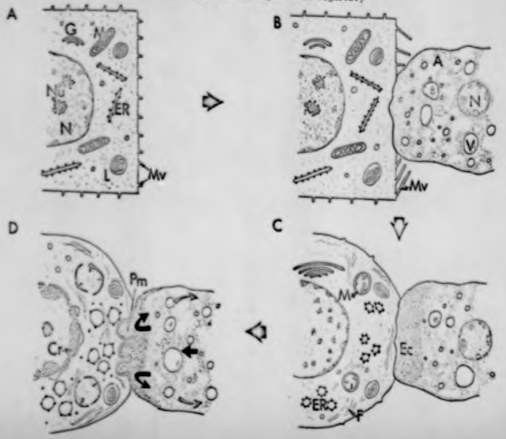
My interpretation, from this study of events in pseudopodial phagocytosis of intact cells is illustrated diagrammatically in Fig. V (A to E).

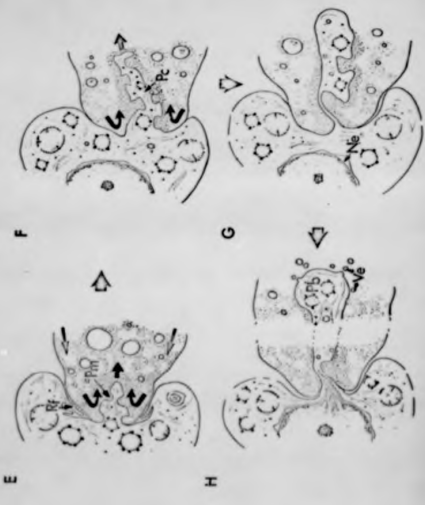
Fig. V (A) Here, the cell, which is not in contact with the amoeba, is unaffected and its organelles are undamaged.

(B) Before and just after the initiation of contact the microvilli of the host cell are seen to extend and point towards the amoeba. Such a finding reflects differences in the surface charge between the culture cells and the amoebae.

(C) Once the contact is made between the amoeba and the host cell, a toxic substance is released onto the surface of the cell. The effect is immediate as cell organelles undergo a

Fig. V (A to H) Diagrammatic illustration of the sequence of events in phagocytosis using information obtained from thin sections. (For explanation of abbreviations on figures see key to lettering used in figures.)




F
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H

rapid pathological changes. The earliest signs of cell injury is swelling of mitochondria and dilatation of the cisternae of the endoplasmic reticulum (see E.3.2 and E.3.3.). The filaments are seen to accumulate in the cytoplasm of the cell. Such a finding may indicate a breakdown in the structure of the microtubules into individual filaments (see E.3.5).

(D) As the cell loses its shape, the plasma membrane of the affected cell becomes more plastic, enabling the micropseudopodia which form at the tip of the pseudopodium to probe into the cell. These micropseudopodia are probably used to act as pincers, and aid in increasing the area of attachment. The contact zone of the amoeba is seen to fill with an electron opaque substance of varying density. No food vacuoles or large particulate matter are seen in this area. Such an area is similar to both the ectoplasm described ultrastructurally by Zaman (1972) and the 'homogeneous border line of fine-reticular cytoplasm' described by Westphal et al. (1972).

(E) Normally, as suggested by Bird (1956) the endoplasmic granules stream towards the tip of the pseudopodium and are deflected towards the sides of the amoeba. After the attachment of amoebic micropseudopodia onto the affected cell, the endoplasmic flow is then reversed with the result that the affected cell cytoplasm is drawn into the cytoplasm of the amoeba aided by the formation of micropseudopodia along the contact zone. The contacted surface of the cell is here crenated and the tips of the ruffles on either side of the amoeba are seen to point towards the tip of the amoebic pseudopodium (Fig. 76c). Such a finding would indicate that the amoebic plasmalemma, which is in good contact with the affected cell membrane is also being reabsorbed into the cytoplasm.

(F) A phagocytotic channel is formed and the area surrounding the channel becomes ectoplasmic. Westphal et al. (1972) regarded an area surrounding ingested material in the process of ingestion as a phagocellar. The micropseudopodia still maintain their grip on the ingested culture cell aiding cell-absorption into the cytoplasm. The size of the phagocytotic channel is found to var-, and the ectoplasm surrounding the channel is well defined as small filaments (Fig. 6b) are visible. These filaments, however, are not similar to the ones described by Pichel and Schupp (1975), who considered the filaments of 9-14 nm in diameter to be myosin-like filaments spread mainly in the uroid of the moving amoeba.

(G) The micropseudopodia extend and engulf the material to form a large vacuole which merges into the cytoplasm.

(H) At the end of the channel, where a vacuole or bulb is formed, vesicles have developed. Such vesicles, containing cell contents are released into the cytoplasm where they soon fuse with lysosomes. An alternative mechanism may operate: the lysosomes may fuse with the bulb, and the hydrolytic enzymes are then discharged into the bulb where further hydrolysis takes place.

When the amoebic toxic effect, however, was blocked by an addition of promethazine hydrochloride, phagocytosis was not completely inhibited (Fig. 19ba). Here, due to the retention of cell rigidity in the present of promethazine hydrochloride, the phagocytotic channel is unusually large and the amoebic micropseudopodia are not fully formed. Such a finding does indicate that the amoeba is also capable of ingesting a healthy cell with an intact plasma membrane.

The phagocytotic activity of trophozoites observed in this

study illustrates two points:

1) Endoplasmic streaming plays a role in the formation of the phagocytotic channel.

2) Adequate attachment between the amoebic plasmalemma and host cell is essential for phagocytosis to occur. Micropseudopodia found at the tip of the pseudopodium act in aiding the attachment.

Normally, as suggested by Bird (1956, 1961), and supported by Zaman (1972) and Proctor (1974) solid particles, as shown in Figs. 23, 63 and 144e, are absorbed through the uroid as a consequence of plasmalemma reabsorption as the amoeba is in motion. In some cases, as demonstrated in this study, the ingestion of food is aided by forcing the endoplasmic stream to flow away from the contact zone of the amoeba and interacted cells. Such a mechanism enables the contact zone to be drawn into the cytoplasm, carrying with it the amoebic plasmalemma in the same manner as small solid particles are taken into the posterior end of the amoeba by the flow of the endoplasmic stream.

B.) CYTOCHEMICAL AND ULTRASTRUCTURAL STUDIES OF CULTURE CELLS IN CONTACT WITH TROPHOZOITES OF *E. HISTOLYTICA*

B.).1 Primary and secondary disturbances

Judah (1969) regarded a cell as a network of structures with specific functions; a strain placed on one primary area of the network will give rise to secondary disturbances throughout the whole system. To differentiate the secondary from primary disturbances time-scale experiments are necessary (Judah, 1969).

Realising the importance of this time factor, cells in contact with E. histolytica trophozoites were fixed after 5, 10, 20, 30, 45, 60 and 120 minutes. It was, however, found that to try plotting the pathological changes according to time-scale was unreliable as the time of lysis varied from 5 to 30 minutes after the addition of trophozoites showing that both the degree of virulence of each individual amoeba within the cultural population and the sensitivity of each host cell to infection must also be taken into consideration. In some cases, the secondary disturbances occurred rapidly and it was impossible to discern the initial attack on the primary site. The time-scale was therefore ignored and the events of both primary and secondary disturbances were mapped out according to the degree and spread of disruption within the injured cells in contact with trophozoites (Tables XIIa, b).

Some of the destructive events in cells damaged by E. histolytica indicated in Tables XIIa, b have also been recorded by previous workers (El-Hashimi, 1975; Knight et al., 1975). The precise sequence of events has not been, however, detailed.

E.3.2 Mitochondrial changes

In all the cell-line monolayers so far described, the most noticeable cellular change in cells injured by amoebae is the degeneration of the mitochondria. The earliest alteration is a loss of matrix and an accumulation of matrix material in the periphery of the mitochondria. This is followed by a swelling of the matrix compartment. The decrease in relative density of the matrix reflects both influx of water and loss of solutes. A similar appearance has been reported in mouse liver during the development of necrosis (Trump et al., 1965) and in toxin-affected Ehrlich

TABLE XIIa

THE SEQUENCE OF EVENTS OF DISTURBANCES WITHIN CULTURED CELLS IN CONTACT WITH TROPHOZOITES OF ENTAMOEBA HISTOLYTICA

The site of disturbances :

Sequence of events (not time related)	SURFACE COATINGS	CYTOPLASMIC MATRIX	NUCLEUS	GOLGI APPARATUS
STAGE 1	1. Prior to contact, and after initial contact, the microvilli of affected cells surrounding the amoebae extend its length and point towards the amoeba. 2. Microvilli are beginning to disappear. 3. The cell becomes rounded up.	1. Matrix less dense 2. Accumulation of filaments	1. Appears normal	1. Hyperactivity
STAGE 2		1. Filaments continue to accumulate	1. Chromatin migrates to the periphery of the nucleus 2. Nuclear envelope is swollen	Disappeared
STAGE 3	1. Plasma membrane beginning to break down.	1. Ribosomal materials accumulate in the cytoplasm	1. Nucleus appears empty leaving behind the nucleolus 2. Nuclear envelope appears very swollen. In some cases, the outer nuclear membrane completely broken down	
STAGE 4	The cell finally lyses releasing the organelles (even the condensed mitochondria) into the extracellular medium.			

TABLE XXIV (continued)

The scale of disturbance :

Sequence of events (not time related)	MITOCHONDRIA	ENDOPLASMIC RETICULUM	INCLUSION BODIES
STAGE 1	1. Matrix less dense 2. Migration of materials to the periphery 3. Fragmentation of the cristae 4. Swelling of the inner mitochondrial membrane In some cells, condensation of the mitochondria occurs. In such mitochondria the cristae are tubular in shape and are [illegible]	1. Vesiculation of the endoplasmic reticulum begins	Unaffected
STAGE 2	1. Swelling of the mitochondria begins 2. Outer membrane beginning to break down	1. The cisternae of the endoplasmic reticulum swollen 2. Degranulated, but polyribosomes are not yet fully disaggregated	1. Some of the inclusion bodies i.e. microbodies and lysosomes are slightly resistant to swelling, some lysosomal disruption may occur at this stage
	In cells with condensed mitochondria, no change in shape of endoplasmic reticulum is evident. The vesicles of endoplasmic reticulum [illegible]		
STAGE 3	Either 1. Mitochondria continue to swell or 2. Swollen [illegible] completely	1. Swelling of the vesicles of cisternae of endoplasmic reticulum continues leading to a complete breakdown in the [illegible] 2. Degranulation [illegible] 3. Some of the polyribosomes still remain attached to the [illegible]	1. The lysosomes either remain intact or broken down releasing the hydrolytic enzymes into the cytoplasm thus accelerating cell death
	Condensed mitochondria [illegible] slight swelling and the endoplasmic reticulum vesicles [illegible] swollen		
STAGE 4	The cell finally lyses releasing the organelles into the extracellular medium		

150

ascites tumour cells (Laiho and Trump, 1975). Poor techniques in tissue-cell processing could also produce mitochondrial swelling but as affected cells are found with others not affected, it is reasonable to assume that this is not an artefact in cultured cell processing.

Swelling of mitochondria in affected cells in contact with E. histolytica, however, may not be the initial response. Some sections show mitochondria in a condensed state. Such condensation may result from a loss of mitochondrial volume control during the initial stages of cell injury, leading to a loss of ion or ions together with water from the mitochondrial inner chamber. Mitochondrial condensation may persist throughout the interaction period even to the time of cell lysis. Similar mitochondrial changes have been reported by Laiho and Trump (1975), who subjected the Ehrlich ascite tumour cells to a non-penetrating membrane-damaging agent, which induced cell-membrane permeability. These authors noticed, during the initial stage of cell injury, a condensation of mitochondrial matrix, and they related such change to loss of ions and water from the mitochondrial inner compartment following inhibition of active accumulation systems. Furthermore they suggested that the appearance of a condensed mitochondrial state could be taken as evidence of the intactness of mitochondrial respiratory and coupling systems - a point at which recovery could take place. Following loss of ability of ATP synthesis, the mitochondrial membrane becomes more permeable and osmotic swelling occurred.

It is known that injury to a variety of tissues is associated with the liberation of enzymes into the cytoplasm or extracellular fluid. By analysing such leakage of enzymes, one can determine a more precise picture of the points of attack of toxic

on cells (Rees, 1962). For the same reason, any variation in mitochondrial enzyme pattern during morphological alteration would provide an additional understanding of the pathogenesis of cell injury. The work of Trump et al. (1965a) gives a good example of correlation between morphological and functional changes in mouse liver during the development of necrosis. Biochemically they were able to demonstrate a correlation between the activities of succinoxidase on mitochondrial membranes and glutamic dehydrogenase in the mitochondrial matrix and changes in mitochondrial morphology. They also showed that the levels of succinic dehydrogenase were much more stable even at times when mitochondrial structure was virtually destroyed. In this study, mitochondrial ATPase, which is a useful marker for studying mitochondria (Sumner et al. 1965) was demonstrated histochemically and the results show an alteration in the pattern of reaction products in the injured culture cells in contact with the amoebae. Although this test does not quantify the degree of enzyme activity, it is significant that the mitochondrial ATPase was still present during mitochondrial structural alteration. Trump et al. (1965a) however pointed out that histochemical tests are not wholly reliable for they found that the pattern and amount of reaction product using histochemical tests bore little relationship to the overall state of mitochondrial preservation. Biochemical tests on the other hand, produced more consistent results.

E.3.3 Alterations in endoplasmic reticulum and Golgi apparatus

It will have been seen that endoplasmic reticulum in cells affected by E. histolytica trophozoites responds to injury by breaking up into small vesicles and swelling of its matrix. The ribosomes are gradually lost from the surface of the cisternae, and

the polyribosomes slowly disaggregate. Such an ultrastructural finding is important because in cells affected by certain toxins, e.g. CCl_4 and ethionine, polyribosomes are seen to disaggregate very rapidly into smaller assemblies and simple particles. Ribosomes are also seen to detach from the membranes of the endoplasmic reticulum (Villa-Trevino et al., 1964; Wood, 1965; Friedman and Stenger, 1966; Reynolds, 1963b). Any disaggregation of polyribosomes promotes inactivation of protein synthesis (Smuckler and Benditt, 1963). Polyribosomes play an important role in introducing amino-acids to the endoplasmic reticulum. Ethionine, a toxin, indirectly suppresses protein synthesis by lowering the level of ATP thus causing a block in messenger-RNA synthesis (Judah, 1969). The messenger-RNA is thought to hold the polyribosomes in an aggregated state. In this study, the polyribosomes of affected cultured cells following amoebic contact are not disaggregated at the early stage of injury. In the very late stage of cell injury when the cell is contact with the trophozoite of E. histolytica is about to lyse, the grossly swollen vesicles of endoplasmic reticulum still retain aggregates of ribosomes (Figs. 52, 71). In that there appears to be no relative increase in detached ribosomes it could be argued that the protein synthesis within the affected cell is not completely suppressed by the amoebic toxic substance. Further evidence that protein synthesis is not completely inhibited is suggested by the morphological finding that there is an apparent hyperactivity of the Golgi apparatus in cells initially infected with Entamoeba histolytica. As such an increase in Golgi vesicles is related to increased secretory activity (Ghadially, 1975) it follows that protein synthesis has not been arrested. Further biochemical studies, however, are

required to determine more precisely the points at which normal metabolic pathways are interrupted.

Cytochemical staining for thiamine pyrophosphatase and inosine diphosphatase demonstrates that both the endoplasmic reticulum and the Golgi apparatus in HTC culture cells are few in number. It is not possible therefore to evaluate the influence of the amoeba on the activity of culture cells' endoplasmic reticulum and Golgi apparatus.

The peripheral or plasma staining of the culture cells, however, can be seen with reaction product for both TPPase and IDPase (Figs. 159, 160). No alteration in enzyme activity of plasma membrane TPPase and IDPase in cells in contact with amoebae is seen (Figs. 195, 161, 162). TPPase staining of plasma membrane has also been reported in secretory cells of the anterior pituitary gland (Pelletier and Novikoff, 1972). The authors stressed that it is not known whether the TPPase is related to the "transport" ATPase commonly associated with the plasma membrane.

E.3.4 Nuclear changes

The earliest nuclear alteration following amoebic contact is the loss of nuclear matrix and condensation of chromatin along the periphery of the nucleus. This is followed by swelling of the nuclear envelope. A complete loss of chromatin leads to the appearance of 'empty' nuclei with intact membranes and nucleoli. This is compatible with the observation of Trump et al. (1965a, b) who studied nuclear changes during necrosis using slices of mouse liver and reported that nuclear changes are secondary to alterations in mitochondrial morphology.

E.3.5 Plasma membrane changes

The plasma membrane of a cell in contact with a

trophozoite of Entamoeba histolytica though initially appearing to remain intact would seem to be susceptible to cell damage and rapidly breaks down before the cytoplasmic contents are completely destroyed. Islands of nuclear contents surrounded by swollen organelles and vesicles without plasma membrane are frequently seen (Figs. 55, 75).

Microtubules have various functions, and one of them is thought to be a cytoskeletal function; that is to say they confer a degree of rigidity to the cell membrane (Chadially, 1975). It has been shown that colchicine can cause a disruption of normal microtubules and an accumulation of cytoplasmic filaments (Wisniewski et al., 1968). It has hence been suggested that the subunits of cytoplasmic filaments and microtubules are probably identical (Wisniewski et al., 1970). Accumulation of cytoplasmic filaments which are observed in the early stage of cell injury would thus indicate that the cell membrane of the cell in contact with E. histolytica has lost its rigidity.

The number of microvilli on the cell surface decreases significantly as shown by scanning electron microscopy, but not, however, during the initial stage of contact. Scanning electron micrographs reveal an extension of the microvilli in the cells surrounding the amoebae. The attraction of the microvilli for the parasite reflects differences in the surface charge between the cultured cells and the amoebae.

Plasma membrane ATPase (Mg^{2+}, and Ca^{2+} activated) is found to be restricted to the plasma membrane of the RW13 cultured cells (Figs. 131, 136a, b). No reaction product is seen on the plasmalemma of trophozoites. Prolonged contact leads to progressive enhancement of Mg^{2+} activated ATPase on the plasma membrane of the

contacted cells. The ingestion of cell debris coated with reaction products for Mg^{2+} ATPase via the uroid end of Entamoeba histolytica by pinocytosis supports the finding of Bird (1961) that uptake of particles can occur through the uroid or tail end. The vesicles and channels containing the reaction products are clearly shown in Figs. 145a, b.

The confinement of the Mg^{2+} activated ATPase reaction products to the uroid of the amoeba during infection reflects the dynamic state of the plasmalemma turnover which is taking place at the uroid. Fucoid threads, more pronounced using histochemical staining for Mg^{2+} ATPase can be seen to spread right across the lesion (Fig. 143).

Although Mg^{2+} ATPase and to a lesser degree Ca^{2+} ATPase on the plasma membrane of the HTLJ monolayer are enhanced during infection with Entamoeba histolytica, it is uncertain whether the results reflect an alteration in the mechanism of active transport of Na^{+} and K^{+}. Active transport is responsible for maintaining ionic gradients between the cell and the extracellular fluid, thus giving the cell control over the internal or external environment. Such active transport processes can be linked directly to ATP hydrolysis. During transport ATP, which is activated by Mg^{2+} ions, is hydrolysed, the hydrolysis of each molecule of ATP being responsible for the movement of approximately 3 Na^{+} ions and K^{+} ions (Finean et al, 1974). Accumulation of ATPase during infection would indicate a rapid hydrolysis of ATP, which in turn leads to rapid movement of ions between the cell and the extracellular fluid.

It has been argued, however, that the Mg^{2+} ATPase localisation cannot demonstrate the transport ATPase (Hugon, 1970).

The lead in the medium, devised by Wachstein and Meisel (1957) strongly inhibits the transport ATPase. Also lead catalyzes a non-enzymatic hydrolysis of ATP which may contribute to artefactual deposits. Such deposits would occur at the same site as for true enzyme activity. The same authors also suggest that in the presence of an inhibitor, ouabain, which inhibits Na-K-ATPase (active transport), the reaction products for Mg^{2+} ATPase are still precipitated. The method used in this study is a modification of Wachstein and Meisel's medium (Jacobsen and Jørgensen, 1969), and the same problems of non-enzymatic hydrolysis leading to artefactual staining occur. Jacobsen and Jørgensen, however, concluded that their medium is more favourable as far as enzymatic hydrolysis is concerned and less favourable for lead catalyzed hydrolysis than the medium of Wachstein and Meisel. Ernst, appreciating the problem posed by non-enzymatic hydrolysis due to lead, has prepared a new medium (Ernst, 1972a, b). This medium avoids the use of lead in the incubation solution, and can localise the Na-K-ATPase enzyme complex (Ernst, 1975). The demonstration of Na-K-ATPase was based on the localisation of an ouabain-sensitive K-dependent phosphatase component of the transport ATPase complex. In this study, Ernst's method has also been used, but the reaction products are scarce as far as the plasma membrane of RV13 monolayer cells is concerned. No reaction product is found on the amoebae. Control mouse kidney sections still show K-NPTase activity when ouabain was used (Fig. 152). Ernst (1975), however, stressed that rat kidney Na-K-ATPase is known to be relatively insensitive to this inhibitor. Jacobsen and Jørgensen (1969), however, have suggested that the two activities, Na-K-ATPase (active transport) and Mg^{2+} ATPase, be considered as

representing two different functional states of a single enzyme.

Although the relationship of Mg^{2+} ATPase to the Na-K-ATPase is not fully understood, the enhancement in the reaction products for Mg^{2+} ATPase on the plasma membrane of HFlj monolayer cells during infection can still be taken to reflect an alteration in cell membrane metabolism.

E.3.6 Involvement of lysosomes in cellular injury

Soon after the discovery of lysosomes by De Duve, it was suggested that lysosomes act as potential "suicide bags" (De Duve and Beaufray, 1956). Such suicide bags contain a number of soluble hydrolases with an optimum acid pH, and are bounded by a lipoprotein complex which prevents leakage of the enzymes. De Duve and Beaufray, working on autolysis, believed that autolysis started at the site of the lysosomes resulting in leakage of lysosomal hydrolases into the cytoplasm. Such a mechanism would result in damage to various cellular components. Several workers have supported De Duve's lysosomal concept. Early studies on lysosomes showed that streptococcal haemolytic toxins, streptolysins, caused release of enzymes from the lysosomes of granular fractions of rabbit liver (Weissman et al., 1963). They found that lysosomal enzymes, cathepsins, acid deoxyribonuclease, ribonuclease, acid phosphatase, and β-glucuronidase were released into the cytoplasm before the mitochondrial enzyme malic dehydrogenase was liberated. Such work indicates that release of lysosomal or lytic enzymes is a primary event in cell damage.

In the course of time, however, investigation has revealed that lysosomes play no role in the early development of cell injury but are involved in the later scavenging process. The works of Slater and Greenbaum (1969) and Rees (1962) have shown, in the

investigation of the biochemical effect of hepatotoxins on liver that there is little change in the overall level or degree of latency in the lysosomal enzymes. Significant changes are detectable in these enzymes only at a late stage of injury when necrosis is well established.

It has also been suggested that De Duve's "suicide bag" concept be applied to the Entamoeba histolytica and host cell interaction (Jarumilinta and Pradolfer, 1964; Rao, 1973). In this case, the amoebic toxic substance which is only transferred after contact with the host cell renders the lysosomal membranes of the cell unstable so that it disrupts releasing lysosomal enzymes into the cytoplasm. In the present study, the lysosomes are intact during the initial stage of cell injury (Figs. 90, 91). During prolonged amoebic infection, light microscopy observation reveals apparent swelling of host cell lysosomes, instanced by the size of the reaction product particles for acid phosphatase (Gomori technique) and aryl sulphatase. This is followed by disruption of the lysosomes releasing the lytic enzymes into the cytoplasm (Figs. 91, 92, 115). The dye techniques for β-glucuronidase, and non-specific esterase initially show no increase in the activity of the host lysosomal enzymes. Eventually, after prolonged infection (Figs. 107, 108, 125, 209) the levels of these enzymes enhance, as seen by the overall increase in the colour of reaction products. Similar appearances such as swelling and disruption of the lysosomes have been observed in the infection of Phytophthora erythroseptica (potato) with Solarum tuberosum (fungus responsible for pink rot disease of potatoes) (Pitt and Coombes, 1968).

The present light microscopical observations, however,

do not establish whether disruption of lysosomal particles is a cause or consequence of cell death. Under the electron microscope, the sequence of events of lysosomal disruption is obvious, and shows that the leakage of hydrolytic enzymes in cells affected by Entamoeba histolytica trophozoites is a late event in cell degeneration. Acid phosphatase, at electron microscopic level, is clearly shown in lysosomes which appear intact as the membranes are unbroken (Fig. 99, 100). Even after cell lysis, lysosomes continue to show enzyme content, and this reflects the belief that the lysosomal membrane can withstand a high degree of osmotic stress.

Such findings can, however, be questioned on the grounds of suspect methodology, as stressed by Brunk and Ericsson (1972). They pointed out that when a slight or moderate release of lysosomal enzyme occurs, it is not necessarily revealed by cytochemical studies in fixed tissues. They also stressed that fixation of in vitro cultured cells under adverse condition of osmotic pressure can result in a diffusion of lysosomal enzymes.

The osmolarity of fixatives (450-500 milliosmoles/kg for 3% glutaraldehyde, 0.066M cacodylate buffer, pH 6.8) used in this study was such that with normal cultured cells incubated for localisation of acid phosphatase and aryl sulphatase, no apparent swelling of the mitochondrial membranes was seen. Furthermore by using this technique the ultrastructural localisation of lysosomal enzymes, especially acid phosphatase was more than adequate; electron dense precipitates were found to be confined to the whole lysosomal structure; indication that diffusion of lysosomal enzymes had not taken place.

E.4 [illegible]

E.4.1 Cell and amoeba contact

Villarejos (1962) proposed that the mechanism of pathogenesis in E. histolytica infections was the result of death and disintegration of amoebae within the tissues and that the dead amoebae released endocellular enzymes which, acting upon the surrounding tissues caused their necrosis. El-Hashimi and Pittman (1970) supported Villarejos' theory in view of the finding of dead amoebae in axenic cultures and some living amoebae fed on dead amoebae. They suggested that the lytic enzymes were not secreted across the plasmalemma, but were liberated after the death of some of the amoebae in the colony.

In this study, using electron microscopy, an addition of homogenized amoebae to the culture cells failed to reveal pathological changes in the host cells. Similar experiments by Knight et al. (1974) using light microscopy also failed to reveal an effect on monolayer culture cells. Also, in this study, the addition of homogenized trophozoites of E. histolytica and E. invadens to labelled Chang cells did not cause any release of chromium from the cells; an indication that Chang cells had not lysed. Such evidence does not favour the view that lytic enzymes are released after the death of the amoebae. The cytopathic effects of E. histolytica would appear to be wholly dependent upon cell contact with an intact protozoon. This has been stressed by Jarumilinta and Kradolfer (1964), Eaton et al., (1970) and Knight et al. (1975). Membrane discontinuities, described by Knight et al. (1975) in contact areas between the amoebae and host cells were also observed in this study. Such apparent discontinuities, however, have been noticed in contact

zones between lymphocytes and target cells, and they were shown to be intact membrane profiles after specimens had been tilted to permit perpendicular viewing (Fiberfeld and Johannesson, 1975).

E.4.2. Viruses

Although rhabdoviruses have been found in all known pathogenic E. histolytica trophozoites of monozenic and axenic strains and in trophozoites from biopsy material of intestinal amoebiasis (Bird et al., 1974; Bird and McCaul, 1976), no rhabdoviruses are seen in contact cells. Viruses thus play no direct part in host cell damage.

In cytocidal virus-cell interaction, where transference of viruses into host cells leads to extensive derangement of normal cellular metabolism and structure and thus to degeneration and death of cells (Tamm, 1975), virus replication in cells would require a latent period of several hours. Cell death due to E. histolytica invasion in this study is seen to occur between 5 and 30 minutes following the addition of trophozoites. Such a time difference would not support the hypothesis that replication of viral genome within the host is the cause of cellular death. The presence of rhabdovirus, however, is of interest and may be related to enhancement of virulence by incorporating the viral genome into the amoebic RNA. As rhabdoviruses are also found in Laredo strain of E. histolytica (Bird and McCaul, 1976), it is probable that rhabdoviruses coexist with amoebae as symbiotic organisms. Further study is necessary to elucidate the relationship between rhabdoviruses and amoebae. Other amoebic viruses such as filamentous and icosahedral forms described by Watters et al. (1970) and Diamond et al. (1972) were not found in the amoebae used in the present study.

E.4.3 Primary target in cellular injury

Swelling of the mitochondria and endoplasmic reticulum of the affected BHK13, CV-1, BD-VI and Rhesus Monkey Brain monolayer cells in contact with E. histolytica suggests that lysis of cells is an osmotic effect. Similar pathological changes have been recorded in hepatocyte exposed to a variety of toxins; CCl_4, ethionine, dimethylnitrosamine and thioacetamide (Rees, 1962; Rees and Spector, 1961; Judah, 1969; Farber and El Mofty, 1975; Slater and Greenbaum, 196[illegible]; Villa-Trevino et al., 1964; Wood, 1965; Frishman and Stenger, 1966; Reynolds, 1963b. Some workers (Rees, 1962; Judah, 1962; Farber and [illegible], 1957), studying the effect of such toxins have suggested that the primary target is the plasma membrane. A breakdown in its selective permeability would lead to serious secondary effects such as accumulation of water, sodium chloride and calcium ions causing injury to various cell organelles notably the mitochondria. Direct evidence for this hypothesis is suggested by the effect of antihistamine drugs interacting on the plasma membrane (Rees, 1962; Rees and Spector, 1961; Judah, 1962). The antihistamine inactivates and inhibits the protein phosphorylation occurring at the plasma membrane. The movement of ions and water across the surface membrane is thus interrupted. The addition of an antihistamine to the liver toxins was found to prevent pathological change in the liver (Rees, 1962; Rees and Spector, 1961; Gallenger et al., 1956).

The effect of promethazine hydrochloride on the interaction between the E. histolytica and BHK13 cells was therefore studied. It was found that promethazine hydrochloride protects the cell against the action of trophozoites (Figs. 13-196a). Electron microscopic studies of the contacted cells reveal no gross

pathological changes in contrast to the unprotected infected cultured cells. This protective effect of antihistamine against cellular injury due to E. histolytica strongly suggests that the primary target in cell death is the plasma membrane.

It is believed that in the initial stage of cellular injury, the damage to the plasma membrane is reversible (Rees, 1962). This idea of reversible plasma injury is supported by Farber and El-Mofty (1975) who were working on the effect of galactosamine on the liver. Galactosamine was found to induce membrane injury by altering the structural components of glycoproteins and glycolipids. Such injury was prevented by the addition of uridine. The time of administration of uridine was found to be critical. If uridine was added before the critical point of irreversible membrane damage, the calcium concentration returned to normal. At the critical point, however, the plasma membrane damage became irreversible giving rise to a markedly increased intracellular calcium and inevitable cell death. That the calcium ion is the key controlling toxicity in hepatocytes was also stressed by Gallagher et al. (1956) and Thiers et al (1960). Whether an increase in calcium ion concentration in cultured cells in contact with the amoebae is the main toxic factor in cell death was not determined. The possibility, however, that accumulation of calcium ions is responsible for accelerating cellular injury in cells in contact with E. histolytica merits further investigation.

As the histochemical relationship of Mg^{2+} activated ATPase to the active transport ATPase is unknown (see E.1.5), it is uncertain whether an increase in the reaction products for Mg^{2+} ATPase on affected culture cells in contact with E. histolytica reflects changes in cellular Na^{+} and K^{+} ion concentration. It is

more likely that such increase in the reaction products indicates an alteration in cell membrane metabolism due to the effect of amoebic toxin interrupting cell-membrane permeability. The changes in mitochondrial configuration in cells in contact with trophozoites are similar to those observed in tumour ascite cells subjected to a non-penetrating membrane-damaging agent; p-chloromercuribenzene sulphonic acid, which induces cell-membrane permeability (Laiho and Trump, 1975). These authors reported a condensation of mitochondria in the initial stage of cellular injury as a result of the breakdown in cell-membrane selective permeability. This is also seen in this study.

E.4.4 Toxin or cytolytic enzyme involvement

The idea that an amoebic toxin is the mechanism by which E. histolytica is able to invade host cells was originally put forward in 1891 by Councilman and Lafleur, but no biochemical evidence was available to establish this. Various workers (Craig, 1927; Neathman, 1932; Menendez, 1932; Westphal, 1938; Villarejos, 1961), however, rejected the toxin theory and supported instead the cytolytic enzyme theory or in the words of Dobell and O'Connor (1921) "The amoebae apply themselves to the mucous membrane and secrete a powerfully cytolytic ferment which destroys the cell". Experimental evidence to support such an hypothesis is lacking (De Lamater et al., 1954; Jarumilinta and Maegraith, 1969; Neal, 1960. See section B.2.3). Nor does this study support Eaton's hypothesis that cytolytic enzymes are stored in the surface lysosomes which lie beneath amoebic plasmalemma (see section E.2.1). Histochemical examination both by light and electron microscopy failed to reveal a spread of cytolytic or lysosomal enzymes from amoebae to adjacent host-cells. The

sequence of events in cellular injury through amoebic contact with CV-1, BD-VI and Rhesus Monkey Brain cultured cells used in this investigation, appears to be the same as that taking place in tissue cells subjected to hepatotoxins (see section E.4.3). The blocking effect of prosethazine hydrochloride on the lytic action of amoebae on cultured cells strongly favours the hypothesis that a toxin is involved in pathogenicity and not proteolytic or other cytolytic enzymes.

It is most likely that the toxin is situated on the surface of the amoeba, as an addition of amoebic homogenate or extract had no effect at ultrastructural level on the RT13 cells (see section E.4.1). If the toxin is cytoplasmic in origin, one would expect the toxin to remain active in the homogenate. The idea that an amoebic surface toxin is responsible for cell death would not be unreasonable when one considers that a mural or surface glycoprotein from Candida albicans is toxin to mice and that a mural mucopolysaccharide obtained from Neurospora causes vacuolation and agglutination of H. ornata cells (Gander, 1974) but it is uncertain whether these are derived from cytoplasmic glycoproteins.

Biochemical assay to identify a surface toxin is difficult when only limited samples of cultivated amoebae are available. The nature of the amoebic surface, however, has been investigated to a certain degree by various workers. The chief components of the glycocalyx when present are acid mucopolysaccharides and glycoproteins, of unknown origin, and of highly electronegative charge densities (Lushbaugh and Miller, 1974; Feria-Velasco et al., 1973). No sialic acid (N-acetyl neuraminic acid) can be detected in the outer coat (Feria-Velasco et al., 1973). The work of Martinez-Palomo et al (1973a, b) may provide

a further step in the determination of the surface properties of the amoebae. They noticed that the surface coat of pathogenic strains of E. histolytica has an affinity for the carbohydrate-binding protein concanavalin A (CON A). Trophozoites so treated formed large agglutinates. In contrast, no agglutination was formed with strains from asymptomatic human carriers illustrating an interesting relationship between the degree of agglutination and virulence of E. histolytica.

Such differences in the agglutination property with CON A have been observed in cultured cells infected with simian virus. Normal 3T3 uninfected cells required high (1000 µg/ml) concentration of CON A to produce half maximal agglutination, but on infection with simian virus, the cells only required 60-100 µg/ml concentration to produce half maximal agglutination (Nicolson, 1972).

In this study, 200:NIH axenic strain trophozoites failed to lyse culture cells and thus it is possible that continuous axenic cultivation may lead to a gradual loss of the factor which governs the affinity of the surface membrane for CON A.

Although in this study no differences in the morphological features of the trophozoite glycocalyx in both non-pathogenic (200:NIH) and pathogenic strains (Rvana, HM-1:IMSS) were found, Lushbough and Miller (1974) were able to show by using special staining techniques that the glycocalyx of axenic amoebae differed from that of monoxenic trophozoites in being more regular and compact in the axenic group. In monoxenic cells, the dense inner portion of the glycocalyx was overlaid by irregularly distributed peaks of more fibrillar material. The authors thought that the increased prevalence of surface projections in the

monoxenic culture is correlated with virulence. If such a correlation does exist, the gradual change in the appearance of the glycocalyx of the trophozoites of axenic strains may perhaps correspond to the alteration in the affinity of the membrane for CON A.

Recent work by Pinto da Silva's group has shown the complexity of the trophozoite (Pinto da Silva et al., 1974, 1975; Martinez-Palomo and Pinto da Silva, 1974). Using E. histolytica to investigate the concept of membrane fluidity which regards the membrane as consisting of integral and peripheral components both of which move independently of each other, these authors treated the trophozoites both with glycerol and with CON A peroxidase, which influence the membrane components. The colloidal (Fe^{+3}) binding sites, negatively charged at pH 1.8 and membrane particles or anionic sites (Pinto da Silva and Martinez-Palomo, 1975) represent the integral components, whereas the CON A surface receptors and acidic sites, negatively charged at pH 4.0 represent the peripheral components. When living trophozoites were pre-treated with glycerol before fixation there was a marked aggregation of the membrane particles and colloidal binding sites (integral components). These components were found to distribute in patches. Such marked induced aggregation was not accompanied by CON A receptors and acidic sites. The distribution of peripheral components was thus unaltered. Similarly, pre-treatment with CON A and peroxidase resulted in the accumulation of CON A surface receptors at the uroid and which was devoid of integral components, while the distribution of membrane particles and acidic sites (integral components) remained unaltered over the whole body. These results clearly underline the structural and organizational

complexity of the plasma membrane of E. histolytica.

This study has provided strong evidence of the existence of a surface toxin intimately associated with the plasmalemma or glycocalyx. Although its identity has not been established there are pointers to indicate that a phospholipase is probably involved (see section F.4.4).

F.4.5 a) Surface-associated phospholipase

b) [illegible]

As the Cr release cytotoxicity test is an accurate indicator of cell lysis it has been used extensively to investigate both the mechanism of target cell lysis by cytotoxic lymphocytes (Perlugo and Allison, 1974, 1975; Erve and Friou, 1975), and the cytotoxic effect of leukocidin from Pseudomonas [illegible] on human polymorphonuclear leukocytes (Johnson, 1975). The ^{51}Cr is taken up by cells as chromate and is thought to bind to cytoplasmic proteins. Its release depends on leaks in the cell membrane large enough for molecules of a high molecular weight to pass through. It was therefore decided to use this test to establish whether a toxin of amoebic origin was damaging the cell membrane in monolayer culture systems.

The fact that homogenates of E. histolytica and E. invadens did not induce release of ^{51}Cr from labelled Chang cells provides further evidence that the cytotoxic mechanism depends on intimate contact between an intact amoeba and a host-cell. The ultrastructural findings also demonstrated that the addition of a homogenate of E. histolytica trophozoites to a BHK cell-line monolayer did not cause any significant morphological alteration in the cells. Furthermore, the failure of the supernatant medium from other amoebic cultures to induce release of ^{51}Cr indicates that a cytotoxic substance is not being secreted - at least in any

harmful quantity.

The protective effect of Rosenthal's inhibitor, which is a synthetic analogue of lecithin and an inhibitor of phospholipase A, against cellular injury would strongly suggest that phospholipid alteration by phospholipase A is the basis for the loss of membrane integrity in such cytotoxic reactions. It is known that phospholipase activity leads to the conversion of lecithin (phosphatidylcholine) to lysolecithin and fatty acid, the former product being a very potent lytic agent which is capable of attacking the phospholipids of intact plasma membranes (Frye and Friou, 1975). Venoms from the bee and from Naja naja (cobra), which contain phospholipase A, has been found to induce rosette formation in human T lymphocytes as a result of phospholipid and lecithin degradation of cell membranes (Hanaumi et al., 1976). It has also been suggested that this enzyme is bound on the cell surface or is actually an integral plasma protein of K-cell (Frye and Friou, 1975). These authors have demonstrated phospholipase A activity as being an initial step in the kill mechanism of K-cell and target cell cytotoxicity. Although the present study on E. histolytica and E. invadens pathogenicity has not demonstrated the involvement of phospholipase A in cytotoxicity, some indirect evidence for a possible connection between this lytic agent and the kill mechanism in E. histolytica and host-cell interaction can be deduced. The ultrastructural studies on culture cells in the initial stage of contact showed that the host cell becomes rounded, and the microtubules dissociate; indication of a change in the maintenance of the cyto-skeleton. Such change in shape may be taken to reflect a loss in membrane stability possibly through depletion of glycoproteins and high molecular

weight polypeptides (Huggins et al., 1976). Thus a breakdown in host-cell plasma membrane in contact with an amoeba might indicate exposure of the host-cell surface to phospholipase A.

The actual site of phospholipase A, if present, is conjectural. As the amoeba is prevented from being killed, the enzyme may either be bound on the amoebic glycocalyx or actually be an integral protein of amoebic plasmalemma. As TPPase was localised within the amoebic vacuoles, and fusing vesicles were demonstrated on both the vacuolar membrane and the plasmalemma (see D.I.[illegible]), it would seem possible that the acid mucopolysaccharides, glycoproteins and glycolipids of the glycocalyx are being transported from the vacuoles to the plasmalemma via the vesicles. It is tempting to postulate that such a mechanism could also be applied to the transport of phospholipase but evidence for such a mechanism is slight. It may be that the enzyme is present in a precursor form, and is activated only by amoeba-cell contact. Frye and Friou (1975) in their work on the inhibition of mammalian cytotoxic cells by phosphatidylcholine and its analogue, stressed that most phospholipases have been found to be incapable of attacking the phospholipids of intact plasma membranes. However, in the presence of membrane-perturbing agents such as detergents, hypotonic saline or direct lytic factor, a small basic peptide found in snake venoms, the enzymes are able to catalyse the conversion of phospholipids into their derivatives (Frye and Friou, 1975). It is thus possible that phospholipase is present on the culture cell surface as a precursor form, and requires activation by a catalytic factor present on the amoebic plasmalemma.

The ^{51}Cr cytotoxicity experiments indicate damage to

the plasma membrane of the cell allowing large molecules such as proteins to leak through. The possibility that prior to this smaller molecules have passed through the plasma membrane in response to osmotic change must now be considered. Green et al. (1959) were able to demonstrate that complement produces holes in the cell membrane. These allow small molecules such as potassium, amino acids and ribonucleotides to pass through but not larger molecules such as proteins and RNA which do not pass through until the holes are enlarged through an influx of water causing swelling of the cell.

Perlugn and Allison (1974) in their studies of tumour cells in contact with T-lymphocytes, and Bohrmann (1976), working on the cytotoxic action of leukocidin from Pseudomonas aeruginosa, supported Green et al's findings. These authors were able to demonstrate that $^{86}Rb^{+}$, a marker of a lower molecular weight, was released from the cells more rapidly than ^{51}Cr, indicating that large molecules are released only after swelling of the cells has led to increased permeability of the plasma membrane. Bohrmann regarded such a phenomenon as a "colloid-osmotic process". Perlugn and Allison (1974) however believed that phospholipase A plays no part in the cytotoxic effect of T-lymphocytes on tumour cells; their conclusion being based on the assumption that if killing of target cells involves disruption of the structure of the cell membrane due to phospholipase, both markers $^{86}Rb^{+}$ and ^{51}Cr should be released simultaneously. They thus regarded lysis due to osmotic change as a totally different phenomenon from that of complete breakdown of the cell membrane due to phospholipase A.

The present morphological studies on the interaction

between E. histolytica and cell-culture systems demonstrate quite clearly that the target cells do undergo a series of internal structural alterations through progressive osmotic change and although fine ultrastructural studies have indicated an alteration in the cell membrane it does remain intact during the initial stage of contact.

From the work recorded in this thesis it can now be postulated that the sequence of events taking place in relation to amoebic pathogenicity may be summarized as follows :

1) In the initial stage of interaction activated phospholipase A generates a potent lytic agent, lysolecithin, which creates holes in the membrane.

2) The cell plasma membrane selective permeability is impaired leading to increased permeability in the cell membrane.

3) The microtubules dissociate leading to a change in the maintenance of cytoskeleton as seen in thin-section preparations.

4) As a result of an influx of water, the cell swells causing further enlargement of the holes, as confirmed by scanning electron microscopy observations and ^{51}Cr release experiments.

5) Further breakdown in cell components due to osmotic changes takes place. The cytoplasmic matrix becomes less electron-dense due to the influx of water. This is followed by swelling of the mitochondria and endoplasmic reticulum. Lysosomes are disrupted at a later stage of contact.

6) Finally the plasma membrane breaks down releasing cell components into the extracellular environment.

It is fully recognized that to substantiate this hypothesis, biochemical evidence for a surface phospholipase must now be sought.

E.5 NOTE ON THE USEFULNESS OF CELL CULTURE SYSTEMS

Continued cultivation of Entamoeba histolytica axenically and monoxenically may lead to a diminution in degree of invasiveness and infectivity in laboratory animals (Phillips and Bartgis, 1954; Phillips et al., 1972; Phillips, 1973; Rae, 1973; Rae and Nagy, 1975; Wittner and Rosenbaum, 1970; Yamamoto et al., 1972; Diamond et al., 1971). Such progressive loss of virulence is, however, found to be unrelated to length of individual strain culture time and strains cultured for the same length of time do not prove to be equally virulent (Diamond et al. 1974). Neal (1971) believed that amoebae are normally avirulent in the intestinal lumen and that in response to some unknown stimulus, they change into virulent organisms. It will be seen from the introduction to this work (E.2.2) that there is inconsistency in published results of attempts, using laboratory animals, to identify host factors which might stimulate the amoeba to invade tissue. Neal (1971) commented that a complex laboratory animal is not the ideal model for the determination of invasiveness.

A cell culture amoebic system has on the other hand been shown to be eminently suitable for the study of early amoebiasis lesion (Knight, Bird and McCaul, 1975) and this alternative technique has been employed in this study in which the determination of degree of virulence provides an admirable example of the superiority of such a method over one using laboratory animals with inevitable variation between hosts.

Two axenic strains, [illegible] and [illegible] were tested for virulence by adding the amoebae to monolayer (MRC5) which is known to be readily affected by monoxenic strains (Knight et al., 1975). The amoebae were left in contact with the cells for two

hours, after which the culture was examined for pathological changes. In one strain, 200:NIH, no alteration in fine structure was noticed in the contacted cells. In contrast the cells responded to the amoebae of HM-1:IMSS axenic strain, and pathological changes were easily detectable. It is apparent that there is a variation in the degree of virulence among axenic strains of E. histolytica. The relation of [illegible] to ^{51}Cr labelled Chang culture cells causing a release of chromium is yet another example of the usefulness of cell-culture systems as an alternative model in the investigation of contributory factors in restoring virulence and in the determination of invasiveness and infectivity in differing strains of E. histolytica.

V. SUMMARY AND CONCLUSION

Experiments recorded in this thesis have illustrated the following points :

1) Fixation in-situ of trophozoites cultivated on glass coverslip has been found to provide a better preservation of extracellular surfaces. Scanning microscopy observations supported by thin-sectioning techniques have revealed a new feature, the existence of filopodia.

2) Thiamine pyrophosphatase is present in the amoebic vacuoles indicating that these vacuoles like a Golgi complex can play an important secretory role in the synthesis of polysaccharides and glycoproteins as well as in the formation of primary lysosomes.

3) Contact between the amoebae and the culture cells is essential for pathogenesis to occur.

4) In pathogenesis of amoebiasis, viruses do not play a primary role in host-cell damage.

5) Changes in cytoplasmic ultrastructure is not an expression of primary damage to the cell.

6) The amoebic enzyme-containing organelles or surface lysosomes do not exist and they are not responsible for cell damage.

7) Disruption of lysosomes or changes in the distribution of lysosomal hydrolases in the host cells occur late following the addition of trophozoites.

8) Promethazine hydrochloride prevents damage to cells in contact with trophozoites by stabilising cellular membrane integrity and by preventing permeability change.

9) A toxin, probably an amoebic surface associated

phospholipase A, impairs the contact cell plasma membrane selective permeability as shown by Cr. release experiments. From this it can be deduced that the toxin breaks down the cell-membrane, lecithin to lysolecithin, a potent lytic acid, and fatty acid. Lysolecithin is capable of attacking the phospholipids of an intact cell membrane causing further breakdown in cell components due to osmotic changes. Once the cytoskeletal control within the contact cell is lost, the amoeba is able to phagocytise the cell and injured cell components are taken into the amoebic vacuoles where intracellular digestion takes place.

10) Promethazine hydrochloride, which prevents damage to cells in contact with trophozoites, does not inhibit amoebic phagocytosis. This work shows micrographs of an amoeba ingesting a healthy cell with an intact plasma membrane.

11) Cell culture systems are in many ways preferable to laboratory animals in the study of amoebic pathogenicity.

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Fine structural changes at *Entamoeba histolytica* rabbit kidney cell (RK 13) interface

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Received 18 June 1974

Several observations suggest that in amoebiasis contact between amoebae and cells precedes tissue damage. Thus in the early caecal lesion in rats, amoebae are found firmly attached to mucosal cells (Bird, 1961). *In vitro* the studies of Jarumilinta and Kradolfer (1964), using blood leucocytes, and those of Eaton *et al.* (1970), using mammalian cell lines grown in Rose chambers, have both shown the necessity of cell contact. Although *Entamoeba histolytica* is well endowed with potentially cytotoxic enzymes (Jarumilinta and Maegraith, 1969) their role in pathogenesis is not clear.

The cell line RK13, grown as a monolayer, provides a suitable 'host' for studies of the cytopathic effect of *E. histolytica in vitro*. It attaches firmly to glass and can withstand for several hours the physiological conditions provided by amoebic Tryptose, Trypticase Yeast (TTY) medium (Diamond, 1968). This paper describes morphological changes that follow the addition of bacteria-free *E. histolytica* trophozoites to this substrate.

Specimen Preparation Methods

(*a*) *RK13 cell monolayer*. A standard RK13 cell line, kindly supplied by Dr. D. Bidwell of the Nuffield Institute, was grown in 10 ml of medium 199, with 5% foetal bovine serum, for five-seven days upon either round (30 mm diameter) cover slips, thicker glass discs (1·0 mm thick) or Millipore filters (25 mm diameter, and pore size 3mμ) placed in airtight flat bottomed sterile plastic containers of 30 ml capacity.

(*b*) *Trophozoites of* E. histolytica. These were cultured in TTY medium with a crithidial associate (Diamond, 1968); the pH being adjusted to 6·8 and osmolarity to 320 millios-moles. After 48 hours the medium was poured off and replaced with fresh chilled medium which rapidly detached the amoebae from the glass. After gentle centrifugation a known number of trophozoites was added to the monolayer; the suspension being virtually free of crithidia.

Just before adding the amoebic suspension to the monolayer, the 199 medium was removed, and replaced by TTY medium containing 5% horse serum. The usual inoculum was 5000 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

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in Fig. 9 the rough endoplasmic reticulum (ER) 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.

Where attachment has been more prolonged, as illustrated in Fig. 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 (Fig. 11); together with very evident disruption of normal ER pattern, mitochondrial swelling and vacuolation.

As the process of cell destruction progresses (Fig. 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 figure, the outer membrane is absent and connections with the ER 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 Fig. 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 RK cell membrane has occurred there is frequently a discontinuity of the amoebic surface membrane with no barrier between the cytoplasm of the cell and parasite (Figs. 8, 9, 10, 11). The small membrane-bound vesicles seen in Figs. 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 membrane of the RK cells.

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 (Figs. 15 and 15a).

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

Bodies closely resembling rhabdovirus particles (Bird *et al.*, 1974) have so far been found in all the strains of typical *E. histolytica* that we have examined. In many trophozoites (Fig. 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.

DISCUSSION

The cytopathic effects of *E. histolytica* seen in this *in vitro* system appear to be wholly dependent upon amoebal contact. As described elsewhere (Knight *et al.*, 1974) extracts of amoebae have no effect; and when a thin layer of agar is interposed between the RK cells and the amoebae no damage occurs. The electron micrographic studies, reported here, show that substantial damage takes place within the cell cytoplasm before the surface membrane is affected, and while the cells are still firmly adherent to the glass. When cell-membrane damage does occur it is localized initially and the cytoplasm of

EXPLANATION OF ABBREVIATIONS ON PLATES

Eh *E. histolytica* trophozoite; Ep Episome; ER Endoplasmic reticulum; FV Food vacuole; GC Golgi complex; L Liposome; Ly Lysosome; M Mitochondrium; MB Microbody; MF Millipore filter; N Nucleus; R Rhabdovirus-like particles; RK Rabbit kidney cell; T Tonofibril.

LEGENDS TO FIGURES 1 – 16

(Figs. 1 and 2 are phase contrast light micrographs; Figs. 3 – 16 are electron micrographs)

FIG. 1. Control RK13 cell monolayer after exposure for two hours to supernatant from an amoebic culture. (× 637)

FIG. 2. *E. histolytica* trophozoites (↗) in cell-denuded areas of an RK13 cell monolayer two hours after addition. (× 637)

FIG. 3. Longitudinal section of healthy RK13 cells from a control monolayer culture. (× 2 720)

FIG. 4. Areas of two adjacent cells from Fig. 3 showing normal cytoplasmic organelles. (× 10 710)

FIG. 5. *E. histolytica* trophozoite (↗) burrowing between a monolayer of RK13 cells and the supporting millipore filter. Note the mitochondrial ballooning in the contacted RK cells. (× 1 420)

FIG. 6. RK13 cells bordering an area of amoebic cell-denudation showing again the mitochondrial swelling and vacuolation seen in Fig. 5 (c.f. Fig. 3). (× 2 272)

FIG. 7. Contact zone between *E. histolytica* and RK13 cell; both cell and parasite membranes are intact and stain densely. Mitochondria in the RK13 cell are vacuolated and there is crowding of microbodies and lysosomes towards the cell margin. (× 14 200)

FIG. 8. After 1½ hours in the presence of and in contact with amoebae, some RK13 cells show patchy loss of membrane, condensation of tonofibrils and early disintegration of cytoplasm adjacent to the amoeba. (× 14 200)

FIG. 9. An area of contact showing interrupted loss of amoebic pellicle and RK cell membrane. The RK mitochondria are vacuolated; the tonofibrils more prominent and condensed, the Golgi complex seemingly active, while the ER shows early degeneration. (× 17 000)

FIG. 10. High magnification of an area where both surface membranes are absent with resulting continuity between amoebic and RK13 cell cytoplasm. (× 68 000)

FIG. 11. A broad contact area. The condensed tonofibril layer is being resorbed and the cell cytoplasm internal to it rarefied. (× 8 032)

FIG. 12. A later stage in RK cell cytoplasmic destruction with nuclear involvement. Note the electron dense small 'liposome' (↗). (× 8 032)

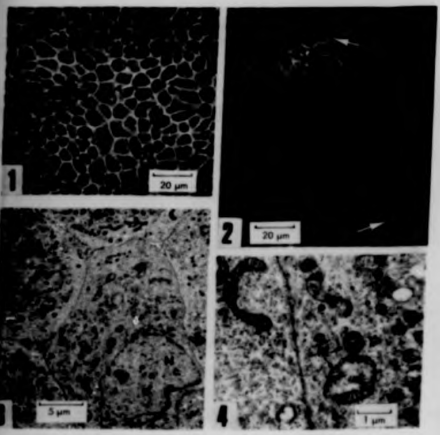
FIG. 13. Amoebic pseudopodia engulfing cell debris prior to ingestion – a late stage in cell destruction. (× 8 820)

FIG. 14. Pseudopodial activity at an earlier stage of cell disintegration with patchy fusion of membranes. (× 35 000)

FIG. 15. Three small 'liposomes' on the pellicle of a trophozoite in contact with an RK cell. Note the degenerating membranes of the nearby mitochondria. (× 35 000)

FIG. 15a. Inset – Two such 'liposomes' in greater detail. (× 84 000)

FIG. 16. Rhabdovirus-like bodies (↗) close to the RK cell contact area and clustered round a possible episome. (× 42 000)



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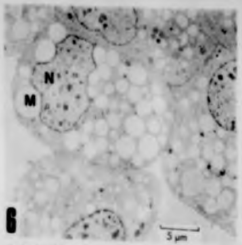
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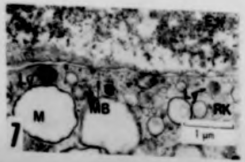
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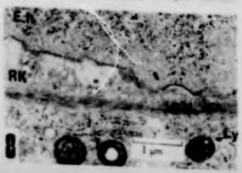
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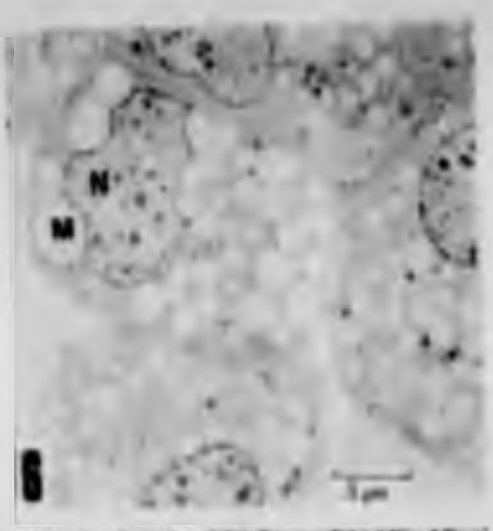

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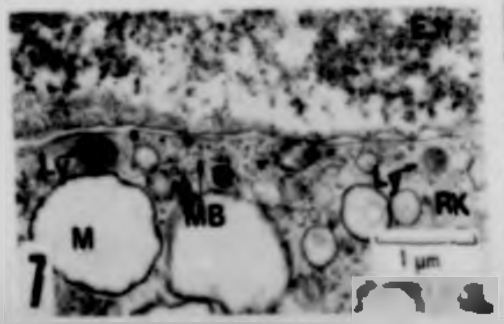

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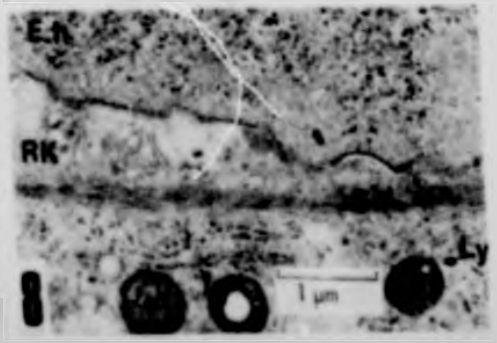

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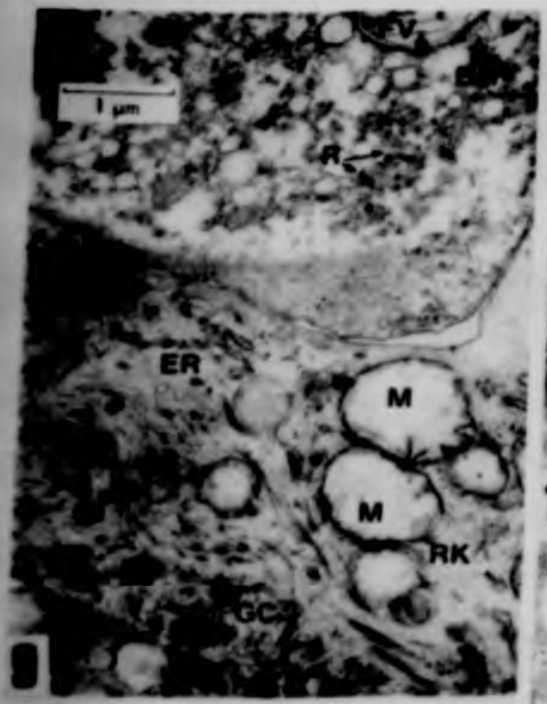

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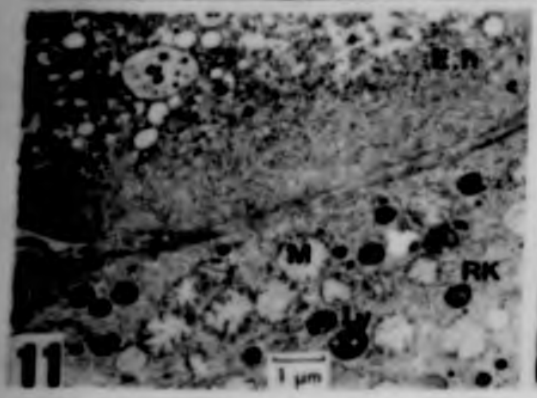

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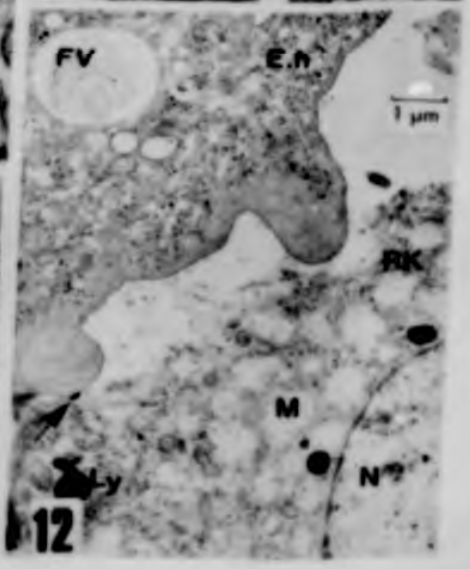

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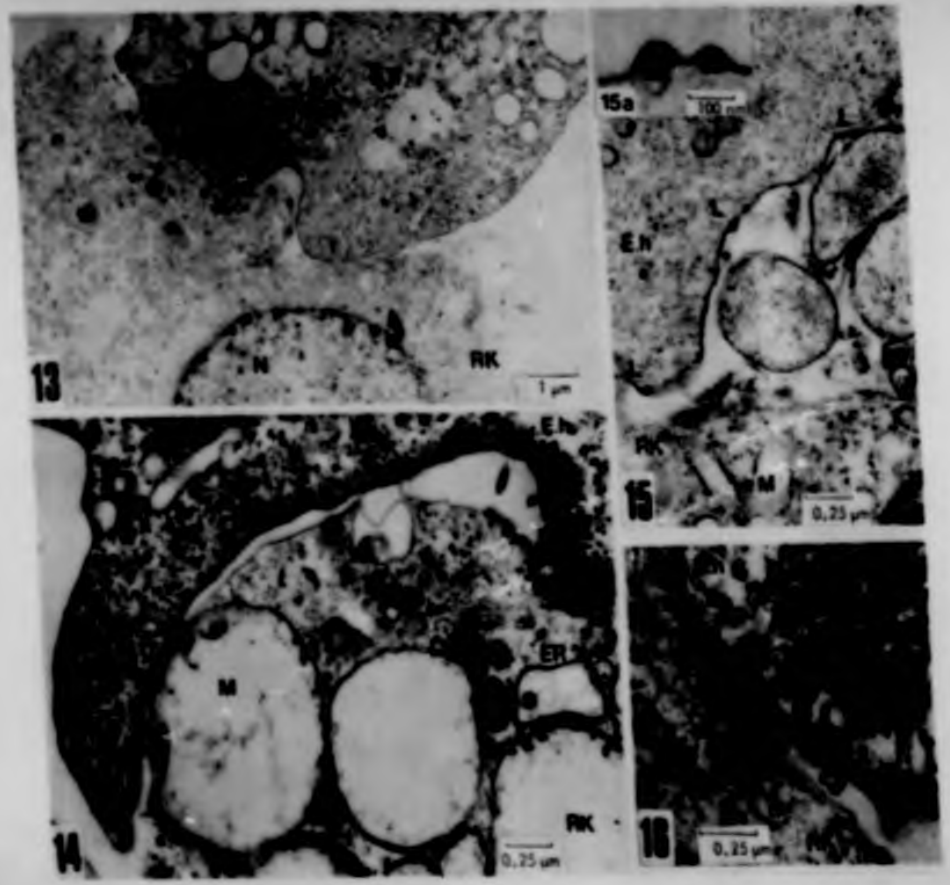
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cell and amoebae appear to become continuous. Fusion with cells may be one of the ways that enable the amoebae to discharge its enzymes. The electron-dense osmiophilic particles seen in the amoebic cytoplasm and beneath the surface membrane may well be small lysosomes (lipid droplets containing enzymes). Certain observations – to be reported elsewhere – suggest that these bodies originate in the nucleus, move across the cytoplasm and so come to lie beneath the internal lamina of the membrane; they later fuse with it and on cell contact appear to be discharged into the cell or surrounding medium.

Our findings of a contact-related effect is in agreement with Eaton *et al.* (1970) who used various cell monolayers, including RK13; however the fine structural changes which they describe differ considerably from ours. They reported that 'the earliest effect on the cell was a loss of stainability of the plasmalemma affecting the entire periphery of the cell'. Figure 8 shows clearly that quite extensively damaged cells still have an 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. We have seen no evidence of the surface lysosomes that they describe, and we suggest that such structures may be digestive vacuoles or other vacuolar structures positioned 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 Fig. 8 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 Griffin (1972) also using colonic material.

Jarumilinta and Maegraith (1961) showed that either extracts of washed amoebae or the amoebae themselves will digest protein substrates. This does not necessarily imply that amoebae liberate enzymes randomly into the medium and it may be that their intact amoebae released enzymes only on contact. Transfer of enzymes or their precursors across points of temporary contact or cytoplasmic fusion would be a less wasteful mechanism. *In vivo*, additional mechanisms may operate, such as release of enzymes from damaged host leucocytes (Griffin, 1972) or perhaps their liberation from dying amoebae themselves (Villarejos, 1962). Because of their physiological requirements, amoebae cannot multiply or persist indefinitely in healthy tissues. Cell death, however, creates conditions suitable for amoebic proliferation and also subjects adjacent cells to anoxic, osmotic and ionic stress. Such stressed cells are likely to be more permeable and susceptible to amoebal enzymes.

The hypothesis that certain strains of *E. histolytica* can become infected with virus particles might account for the instability of certain characteristics, including virulence and ease of cultivation. This idea had originally been suggested by Bird (1961). The situation may 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* (Jones *et al.*, 1969; Grange and Bird, in press). The apparent transfer of episomal material from bacteria to axenic *E. histolytica* has been reported by Wittner and Rosenbaum (1970), but several other sources are possible, as rhabdoviruses in particular are widespread in nature. The finding by Hoingberg and Reed

(1960) that virulence in *Trichomonas gallinae* could be transferred between strains by a cell free homogenate could be explained in a similar way; as could the temporary hybridization, achieved by Entner (1971) between typical *E. histolytica* and the Laredo strain.

Griffin (1972) considered that *in vitro* studies of cell damage were not relevant to pathogenesis because earlier reports were inconsistent with *in vivo* observations. Our results, however, are consistent. Furthermore, we feel that a cell amoebic system is an ideal way of studying the complex problems of the early lesion of amoebiasis.

SUMMARY

When bacteria-free trophozoites of *Entamoeba histolytica* were added to a monolayer of rabbit kidney cells, cellular injury occurred at the sites of contact. Changes appeared within the cell cytoplasm before there was any generalized cell membrane damage. At some points of contact there was apparent fusion of amoebic and cell cytoplasm. Electron-dense bodies, here interpreted as liposomes, were present in the amoebic cytoplasm and beneath the surface membrane. No surface lysosomes were seen. Various modes of cell damage and enzyme transfer from amoeba to cell are suggested, together with the possibility that cytopathic amoebae are infected with virus particles.

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The rhabdoviruses of *Entamoeba histolytica* and *Entamoeba invadens*

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One of the major factors implicated in the pathogenesis of amoebiasis is the oxidation-reduction potential in the environment of the amoebae (Eaton and Meerovitch, 1973). Some of the more remote factors which may influence this potential were discussed by Hoare (1953) but other major factors and the sequence of events involved in pathogenesis still remain undefined.

The possibility that carriage of virus by *Entamoeba histolytica*, or a response by intestinal epithelial cells to viral infection, might be among these major factors was mooted by Bird (1961) in a thesis in which two distinct types of particle (Fig. 1) were described in trophozoites invading a biopsy specimen from an established case of amoebic dysentry. However, opportunity to investigate the nature of these particles more fully did not arise until recently when material suitable for an electron microscope study (Knight *et al.*, 1975) became available through the work of Knight (1975).

Interest was focused on the osmiophilic, cylindrical, sometimes corrugated, particles (Fig. 1 GB_1) because unmistakably similar ones have been described with progressively greater detail by Lowe and Macgraith (1970), Proctor and Gregory (1972) and Feria-Valesco and Trevino (1972). Their likeness to rhabdoviruses was demonstrated at a laboratory meeting of the Royal Society of Tropical Medicine (Bird *et al.*, 1974) with due acknowledgement to Dr. J. Grange, Middlesex Hospital, for drawing our attention to the similarity of particles in one of our micrographs to those of the plant rhabdovirus—sowthistle yellow (STYV) (Özel, 1973).

This paper describes in greater detail the formation of these virion-like particles in trophozoites of *E. histolytica* and *E. invadens*, also their appearance when negatively stained. Their inclusion among the rhabdoviruses, probable commensal state and possible role in pathogenicity is discussed.

MATERIALS AND METHODS

Trophozoites of *E. histolytica*

Thin sections were prepared and examined from 12 pathogenic strains as well as the Laredo strain of *E. histolytica*. Eleven of the pathogenic strains were cultured in TTY medium with a crithidial associate (Diamond, 1968*a*) as described by Knight *et al.* (1975) when studying the fine structural changes at the amoeba-rabbit kidney cell (RK13) interface.

The remaining pathogenic strain, Axenic 200, kindly supplied by Dr. R. A. Neal of the Wellcome Laboratories (Beckenham), was cultured in TPS-1 medium with a bovine

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serum supplement (Diamond, 1968*b*). The Laredo strain was cultured in a BRS medium supplemented by starch (Robinson, 1968). Negative contrast preparations were made of the Axenic 200, Evans and Laredo strains.

Trophozoites of *E. invadens*

The same strain (BAH) of *E. invadens* used by Bird (1961) and known to contain the particles was kindly supplied by Dr. R. A. Neal of the Wellcome Laboratories for this investigation. Trophozoites were grown in Neal's modification of Jones' medium (Neal and Vincent, 1955) and used to make both thin section and negatively stained preparations for comparison of the virions from the two species of *Entamoeba*.

Method of Fixation and Embedding of Trophozoites

The fluid medium in 48–72 hours old cultures was replaced with fresh chilled medium, and the tubes put in ice water for five minutes and inverted several times to detach the amoebae from the glass. Three minutes' centrifugation at 1500 r.p.m. loosely pelleted the organisms which were then fixed with 3% glutaraldehyde in 0·066 M cacodylate buffer (pH 6·8) at 4°C for 45 minutes. This primary fixation was followed by a cacodylate buffer wash to remove all traces of glutaraldehyde, and secondary fixation for one hour with 1% osmium tetroxide also in 0·066 M cacodylate buffer (pH 6·8) at 4°C. Excess osmium was removed by second cacodylate buffer wash and the pellets embedded in 2% Difco Nobel Agar (Gowans, 1973). Agar blocks were dehydrated through serial concentrations of ethanol, 0·25% uranyl acetate being used as a stain in the 10% ethanol. Finally, the agar blocks were embedded in either Araldite or Spurr resin using Beem capsules.

Trophozoites on thick coverslips were selected by phase contrast microscopy for removal by the specially designed microscope boring attachment described elsewhere (Bird and Chapman, in press) and the plug of Araldite and amoeba mounted on perspex rod.

Sections from both pellets were cut on a Reichart OMU2 microtome, collected on uncoated Smethurst New 200 grids, stained with lead citrate (Venable and Coggeshall, 1965), and examined in an EM 801 GEC microscope provided by the Wellcome Trust.

Method for Negative Staining

Suspensions of cultured organisms in P.B.S. (pH 7·2) were homogenized in a glass tissue blender, centrifugated at 3000 r.p.m. for 15 minutes to remove starch grains and heavy cell debris. The supernatant fluid was submitted to high speed centrifugation at 25 000 G. for one hour to provide a pellet which was washed with buffer to remove media components before recentrifugation to provide the final pellet for negative staining.

A 2% phosphotungstic acid solution adjusted with N/10 potassium hydroxide to pH 6·8 was used as the negative stain and 0·1 ml of this solution added direct to the pellet in the centrifuge tube. One drop of the resuspended pellet was put onto each of two grids. Excess fluid was removed after 30 seconds and the grids allowed to dry thoroughly before examination in the microscope. Slight variations on the technique included the use of 2% ammonium molybdate instead of the P.T.A. and the inclusion of 30 μg/ml bacitracin to act as spreading agent (Gregory and Pirie, 1973).

RESULTS

Fixed Material

Cut sections of all 12 strains of *E. histolytica* including the Laredo strain as well as the B.A.H. strain of *E. invadens* contained the rhabdovirus-like particles scattered throughout

the cytoplasm or arranged in rosettes around an area of specialized cytoplasm described below (Figs. 2, 3). The particles varied in size up to 250 nm in length and 90 nm in diameter, also in appearance according to their state of maturity and the angle of cut, some incomplete ones were rounded at both ends (Fig. 4). These lacked a definite surrounding membrane, possessed a fine granular homogenous content with a single osmiophilic limiting boundary. Fully formed particles were typically bullet shaped and possessed an outer, possibly spiked, membrane and two internal concentric helices, as diagramatically represented in text-fig. A and seen in Figs. 5a, b, c, d.

Complete transverse sections (Figs. 6a, b) showed an electron dense midzonal spot 15–20 nm diameter located by 1/3 about the mid point of the particle.

Negative stain preparations did not demonstrate this feature.

Where seen within cytoplasmic vacuoles with no other material in the plane of sectioning, the particles appeared to be losing the outer membrane and fully justify the description bullet shaped (Figs. 7a, b). Some particles were clearly associated with RNP-like helical strands, (Figs. 8a, b). Others showed variation in shape, (Figs. 9a-c).

No difference was detected between particles in the various strains of *E. histolytica*; nor between those found in *E. histolytica* and the B.A.H. strain of *E. invadens*.

The specialized areas of cytoplasm, rather like an episome, around which the particles were being elaborated, referred to by Lowe and Macgraith (1970) as a *rosette*, were easily distinguishable from the rest of the cytoplasm, being granular, more compact and with a slightly higher electron density than the rest of the cytoplasm. There was no limiting membrane or vacuolation apart from where the particle membrane appeared to be forming *de novo*, (Fig. 10). One section showed a line of particles in formation (Fig. 11).

Negative Stained Material

Particles were easily identified among the few remaining cell fragments by their distinct similarity to the negative stained appearance of known rhabdoviruses.

Profiles (Figs. 12a, b; 13a, b; 14a, b; 15a, b) accorded well with those seen in fixed material but failed to demonstrate clearly spikes on the outer envelope as seen for instance *on vesicular stomatitis virus (Simpson and Hauser, 1966)* or the dense spot seen in sections. By measuring a number of particles it was possible to give an average size range—see text Fig. A.

DISCUSSION

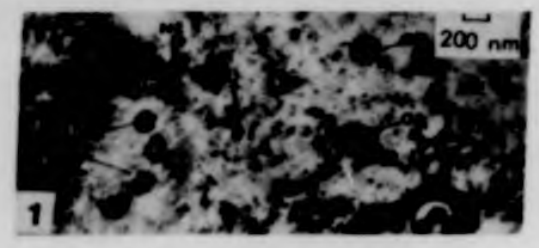
The presence of the GB1 particles in pathogenic strains of *E. histolytica*, collected from various areas of the world also in the B.A.H. strain of *E. invadens* has been known for nearly fifteen years. It is their recent recognition as a probable member of the rhabdovirus group which is of importance.

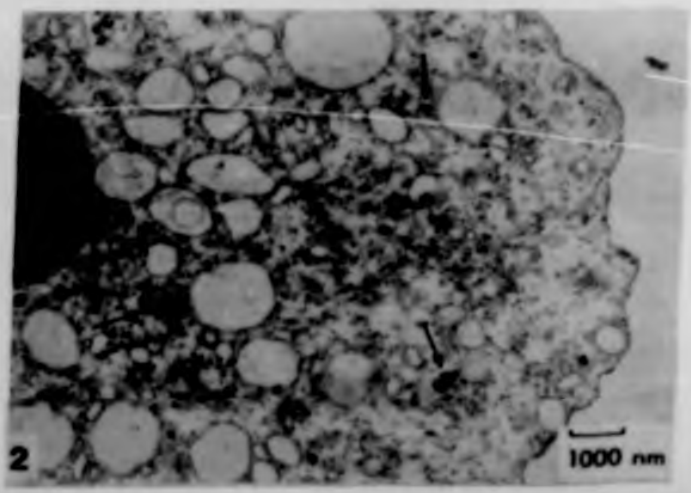
Howatson (1972) reviewed rhabdoviruses of vertebrates, and compiled a useful list of references; Hummeler and Tomassini (1973) pointed out that these and others constitute a genus based on structural similarities. Because they are now known to parasitise plants and invertebrates as well as vertebrates, and that some members of the group are of medical, veterinary and agricultural importance, they are of particular interest; this first recognition of rhabdovirus parasitizing a parasitic protozoa from man and snake adds to that interest.

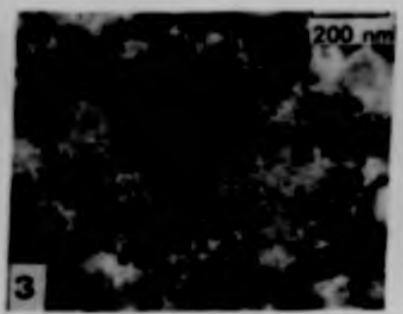
CAPTIONS TO FIGURES

Explanation of abbreviations on Figures 1–6

Ds Dense spot; E Envelope; H Helical strand; Hi Inner helix; Ho Outer helix; N Nucleus; V Vacuole.







1. Two types of granule noted in pathogenic strains of *E. histolytica* and *E. invadens* (Bird, 1961).
2. Rhabdovirus particles (indicated by arrows) arranged in rosettes or scattered throughout the plasm.
3. A cluster of virions at higher magnification.
4. Incomplete forms with uniform granular appearance and no surrounding membrane.

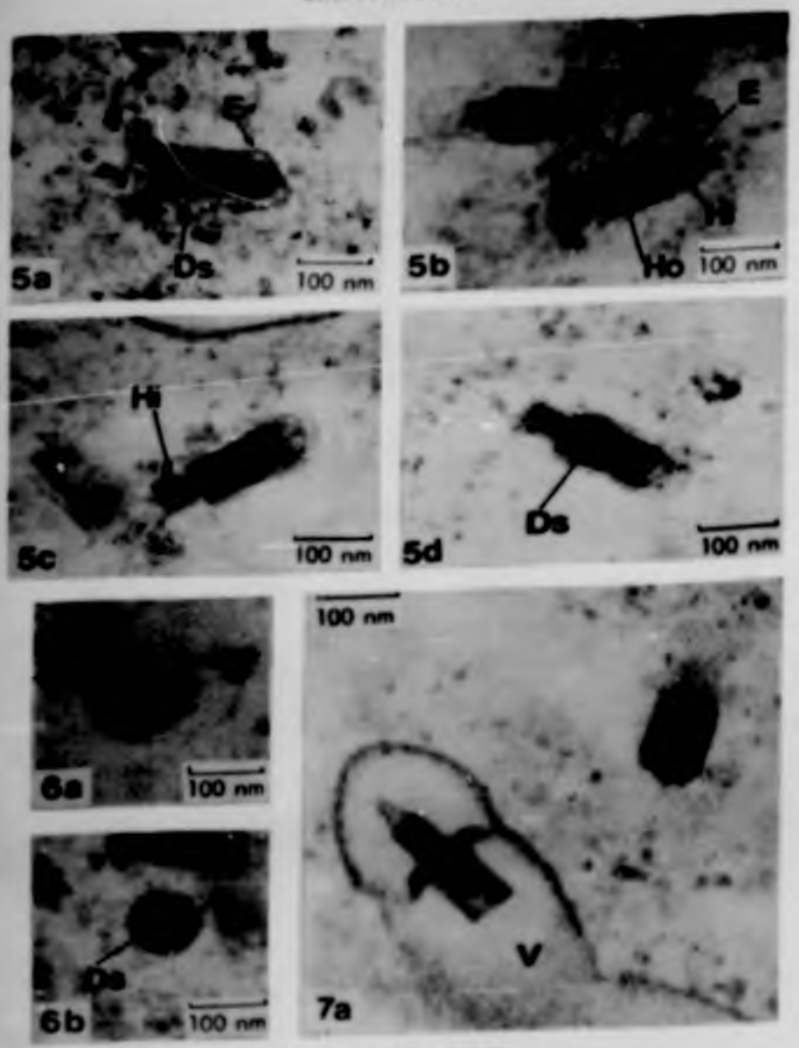


Fig. 5. (a–d). Selected particles to show structural characteristics.
Fig. 6. (a & b). Complete transverse sections of particles.
Fig. 7. (a & b). Particles seen within cytoplasmic vacuoles.

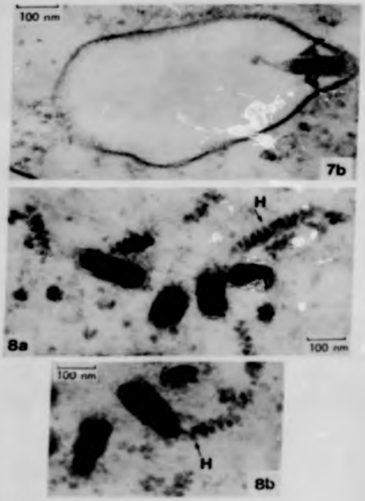


Fig. 8. (a & b). Particles associated with RNP-like helical strands.

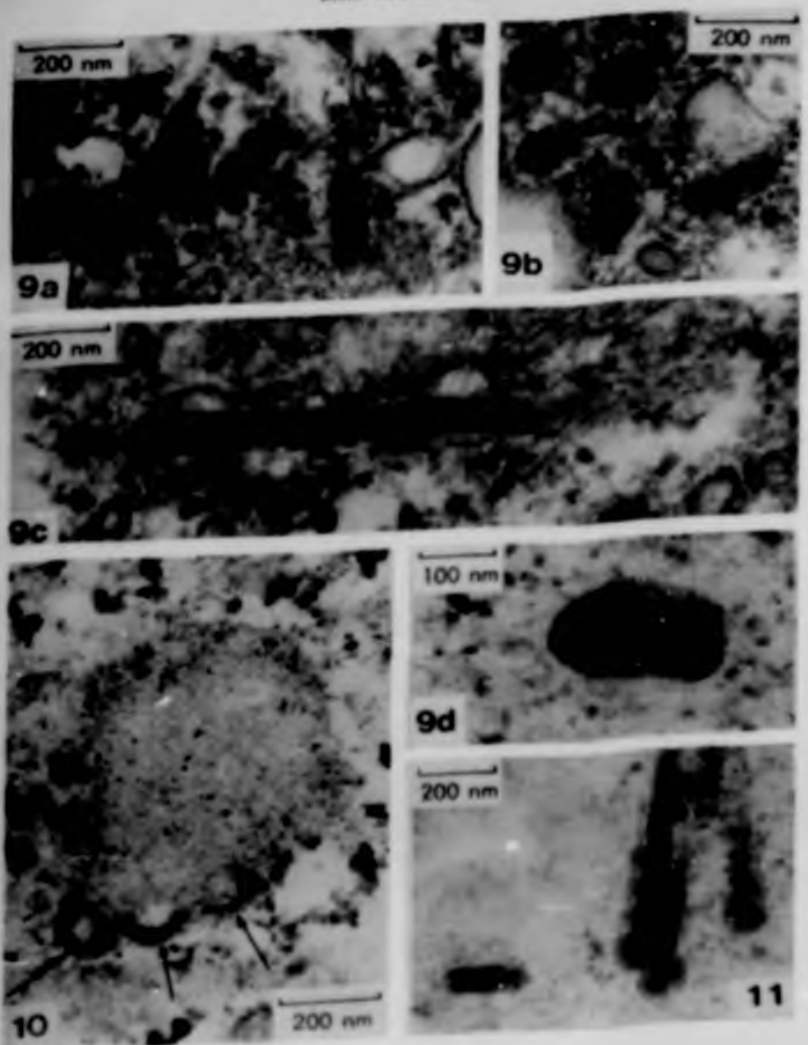


Fig. 9. (a–d). Bizarre forms.
Fig. 10. Specialized area of cytoplasm with particle membrane forming in new
Fig. 11. A line of particles in process of formation.

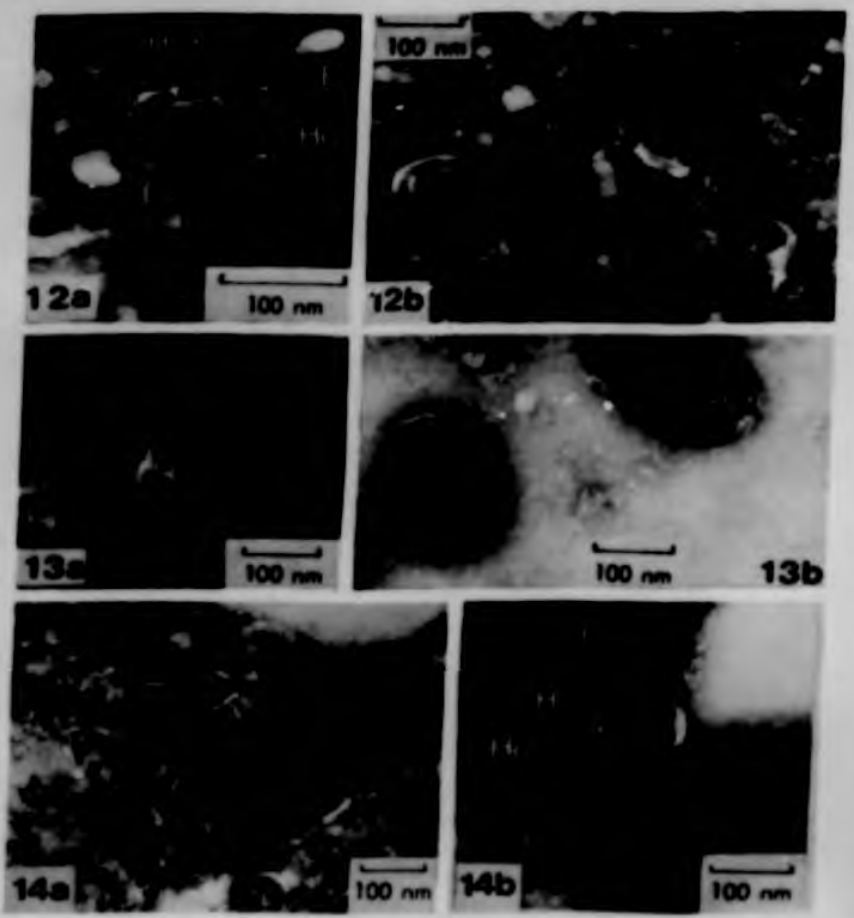


Fig. 12. (a & b). Negative contrast preparations of *E. histolytica* Evans strain.
Fig. 13. (a & b). Negative contrast preparations of *E. histolytica* Laredo strain.
Fig. 14. (a & b). Negative contrast preparations of *E. histolytica* An 200 strain.

Fig. 15. (a & b). Negative contrast preparations of *E. invadens*.

Although, in common with many members of the group, there are, as yet, no data on biological, physical and biochemical properties of this virus, it is possible to point out the characteristic features which led us to suggest classification within the Rhabdovirideae.

Morphogenesis

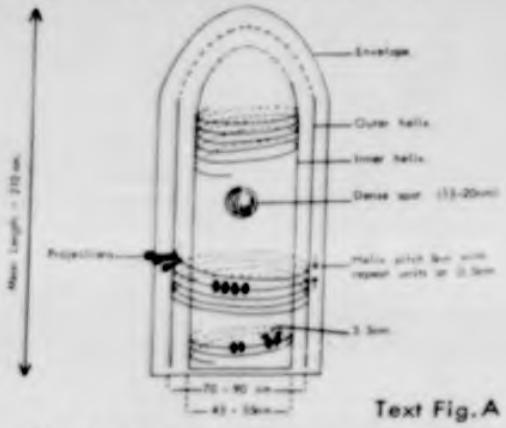
The site of origin of most rhabdoviruses is in the cytoplasm, though some in plants are formed in the nuclei (Ozel, 1973). As yet no DNA rhabdoviruses have been described, and the RNA nucleoprotein as occurs in rabies, Mokola, Lagos and Bat viruses (Howatson, 1972; Murphy *et al.*, 1973) is usually synthesized in discrete granular, but sometimes filamentous, cytoplasmic inclusions which initially are not membrane bound (Hummeler and Tomassini, 1973). Figs. 2 and 3 already show this to be the case in the *Entamoeba* where the virions commonly form a rosette around the inclusion.

The nucleoprotein of most rhabdoviruses exists in the cytoplasm in single strands but in the case of rainbow trout viral haemorrhagic septicaemia (VHS) (Zwillenberg *et al.*, 1965) it is present in small helices of 10 nm diameter. Figs. 8a and 8b would indicate that the nucleoprotein of *Entamoeba* virus is also made up of small helices with diameter approximately 16 nm.

Whereas most rhabdoviruses develop in association with pre-existing cell membranes, the rabies group (Howatson, 1972) can exhibit a *de novo* synthesis of membrane within the nucleoprotein inclusions. This is seen to occur with the elaboration of some of the virions in the *Entamoeba* (Fig. 10).


15a
100 nm


15b
100 nm



Text-Fig. A. Diagramatic representation of the rhabdovirus using information obtained from thin sections and negative contrast preparations. Measurements up to 250 nm × 90 nm have been made.

There is a wide range of replication rates among the rhabdoviruses. This would seem to be low as far as the *Entamoeba* virion is concerned (Fig. 2); rarely are more than 40 particles seen in any one section and we have never seen large numbers in negative contrast preparations. Bos (1973), however, shows a section of axenic strain *E. invadens* containing up to 100 particles.

Dimensions

Most viruses within the group are within the range of 130 nm–230 nm long by 70–80 nm wide (Hummeler and Tomassini, 1973). Measurements up to 250 nm long and 90 nm wide have been obtained from *E. histolytica* particles, which, though slightly on the large side, would not exclude them from the group. The mean length of the particles obtained from both *E. histolytica* and *E. invadens* was 210 nm and mean width 80 nm.

Measurements were made on micrographs printed from Ilford negatives taken at × 63 000 magnification on an AEI EM 801 microscope, whose specification on magnification allows for an error of 5%.

Morphology

Both thin sections and negatively contrasted specimens show the virions to be typically bullet shaped, remarkably so when seen within a membrane bound vacuole and possibly in a state of degradation (Fig. 7a, b). The fully formed particle consists of an outer membrane and two concentric helices—see text figure A. However, some incomplete forms are rounded at both ends and appear like particles of corn virus (Herold *et al.*, 1960).

The absence of surface spikes as exhibited by most viruses of the group is of note but

not of significance as negative contrast materials are known to destroy surface structural details with comparative ease, and there is some evidence that spikes are present in some of the particles seen in fixed material. Further studies changing the characteristics of the negative contrast solutions are being carried out to obtain precise knowledge of this particular morphological detail. The helical arrangement of the nucleocapsid is clearly seen in both thin sections and negative contrast preparations, so that it has been possible to represent diagramatically all the structural details so far known (Text Fig. A).

Aberrant Structures

As well as incomplete forms, bizarre, short and long forms are well documented for most members of the genus and have been particularly well investigated in vesicular stomatitis virus (Holland, 1974), where the short defective interfering particles inhibit the reproduction of complete infective virions—some aberrant forms found among the *Entamoeba* virions are illustrated in Fig. 9a, b.

Efforts to identify the virions have so far failed. They are not pathogenic to suckling mice and apparently will not adapt to a *Xenopus* cell line (Bruce Johnson, Arbovirus Unit, London School of Hygiene and Tropical Medicine—personal communication), and we ourselves have been unable to establish replication in a BHK-21 cell line. This lack of success might indicate a very limited host cell specificity, or even a state of symbiosis within these *Entamoeba* species, achieved over innumerable years of generation to generation transmission; and comparable to the difficulty experienced in cultivating Wolbachia from Argas ticks (Suitor and Weiss, 1961). It may, on the other hand, indicate the existence of defective interferring virions as occurs in the case of VSV (Hackett, 1964).

The slow rate of elaboration and inability to establish virions from either *E. histolytica* or *E. invadens* in the available cell lines has held up progress towards purification and characterization, and indicates how much more work is necessary to solve the problems presented.

Intestinal parasitic amoebae live in close contact with gut epithelial cells, a host of bacterial species, other intestinal protozoa and possibly helminths, as well as a continual flow of mixed animal and vegetable cells. It is not surprising, therefore, that Hruska *et al.* (1972) were able to detect viral agents in nine strains of *E. histolytica*. These were of two types, polyhedral and filamentous. Metabolic inhibitors indicated that the nucleic acids of both types were composed of DNA. It would be interesting to know whether these viruses relate to any known bacterial phages.

The presence of a probable RNA rhabdovirus in *E. histolytica* now extends the known possibilities of genetic transfer and provides a wider base for varying enzyme patterns, as has been shown to occur by Reeves and Dischell (1968). A situation similar to the state of lysogeny in bacteria can now be envisaged bearing with it the same possibilities as 'phage conversion' and 'phage transduction'—see discussion Knight *et al.* (1975). There is obvious need for much more thorough investigation of this aspect of *Entamoeba* bacterial relationship, especially with regard to pathogenicity. Experiments by Wittner and Rosenbaum (1970) already suggest that increased virulence is associated only with contact of amoebae with live bacteria; moreover, that this is likely to be due to the transfer of a factor from the bacteria to the amoeba.

Slow, inapparent and recurrent viruses are known to persist in their host without giving rise to the usual signs of infection, also that the 'DI' virus competes with its intact virus in the replication process (Holland, 1974). Such a mechanism could well be involved in virulence changes in strains of *E. histolytica* or *E. invadens*. The presence of virus in the *Entamoeba* could also account for transfer of genetic markers by mating as put forward by Entner (1971). It would appear, therefore, that the involvement of virus in the pathogenesis of amoebiasis may be as important a factor as the redox potential.

SUMMARY

Rhabdoviruses have been described in plants, arthropods and vertebrates including man. Members of the group are of agricultural, veterinary and medical importance. The presence of a rhabdovirus in *Entamoeba histolytica* and *Entamoeba invadens* is the first record of their existence within protozoa.

The morphology of this virus is described and its significance discussed, in relation to a possible lysogenic state and pathogenecity of *Entamoeba* species.

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