Studies on the factors which determine the survival of *Entamoeba histolytica* in cryopreservation

by

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Dedicated to my husband
Ademola
and my children
Eniola and Aramide

ABSTRACT

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Maintenance of *E. histolytica in vitro* requires frequent subcultures which are time-consuming and may lead to changes in the biological characteristics of stocks. Cryopreservation avoids these problems, since amoebae can be stored at low temperature soon after isolation with their biological characteristics relatively unaltered.

To investigate those factors which may influence survival after cryopreservation, a standard, reproducible method of cultivation was developed using Robinson's medium, which was found to be the best culture medium for growth of amoebae from low inocula. It was possible, using this medium, to prepare genetically homogenous populations of *E. histolytica* by cloning.

A sensitive viability assay, based on titration of infectivity to cultures, was developed to measure the success of cryopreservation.

The effect of various cryoprotectants on the viability of *E. histolytica* stocks before and after freezing was studied. It was found that polyvinylpyrrolidone (PVP), dimethylsulfoxide (DMSO) and glycerol, though cryoprotective, are toxic to amoebae. Although sorbitol, methanol and ethanol at the concentrations used were not in themselves toxic, they conferred no cryoprotection.

Up to 12.5% of the amoebic population survived when the amoebae were equilibrated for 15 minutes at 37° C with

7.5% (v/v) DMSO and then cooled at 1° C/min to -196° C followed by thawing at 37° C and inoculation into medium in less than two minutes. However, there was a partial loss of viability over a storage period of six months in liquid nitrogen.

Conditions which influenced survival were: the protectant, its concentration, the temperature and period of equilibration, freezing rate, suspending medium, culture medium and thawing temperature. A period of 'structural reconstitution' after thawing led to a decrease in viability.

It was possible to show, by characterization of isoenzyme type, observation of concanavalin A-induced agglutination, reaction with antibody in the indirect fluorescent antibody test and studies on leucocytotoxicity, that the biological characteristics of *E. histolytica* had been retained during cryopreservation. The generation-time of some amoebal stocks was found to change after cryopreservation. It was not possible to demonstrate selection of a population with reduced sensitivity to freezing damage.

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A INTRODUCTION AND LITERATURE REVIEW

A.1 Historical review

The word 'amoebiasis' (Musgrave and Clegg, 1904) was first used to denote 'a state of infection with amoebas'. Today, it denotes the condition of harbouring *Entamoeba histolytica* with or without clinical manifestation (WHO, 1969). However, the term amoebiasis is often used to describe a clinical condition attributable to *E. histolytica* alone.

Entamoeba histolytica is a sarcomastigophoran protozoon belonging to the class Rhizopoda von Siebold, 1845 and genus *Entamoeba* Casagrandi and Barbagallo, 1895.

The trophozoite lives and multiplies in the contents of the large intestine of man but under certain conditions, yet unknown, can invade the tissues. Invasion can spread to the liver, brain, lungs and other organs.

The trophozoites (size 10-40 µm) have a characteristic nucleus (2.8-4.5 µm) with a small central karyosome and peripheral chromatin (see Fig. 1). The cytoplasm consists of an outer clear ectoplasm and an inner granular endoplasm enclosing food vacuoles which in pathogenic 'strains' usually contain ingested erythrocytes.

Reproduction is by binary fission. Locomotion is sluggish and is achieved by the use of lobose pseudopodia. Feeding is by phagocytosis. The trophozoite feeds on the content of the gut, on bacteria and on mucus.

The cyst, which is the only known infective form, is spherical (average size 12 µm) and is more compact than the trophozoite. Its nucleus divides by mitosis to produce two, and then four, nuclei which have the same characteristics as those of the trophozoites but are smaller. The

cyst wall is relatively tough; within it are chromatoid bodies which stain black with iron-haematoxylin. Cysts are usually found without the trophozoites in the faeces of symptomless individuals and have never been found in extra-intestinal lesions.

Before the classical work of Lösch (1875), many people had reported finding amoebae in association with man but were not appreciative of the significance of their findings. Gros, 1849 (Dobell, 1919) found amoebae in the mouth; this amoeba was to be known in later years as *Entamoeba gingivalis*. Lambl, 1859 (Councilman and Lafleur, 1891) discovered amoebae in the stools of children suffering from enteritis. Lewis, 1870 (Dobell, 1919) found amoebae in the intestine of man and Cunningham, 1871 (Dobell, 1919), in a sanitary report on cholera to the Governor of India, claimed to have found amoebae in the stools of healthy as well as sick people; the amoeba he found was later considered to be *Entamoeba coli* from the description he gave.

It was Lösch (1875) who first recognized that amoebae, found in association with man, could give rise to clinical conditions. In his classical work at St. Petersburg in 1875, he isolated trophozoites of amoebae from the dysenteric stool of his patient and successfully established dysentery in one of three experimental dogs which he inoculated with the amoebae. His experimental success made it possible for him to record a vivid description of the amoeba's morphology. He made what many people later recall as 'accurate and careful observations' and description of the clinical condition and autopsy findings. After rigorous histological investigations, he became convinced that he was dealing with a true parasite. He called it *'Amoeba coli'*, after the site at which it was found. He was cautious and considered that amoebae alone could not have been responsible for his patient's condition but that there must have been a pre-existing disease, probably dysentery, of other origin, before the infection with amoebae which later sustained the inflammation.

Kartulis (1886) and Quincke and Roos (1893) amongst many others described dysentery amoebae exactly as did Lösch, with only minor differences in the sizes of the trophozoites which they observed, e.g. Kartulis reported the size of his amoebae as 0.012-0.03 mm in diameter, Lösch recorded a diameter of 0.02-0.035 mm.

For many years after Lösch, the position of *Amoeba coli* as the causative agent of dysentery was uncertain. Different views were held at different periods by different workers, the concensus of opinion swung from one side to the other (Elsdon-Dew, 1968). The controversy might not have arisen if only people had known that dysentery can be a symptom of several pathological conditions which could be caused by viruses or bacteria.

It was not until the work of Councilman and Lafleur (1891) that it became clear that other dysentery conditions exist that bear no relationship to that caused by amoeba. They showed that the dysentery amoeba was the causative agent of 'tropical dysentery' because amoebae were constantly present in the stools and in the anatomical lesions from patients and corpses; that its clinical manifestation was different from any other form of dysentery and should therefore be regarded as a distinct disease. They decided that the *Amoebae coli* of Lösch deserved a new name - *'Amoeba dysenteriae'*. They showed further that amoebic dysentery may be followed or accompanied by the formation of a hepatic abscess which is bacteriologically sterile, as earlier observed by Kartulis (1887). In such cases amoebae apparently identical with those found in faeces may be present in the liver (Koch and Gaffky, 1887; Councilman and Lafleur, 1891).

In 1893, Quincke and Roos were not only able to produce dysentery in cats by injecting faeces containing amoebic trophozoites into their recta but also by feeding cats with faeces containing cysts. They were the first to show that 2 kinds of amoebae exist in man, that which caused dysentery in cats,

which they called *'Amoeba coli mitis'*, and that found in the faeces of apparently healthy people, which they called *'Amoeba intestini vulgaris'*.

Casagrandi and Barbagallo (1895) distinguished between *Entamoeba* of man as distinct from *Endamoeba* Leidy, 1879 of cockroaches (Dobell, 1919). In 1897, the same workers proved that the intestinal amoebae were distinct morphologically and culturally. *Entamoeba* from healthy individuals was called *Entamoeba hominis*.

Celli and Fiocca (1895) observed that not all intestinal amoebae caused dysentery in man. Casagrandi and Barbagallo (1897) made similar observations.

By the end of the 19th century, it had been confirmed that there is a particular kind of dysentery caused by amoebae, as Lösch (1875), Kartulis (1887, 1889), Councilman and Lafleur (1891), Quincke and Roos (1893) had demonstrated, and that not all intestinal amoebae cause dysentery in man (Casagrandi and Barbagallo, 1897; Celli and Fiocca, 1895).

Work on the establishment of the life history of *E. histolytica* and further advances took place at the beginning of this century with the work of Huber (1903) who rediscovered (first discovered by Quincke and Roos; 1893) the cyst of the dysentery amoeba. He showed that the cyst of dysentery amoebae may have one, two or four nuclei. Hartmann (1908) also described the quadrinucleate cyst of *E. histolytica* but he believed it was a different species.

Elmassian (1909) described, from the faeces of a quiescent case of recurrent dysentery, amoebic trophozoites closely associated with quadrinucleate cysts and differing from the forms encountered in dysenteric stools only in their size (12-14 μm) and in the fact that they contained ingested bacteria. He thought he was dealing with a new species and so called it *'Entamoeba minuta'*.

Schaudinn (1903) confirmed the observations of earlier workers on the existence of at least 2 different amoebae inhabiting the bowels of man - one pathogenic and the other harmless. He differentiated *E. coli* found in healthy people from the haematophagous amoeba found in dysentery cases, which he called *E. histolytica*. In an attempt to describe the development of *E. histolytica*, he misled a great many workers by stating that *E. histolytica* propagates itself by sporulation and that completely dry spores gave infection to cats. He also stated that *E. coli* reproduced by schizogony. He believed that the quadrinucleate cyst described by Huber (1903), which he himself had observed, was a different species, naming it *Entamoeba tetragena*.

Walker (1911), in his extensive research on the comparative study of the amoebae in Manila's water supply, in the intestinal tract of healthy persons and in persons with amoebic dysentery concluded that amoebae from the intestinal tract which were cultivable in Musgrave and Clegg's medium (Musgrave and Clegg, 1904), were probably derived from the cysts of amoebae that had been ingested with water or food and had passed unchanged through the gut. He concluded that the amoebae parasitic in man were strict obligate parasites which were incapable of multiplication outside their host: infection from them must always come directly or indirectly from another infected person. He recognized *E. coli* as a non-pathogenic species which produced cysts with as many as eight nuclei. He showed that *E. tetragena* and *E. histolytica* produced quadrinucleate cysts and were associated with clinical disease in man. He demonstrated that tetragena and minuta forms referred to a phase of *E. histolytica* intervening between tissue stage and the formation of cysts and that *E. histolytica* has the same life history as *E. coli*.

In 1913, Walker, in collaboration with Sellards, proved all the statements made in an earlier paper (Walker, 1911) by experimenting with human volunteers. These human 'guinea-pigs' were fed with all the different species of amoebae that could be cultivated in Musgrave and Clegg's medium from

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stools of healthy persons and stools of cases of amoebic dysentery: *E. coli* from healthy persons, cysts of *E. tetragena* from carriers and motile *E. histolytica* from acute cases of amoebic dysentery and amoebic liver abscesses. They observed that:

(i) amoebae which could be cultivated in Musgrave and Clegg's medium did not infect volunteers or cause dysentery, and so play no rôle in the aetiology of tropical dysentery;

(ii) *E. coli* and *E. histolytica* are strict or obligate parasites uncultivable in the artificial media then available;

(iii) *E. tetragena* was identical with *E. histolytica* and its cysts represented a stage in the life history of *E. histolytica;*

(iv) *E. histolytica* was the essential aetiologic factor in endemic tropical dysentery, carriers of *E. histolytica* pass large numbers of cysts in their stools and they constitute the chief agent in the dissemination of amoebic dysentery;

the motile forms of the parasite passed in bloody mucous stools in acute dysentery quickly die and disintegrate and are probably, under natural conditions, incapable of withstanding passage through the human stomach.

Walker and Sellards' work showed conclusively that *E. histolytica* could live as a commensal in the gut lumen of man. It appeared that only when conditions became abnormal owing to depression of the natural resistance of the host or his tissue, or when there was pre-existing inflammation or lesions, could amoebae penetrate the intestinal epithelium to produce characteristic lesions of amoebic dysentery.

Kuenen and Swellengrebel (1913) recognized the commensal phase of *E. histolytica* as an integral and essential part of its life history. They found that in healthy individuals, and in dormant periods of chronic infections, the amoebae correspond to Elmassian's *E. minuta*, and they called it the 'minuta' stage. At this stage, the amoeba lives as a commensal in the lumen eventually producing quadrinucleate cysts. On invading the tissue these amoebae assume the appearance

and haematophagous habits of the large *E. histolytica* forms. The commensal phase was called the 'saprozoic' phase.

It was reported (Elsdon-Dew, 1968) that, at the 'Reunione d'Information sur l'Amibiase' in Paris 1961, Swellengrebel explained that he and Kuenen had retained the name *'Minuta'* not as a different species but to show that in the evolution of *E. histolytica* there is a stage that is independent of the tissue phase. However, Walker thought this should not be, because, he explained, 'the *minuta* form is a direct descendent of the tissue form, the absence of pathology in some cases was due to the good immune response of the host'. Kuenen maintained that, although the *minuta* forms are direct descendants of the commensal form, they have variable tendencies to metamorphose into tissue forms and therefore invasion would depend on the quality of host defence and the inclination of the amoebae in question to metamorphose.

In 1917, Wenyon and O'Connor showed that a great many more symptomless than symptomatic people are colonised by amoebae, that the cysts produced by carriers could give dysentery to kittens when artificially inoculated, and that stages of acute dysentery may alternate with quiet infections with *E. histolytica*. At this stage the amoeba lives as a commensal. They confirmed that there is no evidence of pathogenicity in *E. coli*.

Dobell (1918) disagreed with the concept that the *histolytica* tissue-invading phase plays no part in the normal life history of *E. histolytica*, as concluded by Kuenen and Swellengrebel (1913) and Walker and Sellards (1913). In his review of the work of Wenyon and O'Connor (1917) he wrote "A parasite which feeds upon its host may obviously do so to a greater or less extent. The ideal condition for host and parasite alike is a state of equilibrium like that between Prometheus and the Eagle - the former generating sufficient tissue each day to compensate the ravages of the latter. The

natural condition of man infected with *E. histolytica* is similar . . . A human being in the state of Promethean equilibrium with his amoebae is called the carrier and there can be little doubt that this is the normal or most common condition of infection of humans with *E. histolytica* in nature. When amoebae devour more tissue than the man can regenerate, destruction outruns construction and disharmony or disturbance of equilibrium results . . . The ravages of the amoebae soon produce a state of dysentery in their host, blood and mucus flowing from the destroyed surface carrying away many of the parasites and being painfully evacuated by the host at frequent intervals in his attempt to get rid of the irritation."

Thus, by the end of the second decade of this century, there existed two schools of thought - the Prometheans, Dobell's school who held that the carrier state was a disease state, and the commensalists, Swellengrebel's school, who held that this was not necessarily so (Elsdon-Dew, 1968).

Dobell and Jepps (1918) studied the diverse races of *E. histolytica* by measurement of their cysts and from the size-distribution curves for many cysts from seven cases, they concluded that *E. histolytica* is a collective species comprising an unknown number of distinct races, strains or pure lines of which five had been demonstrated by them. At about the same time, Smith (1918) accurately described the morphological criteria for differentiating *E. histolytica* from *E. coli*. He also constructed a size-distribution curve based on the measurement of 1000 cysts and showed that the size of the cysts divides *Entamoeba* with quadrinucleate cysts naturally into two strains, the small and the large. He studied the two groups extensively and was able to distinguish between the two strains by the frequency of the number of nuclei which their cysts contained and by the differences in their cytoplasmic inclusions.

In 1919, Dobell published a book "The amoebae living in man". In his book he stated that the species of the genus

Entamoeba parasitic in man include *E. histolytica*, *E. coli* and *E. gingivalis*. He also mentioned that other genera existed - *Endolimax*, *Iodamoeba*, and *Dientamoeba*. He emphasized that the name *Entamoeba* should apply to amoebae similar to those of man.

Brumpt (1926) maintained that three species of amoebae parasitic in man produce quadrinucleate cysts: *E. dysenteriae*, *E. dispar* and *E. hartmanni* von Prowazek, 1912. He described the cyst of *E. hartmanni* as being small, less than 10 µm in diameter, and those of *E. dysenteriae* and *E. dispar* as being large being 10-16 µm in diameter. *E. dysenteriae*, according to him, differs from *E. dispar* only in the sense that *E. dispar* is never pathogenic, and *E. dysenteriae* can be invasive and cause dysentery.

The life history of the amoebae in man remained confused for many years until a suitable culture medium for *E. histolytica* was discovered by Boeck and Drbohlav (1925). This medium was used by Dobell to carry out extensive researches on the growth and development *in vitro* of *E. histolytica* of man and of intestinal protozoa of monkeys, concentrating largely on the influence of bacterial flora on growth (Dobell and Laidlaw, 1926a, 1926b; Dobell, 1927, 1928; Dobell and Neal, 1952). These researches led Dobell to revoke his earlier view that *E. histolytica* could not exist as a harmless commensal in man, feeding on bacteria. He observed that the *E. histolytica* of monkeys lived in this manner.

According to Dobell (1928), there are four stages in the life history of *E. histolytica* - the trophic amoeba, the precystic amoeba which is capable of encystation, the encysted amoeba, and the metacystic amoeba which ultimately gives rise to the trophic forms. The cycle, he said, can be induced to occur *in vitro "ad infinitum"* or *"ad libitum"* (see Figs. 1-3).

Sapero *et al* (1942), from the results of their statistical studies on over 3,000 cysts alongside with the data of Dobell and Jepps (1918) and Smith (1918), were able to show conclu-

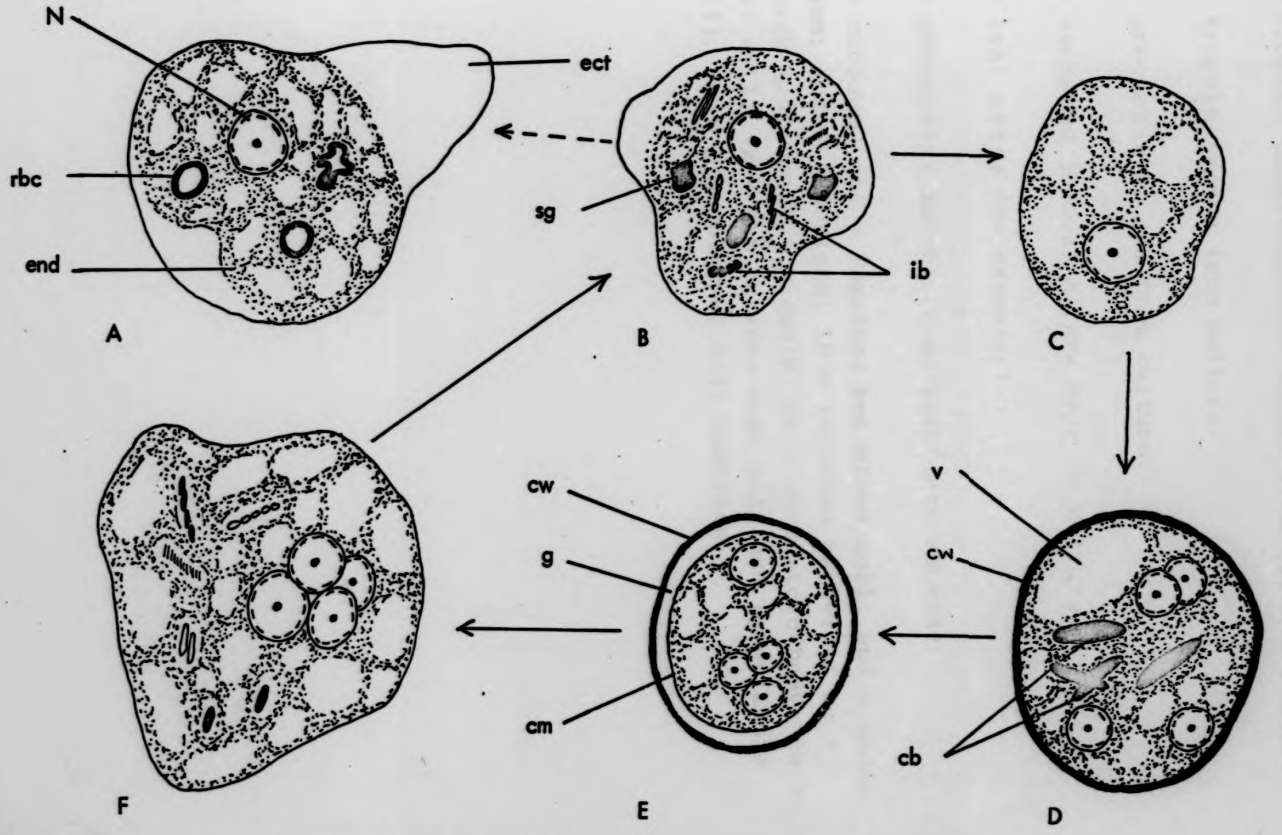

Fig. 1 The life cycle of Entamoeba histolytica after Dobell, 1928

Legend to Figure 1

A = trophic haematophagous amoeba from dysentery stool.

B = trophic amoeba from culture.

C = precystic amoeba from culture.

D = encysted amoeba, mature cyst in culture.

E = cyst ready for excystation.

F = metacystic amoeba (quadrinucleate amoeba)

n = nucleus; rbc = ingested red blood cell; end = endoplasm; ect = ectoplasm; ib = ingested bacteria; sg = starch granule; v = vacuole; cb = chromatoid body; cw = cyst wall; (g = gap between cyst wall and cytoplasmic wall); cm = plasmalemma, cell membrane.

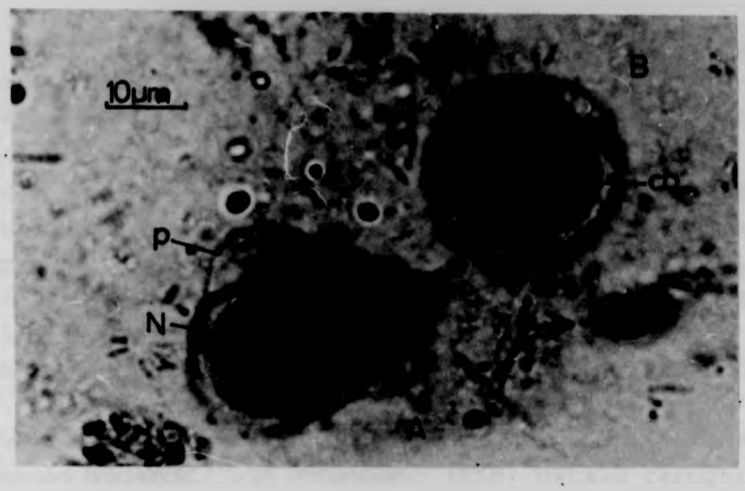

Fig 2 Two stages in the life history of E. histolytica in Robinson's medium
A = trophozoite; B = immature cyst; N = nucleus;
P = pseudopodium; cb = chromatoid body.

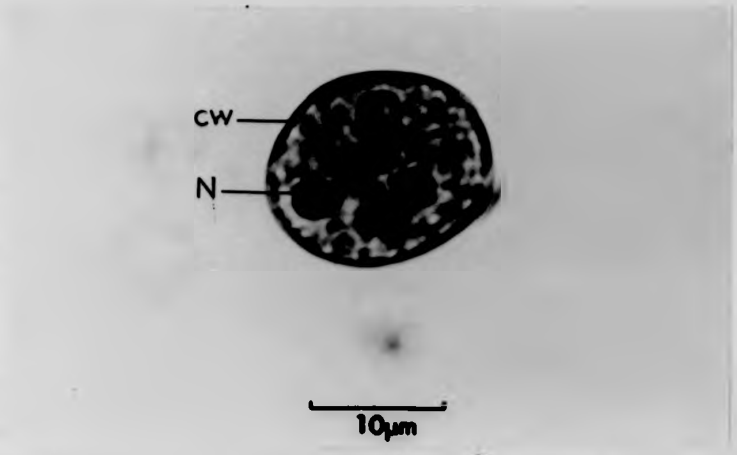

Fig 3 A mature, tetranucleate cyst of E. histolytica
N, nucleus; cw, cyst wall.

sively that two races of amoebae producing quadrinucleate cysts occur in man - the small and the large races. In 1949, Hoare, following the information provided by statistical studies of Sapero *et al*, suggested that the large race and the small race remain subspecies rather than separate species in view of the overlap in the size of their cysts. He suggested that the small race be designated *E. histolytica hartmanni* and the large race *E. h. histolytica*. Goldman (1960), used fluorescent antibody technique (FAT) to establish that there was a difference in antigenic constitution between *E. histolytica* and *E. hartmanni*. Hoare (1961) concluded that the small race genuinely deserved a species rank and so *Entamoeba hartmanni* von Prowazek, 1913 became recognized as a separate species.

To confuse the issue even further, Dreyer (1961) reported that another strain of *E. histolytica* had been isolated by Dr. F.H. Connell (1956) as trophozoites from the diarrhoeal stool of a patient in Laredo, Texas. It was called the Laredo strain. It differs from *E. histolytica* (large race) only in its ability to grow at a temperature of 25° C *in vitro*. The difference between Laredo strain and *E. histolytica* was confirmed by Entner and Most (1965) who showed that they differ in their susceptibility to various chemotherapeutic agents. Goldman and Gleason (1962) and Richards *et al* (1966) also showed differences in their antigenic make up.

At least five strains of *E. histolytica*-like amoebae which have essentially the same antigenic properties as the Laredo strain have been isolated (Richards *et al*, 1965,1966), all from North America. The inability of Neal and Johnson (1966 and 1968) and of Robinson and Sargeaunt (1969) to isolate similar strains of amoebae in England led them to suggest that this type of amoeba may be region-specific. All Laredo-type *E. histolytica* strains have been shown to be avirulent in young laboratory animals (Neal and Johnson 1966 and 1968; Richards *et al*, 1965).

The classification of *Entamoeba* has been based primarily on morphology, host specificity and pathogenicity but these are not reliable because, for example, there are few consistent morphological differences between species other than size (Gelderman *et al*, 1971), and because host specificity and pathogenicity are unstable characteristics (Neal, 1971). It is unfortunate that after a century of research in amoebiasis a suitable method has not been developed to distinguish between strains of typical *E. histolytica* - especially between pathogenic and non-pathogenic strains.

Several biochemical, physiological and drug-sensitivity methods have been sought to differentiate between strains of *Entamoeba*. For example, Gelderman *et al* (1971) demonstrated that there is a significant difference in deoxyribose nucleic acid base composition in strains of *Entamoeba* thought to be of the same species indicating the existence of more than one genostrain within the typical *E. histolytica* group. Trissl *et al* (1977) showed that strains of amoebae considered to be pathogenic formed much larger agglutinates than other strains in the presence of 50 µg/ml of concanavalin A. The same authors also showed that 'non-pathogenic' strains showed negative surface charge and 'pathogenic' strains showed a very low charge which was impossible to measure by the technique of cell electrophoresis under their experimental conditions.

By immunofluorescence after cross absorption of antisera between typical *E. histolytica* strains, Nayebi (1971) demonstrated that there existed some minor differences. Serological methods of this nature have been unquantitative and are therefore of little value; there is therefore an indication for an extra taxonomic tool.

Krupp (1966) analysed the antigens of typical *Entamoeba* strains and showed that there existed some minor differences in their electrophoretic mobilities. Montalvo and Reeves

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Krupp (1966) analysed the antigens of typical *Entamoeba* strains and showed that there existed some minor differences in their electrophoretic mobilities. Montalvo and Reeves

(1968) and Reeves *et al* (1967) developed a technique by which amoebae could be characterized by their isomerase and glucokinase enzymes by electrophoresis on cellulose acetate strips. They indicated that their method might be a convenient means of classifying amoebae and of distinguishing typical *E. histolytica* from atypical forms and related species.

More recently, Sargeaunt and Williams (1978) have extended this use of isoenzymes (malic enzyme, glucose-phosphate isomerase and phosphoglucomutase) in characterizing amoebae by thin-layer starch-gel electrophoresis. Intra- and inter-specific differences have been shown. *E. histolytica* strains fell into three groups based on their isoenzyme electrophoretic patterns. There appears to be an association between clinical amoebiasis and the presence of amoebae in isoenzyme group II. Isoenzyme characterization may very well be an important tool in taxonomy of *E. histolytica*.

A.2 Culture methods

Cultivation of organisms *in vitro*, first unequivocally achieved for *E. histolytica* by Boeck and Drbohlav (1925), is an attempt to reconstruct the conditions in the natural habitat of the organism by artificial means. Anaerobic and reducing conditions which appear to be important in the survival of *E. histolytica* in the colon are the result of the presence of a very large bacterial flora. Nutrients such as starch and other food materials including bacteria and fungi are available to the amoebae in endless supply. A rich supply of vitamins is available from the food ingested by the host or synthesized by the bacteria. The colon appears to be well buffered so as to maintain an optimum pH at which the amoeba thrives.

These anaerobic and reducing conditions are achieved in the artificial medium through consumption of available oxygen by the concomitant organism such as bacteria, chick-embryo extract, trypanosomes or *Crithidia*, or by inclusion of

reducing agents such as cysteine, thioglycollate or ascorbic acid in axenic media. Starch granules, peptides, amino acids or chick-embryo extracts are the chief sources of food in amoebic media in which concomitant organisms exist. But in the axenic medium glucose is the main source of carbohydrate. Vitamins are essential constituents in axenic cultivation, being supplied by the serum and liver components and also added separately. In other media they are provided by the concomitant organisms and are also available from serum and liver extracts when used.

A good buffer system is needed in an artificial medium to maintain optimum pH for amoebal growth. Good buffer systems are provided in diphasic media partly by the solid part of agar, coagulated egg or inspissated serum and partly by the presence of phosphate and/or phthalate salts in the overlay. The buffer systems in monophasic media are generally of the phosphate type.

The original medium of Boeck and Drbohlav is essentially a diphasic medium consisting of a solid egg slant overlaid with dilute egg-white or serum in Locke's physiological solution. Dobell and Laidlaw (1926b) were the first to exploit and improve on this technique. They found that the original Locke-egg-serum or LES (of Boeck and Drbohlav, 1925) could not maintain *Entamoeba* continuously for more than three days because Locke's solution contained dextrose, which is readily split by accompanying bacteria with production of acid which is highly detrimental to the amoebae. They replaced dextrose by starch and found that growth was greatly improved. After several experiments, they were able to conclude that rice-starch was the optimum source, accessible only to the amoebae without the risk of production of acid by the usual faecal bacteria, although starch-splitting bacteria still cause trouble in the isolation of amoebae.

In the years that followed, Dobell (1931) was able to show that inspissated horse serum was as good as whole egg

for the solid phase and that diluted egg-white in Ringer's solution was more effective as an overlay. He was thus able to produce a culture medium (HSre) in which amoebae could thrive for several days and sometimes weeks without serial passages. However, passages at 2-3 days intervals were shown to support growth *in vitro* indefinitely.

Additional culture media have been developed empirically for the isolation and cultivation of intestinal amoebae, especially *E. histolytica*. These media have been prepared from a variety of materials and may be diphasic with a solid slant overlaid with fluid, semi-solid, liquid or more rarely entirely solid. A summary of the most commonly used media is given in Table I.

Basically, the conventional diphasic medium invariably has one or more bacterial components, rice-starch and a protein mixture, usually serum. The development of bacteria-free media for *E. histolytica* was first achieved by Shaffer and Frye (1948) who grew amoebae in a medium containing chick embryonic extract. As an outgrowth of the development of Shaffer and Frye medium (S-F), Phillips (1950, 1951) developed a procedure for the monoxenic cultivation of *E. histolytica* with *Trypanosoma cruzi* or other trypanosomatids. Diamond (1968a) developed a method for the cultivation of *E. histolytica* and *E. histolytica*-like amoebae in association with either *Crithidia* species (isolated from a hemipteran, *Arilus cristatus*) or *T. cruzi*.

In 1968, Diamond unequivocally developed a medium for the indefinite cultivation of amoebae in an environment free of metabolizing bacteria, fungi, protozoan or metazoan cells, i.e. the axenic medium (Diamond, 1968b).

One of the difficulties of studying protozoa *in vitro* is the maintenance of strains. To maintain a rich and healthy growth of amoebae indefinitely, whatever the culture medium, it is necessary to passage the cultures frequently at between 48-96-hour intervals. Such serial passages have

Table I

Culture media most frequently used in the cultivation of *Entamoeba histolytica*

Name of medium	Type of medium	Components	Concomitant organism	Reference
L-E-S	Diphasic	Solid part - whole egg and Locke's solution Overlay - dilute egg-white or serum in Locke's plus starch	Bacteria	Boeck and Drbohlav (1925)
Ehsr	Diphasic	Solid part - coagulated egg and Ringer's solution. Overlay - horse serum, Ringer and starch	Bacteria	Dobell and Laidlaw (1926)
LIA	Diphasic	Solid part - liver infusion agar Overlay - horse serum, Ringer and starch	Bacteria	Cleveland and Collier (1930)
HSre	Diphasic	Solid part - inspissated horse serum Overlay - dilute egg white, Ringer's solution and starch	Bacteria	Dobell (1931)

Table I contd.

St. John's	Monophasic (fluid)	Beef heart extract in modified Locke's solution	Bacteria	St. John (1932)
Semi-solid		3% agar in serum diluted in Locke's physiological solution	Bacteria	Adler and Foner (1941)
EY1	Monophasic (fluid)	Buffered aqueous egg-yolk infusion without serum	Bacteria	Balamuth (1946)
Solid medium (anaerobically cultivated)	Monophasic (solid)	Horse serum, yeast, peptone, agar, cholesterol and Ringer's solution	Bacteria	Snyder and Meleney (1946)
Jones's	Monophasic (fluid)	Dilute horse serum or dilute horse serum supplemented with yeast extract	Bacteria	Jones (1946)
Nelson's	Diphasic	Solid part - egg yolk alcoholic extract and agar Overlay - plain buffered solution	Bacteria	Nelson (1947)
S-F (original)	Monophasic (fluid)	Thioglycollate agar - containing medium and horse serum	Bacteria (only for preconditioning)	Shaffer and Fry (1948)

Table I contd.

Amoeba-Trypanosome medium	Diphasic	Solid part - NIH blood agar Overlay - thioglycollate preparation and horse serum	Live or heat treated *T. cruzi*	Phillips (1950)
EY1	Diphasic	Solid part - liver and agar Overlay - egg yolk infusion and horse serum	Bacteria	Balamuth (1951)
S-F (modified)	Monophasic (fluid)	Chick embryonic material	None	Shaffer *et al* (1953)
Whole blood medium	Diphasic	Solid part - agar in Ringer's solution Overlay - whole human or rabbit blood in buffered saline	Bacteria	Inoki *et al* (1953)
McQuay's	Diphasic	Solid part - agar charcoal and cholesterol Overlay - plain buffered solution	Bacteria	McQuay (1956)
MS-F	Monophasic (fluid)	Enzymatic casein digest, glucose thiomalate and salts in demineralized water	None	Reeves *et al* (1957)

Table I contd.

Robinson's BRS	Diphasic	Solid part - saline agar Overlay - buffered phthalate solution and serum with peptone and antibiotic	Bacteria *(Escherichia coli)*	Robinson (1968)
TTY	Monophasic (fluid)	Tryptose, trypticase, yeast extract and blood	*Crithidia* species or *T. cruzi*	Diamond (1968)
TP-S-I	Monophasic (axenic)	Trypticase-Panmede broth, serum and vitamins	None	Diamond (1968b)
Modified Asami	Monophasic (solid)	Meat extract peptone, agar, glucose and horse serum	Bacteria	Myjak (1971)

the disadvantage of being tedious and time consuming (Diamond, 1964). If several strains are being passaged, this calls for the expenditure of time and material (McEntegart, 1954; Kasprzak and Rydzewski, 1972). There is also the risk of contamination by bacteria and fungi of axenically cultivated amoebae (McEntegart, 1954). Quite apart from the disadvantages mentioned above and the materials involved, frequent passages *in vitro* may lead to changes in the strain until it bears little resemblance to the freshly isolated one (McEntegart, 1954; Gordon *et al.*, 1969; Kasprzak and Rydzewski, 1972). It is a well known fact that changes in the biological characteristics of amoebae can occur when they are repeatedly passaged (Wenyon, 1926), e.g. virulent strains invariably become attenuated (Thompson *et al*, 1954; Elsdon-Dew, 1958; Hoare, 1958; Neal, 1958; Vincent and Neal, 1960; Biagi and Marwin, 1964; Wittner and Rosenbaum, 1970; Tanimoto *et al*, 1971; Neal, 1971; Phillips, 1973 and Zaslavaskaya, 1975); there may also be a loss of infectivity, that is, the ability to establish itself in a host, and of pathogenicity, that is, the ability to produce detectable disease in a host (Chang, 1945).

These difficulties call for methods by which strains of amoebae can be maintained under conditions which will prevent or minimize modification of their original characteristics. Cryopreservation appears to be an obvious solution to these difficulties (Weinman and McAlister, 1947; McEntegart, 1954; Diamond, 1964; Gordon *et al*, 1969; Kasprzak and Rydzewski, 1972 and Dalgliesh, 1972).

A.3 Cryoprotection

1 What is cryopreservation?

Cryopreservation is the viable preservation of living organisms at very low temperature (Lumsden and Hardy, 1965) in a state of suspended animation in which ageing or death does not occur and in which metabolic processes are arrested and can be restarted. The term was derived from cryogenics - the study of ice (Parkes, 1964). A new term was needed to

designate populations whose reproduction was arrested by viable preservation, so the term 'stabilate' was suggested (Lumsden and Hardy, 1965) and this has come into general use. A stabilate is defined as a population of organisms viably preserved on a unique occasion (Lumsden and Hardy, 1965).

But cryobiology - low temperature biology - was first studied in 1884 when Fol succeeded in culturing amoebae and ciliates from earth which had been cooled to -100° C (Luyet and Gehenio, 1940). In 1936, Becquerel recovered living amoebae of various species, an *Euglena* and a *Paramecium*, from completely desiccated soil which had been exposed to liquid helium at -269° and -271° C.(Smith, 1961). However, Taylor and Strickland in 1936 (Smith, 1961) suggested that some organisms may have encysted before exposure to intense cold in the soil used by Fol and Becquerel. In 1912, Laveran and Mesnil showed that pathogenic trypanosomes survived and retained their virulence after 15 minutes exposure to liquid air at -191° C. Coggeshall (1939) recovered *Plasmodium* species from the frozen state in CO_2 and alcohol at -72° C to -80° C after 70 days. Wienman and McAlister (1947) cryopreserved various protozoa including *Trypanosoma*, *Leishmania*, *Trichomonas*, *Plasmodium* and amoebae in a CO_2 ('dry-ice') cabinet at -15° C and -70° C for varying lengths of time. They were unable to recover *Balantidium* after freezing. *E. histolytica* survived freezing to -15° C just once (but not to -70° C). At this temperature, it is likely that the *E. histolytica* was supercooled but not frozen even though the suspending medium was. Smith *et al*(1951) demonstrated that free-living amoebae appeared not to be frozen when cooled to -80° C even though the surrounding medium was; neither was there a change in their size during the short period of observation. Weinman and McAlister froze both trophozoites and cysts in their experiments but did not indicate which actually survived the freezing to -15° C.

Later workers were able to achieve more success when principles governing the survival of protozoa at low tem-

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Later workers were able to achieve more success when principles governing the survival of protozoa at low tem-

peratures were better understood. These will be discussed later.

In parasitology research, it is necessary to isolate and maintain parasites in culture bottles and/or in laboratory animals. Such serial passages are tedious and time-consuming but, more important, lead to changes in the characteristics of the strain. It was soon realised that cryopreservation had a great many advantages over maintenance by serial passage. Cryopreservation is a convenient way of storing animal populations over very long periods of time until they are needed again for experimentation (Lumsden, 1972; James and Farrant, 1976). It is a convenient way of transferring them from laboratory to laboratory without the need to send animals; it is a way of arresting the continuous reproduction of a population which occurs when it is maintained by serial passage in animals or cultures (Weinman and McAlister, 1947; Kasprzak and Rydzewski, 1972; Lumsden, 1972). That changes can take place in the biological characteristics of amoebal populations during serial passage is well known and has been discussed above: cryopreservation is a means of avoiding this.

"Cryopreservation at a low passage level as nearly related as possible to the original wild population offered therefore a way of setting up a comprehensive collection of materials closely representative of those circulating in nature. Equally, it offered the possibility of comparing critically, in the same experiment and at the same time, the biological behaviour of populations separated more or less in a passage series" (Lumsden, 1972). It prevents adaptation to laboratory animals of newly isolated strains (Walker, 1966). Virulence, it is said, may even be conserved (Weinman and McAlister, 1947; Lumsden and Hardy, 1965; Schneider *et al*, 1968).

Freezing and thawing of cells, however, generally causes death (Dalgliesh, 1972; Farrant, 1972). Intracellu-

lar freezing is not the sole cause of death at low temperatures (Smith, 1961) but the presence of large internal ice-crystals almost invariably appears to be lethal (Mazur, 1960, 1961).

ii The physiology of freezing

Living cells contain water up to 90% of their total weight and are also surrounded by it. Water surrounding cells usually contains various salts and perhaps proteins and other chemicals. When water freezes, several important physico-chemical changes occur, e.g. alteration in its viscosity, its ability to dissolve gases and its thermal conductivity as well as osmotic and chemical effects associated with phase changes, as salts separate from water before the lowest temperature at which salts can exist in solution is reached (Meryman, 1966; Ashwood-Smith, 1970). When water in a solution freezes, the solute is concentrated in the water remaining and the melting point of this remaining solution is lowered. Ultimately, all the water that can be crystallized as ice freezes, leaving only the solute and its water of hydration. Further reduction in temperature results in the solidification of the remaining solution (Meryman, 1960).

When water in the external medium freezes, the vapour pressure of the solution of salts remaining unfrozen outside the cell is lowered below that of supercooled water (water cooled below its freezing point without solidification) within the cell. This vapour-pressure differential draws water out of the cell, where freezing has not yet taken place, and further freezing takes place externally as cooling progresses (Mazur, 1960; 1963; Mazur *et al*, 1972). If the cooling-rate is slow or the permeability of the cell to water is sufficiently high, there is sufficient time for an appreciable quantity of water to leave the cells before they cool to the temperature at which intracellular freezing would ordinarily occur. By the time that temperature is reached, the

residual intracellular solution is incapable of freezing so that crystallization becomes impossible (Mazur, 1960, 1963). When cooling-rate is too high, or the permeability of the cell to water is too low, the water in the cell becomes increasingly supercooled and eventually freezes intracellularly - because there is no time for sufficient water to leave the cell to prevent intracellular freezing. (Mazur 1960, 1963).

Living cells are usually not damaged during short periods in the supercooled state (-30° C) but as soon as freezing takes place, damage is likely to occur in a high proportion of cells of most types (Farrant, 1970). Freezing also causes profound changes in the physical environment which are sufficient to denature the more sensitive lipoprotein complexes of the cell membrane, which are bound by weak association and are unstable. As mentioned above, when the medium in which a cell is suspended freezes, ice separates out as pure substance and the solutes and cells are concentrated in the spaces remaining between the ice crystals. This process of concentration changes the physical environment of the cell in several harmful ways (Lovelock, 1957). But death from freezing is caused not by freezing itself but by the following combinations of factors:

i. mechanical injury by ice crystals (Nei, 1970; Farrant, 1970);
   a. extracellular ice from slow cooling;
   b. intracellular ice from fast cooling (Farrant and McGann, 1975);

ii. chemical injury from concentration of salts (Lovelock, 1953; Farrant and Woolgar, 1972);

iii. dehydration (Luyet and Gehenio, 1940; Lovelock, 1957 and Meryman, 1968);

iv. metabolic imbalance (Farrant and Morris, 1973; Farrant and McGann, 1975);

v. cooling rate (Mazur *et al*, 1972).

When temperature falls below freezing point ice forms initially outside the cell; once ice begins to form, latent heat is released that has to be removed as cooling progresses (Meryman, 1966). There is concentration of all dissolved substances in the extracellular liquid phase. The raised concentration of solutes, particularly electrolytes, creates osmotic changes which may damage the cell by interacting with its lipo-protein membranes (Lovelock, 1954; Farrant, 1970). Increase in salt concentration increases membrane permeability in red blood cells, with a loss of phospholipids from the cell; this loss makes the cell membrane permeable to cations. When the ionic strength is suddenly reduced with thawing, the cells suffer osmotic lysis (Lovelock, 1953). The buffer systems are disturbed when salts precipitate and severe changes in pH can take place both inside the cell and in the surrounding medium (Lovelock, 1957; Van den Berg, 1959; Farrant, 1970). Change in pH is said to be responsible for denaturation of proteins within the cell (Mazur, 1966).

The concentration of solutes outside the cell during ice-formation tends to dehydrate the cell by causing water-flow outwards under an osmotic-pressure gradient. This dehydration brings about molecular contact within the cell (Lovelock, 1957). Lovelock investigated β-lipo-protein denaturation in human plasma-cells and he obtained evidence that denaturation was caused by the progressive removal of water which concentrated the lipo-protein molecules until they were in actual physical contact. There is the possibility that physical contact between protein molecules permits the formation of undesirable cross-linkages leading to distortion or rupture on rehydration.

Levitt (1962) proposed that damage may be due to oxidation of sulfhydryl groups, resulting in the formation of disulphide bonds between adjacent proteins or portions of the same protein. On rehydration, these SS bonds with 50-60 kilocalories of bond-energy remain unbroken and the protein molecule is distorted, or weaker bonds are ruptured.

His evidence was based on finding an increased SS content when freezing produced injury.

Damage during freezing can be prevented by avoiding freezing within the cell, that is, avoiding crystallization of ice, by cooling very rapidly in such a way that there is no time for water molecules to assume an ice-like configuration so that the temperature quickly falls below that at which vitrification of water occurs or, if micro-ice-crystals do form, that there is not enough time for them to grow (Mazur, 1963; 1966). Otherwise freezing should proceed infinitely slowly. Ultra-rapid cooling was at first thought not to be practical for biological purposes as it was estimated that a rate of 5,000° C/sec. would be required (Farrant, 1970). Later, Farrant (1975) showed that if cell systems were held at a subzero temperature for some time and then cooled rapidly to -196° C some protection was afforded the cell systems. Walter *et al* (1975) showed that during the period at the subzero temperature, the formation of extracellular ice leading to outflow of water from the cells caused lymphocytes to shrink and their content of intracellular ice to be reduced. There is also the possibility that the time in the subzero condition alters the sensitivity of the cellular membranes in some way so that they become insensitive to injury on subsequent freezing (Farrant and Walter, 1976). Many cell components, e.g. nucleus, lysosomes, mitochondria, etc., possess semi-permeable membranes similar to the external cell membrane and composed of lipo-protein complexes. The high sensitivity of lipo-proteins to freezing suggests that the inner membranes and the external membrane suffer irreversible damage during freezing (Lovelock, 1957).

The fact that many living cells and tissues have now been successfully stored at very low temperatures in spite of all the above-mentioned formidable hazards is due to the discovery of cryoprotectants (Polge *et al*, 1949; Lovelock and Bishop, 1959; Bricka and Bessis, 1955; etc.). These substances alone, at concentrations which are not in them-

selves harmful, are able to prevent many of the destructive effects of freezing from taking place.

iii Cryoprotectants

Chemicals which aid cryoprotection have been called cryoprophylactic agents and solute moderators (Karrow and Webb, 1964). But the word 'cryoprotectant' was suggested at the second annual meeting of the society for cryobiology (1965) to mean a chemical that aids cryoprotection.

The historic discovery of the protective effect of glycerol was made by Polge *et al* (1949), when they found that avian spermatozoa that had been cooled to -79^{o} C in Meyer's histological albumin solution recovered with little damage. Further investigations revealed that glycerol was the active ingredient.

Lovelock (1953) demonstrated that the red-cell haemolysis produced by freezing and thawing was related to the increase in concentration of electrolytes as pure water was removed from the solution as ice. The protection afforded by glycerol or any other protectant is related to properties which are independent of its nature and dependent on the number of particles present (i.e. its colligative properties). Since the first use of glycerol, cryopreservation of living materials has become a more practical proposition and various workers have utilized it in preserving a variety of living materials from tissues to bacteria. Lovelock (1954) found that a variety of other mono- and polyhydric alcohols, sugars and amides protect human red-blood cells during relatively slow cooling.

In 1959, Lovelock and Bishop reported the use of dimethylsulphoxide (DMSO) as a protectant for erythrocytes and spermatozoa. In 1961, Diamond *et al* used DMSO to protect intestinal protozoa from the damaging effects of freezing. It was also found to be protective for blood parasites, e.g. trypanosomes (Walker and Ashwood-Smith, 1961). Chang and Simon (1968) evaluated the effect of DMSO on cellular sys-

tems: they showed that it was a more effective cryoprotectant than glycerol in that it diffused more rapidly and freely through cell membranes when used in the same percentage concentration. DMSO also required a shorter equilibration period: 30 seconds was required for bovine erythrocytes while 2 hours was required with glycerol (Lovelock and Bishop, 1959).

Other chemicals, some related to and some dissimilar from glycerol have been established as cryoprotectants (Doebbler, 1966; Karrow, 1969; Callow and Farrant, 1973).

Bricka and Bessis (1955) and Doebbler *et al* (1966) demonstrated the extraordinary effectiveness of dextran and polyvinylpyrrolidone (PVP) at concentrations of 40% (w/v) in permitting the recovery of nearly 100% of human erythrocytes after low-temperature treatment. Persidsky and Richards (1962) and Persidsky *et al* (1965) reported the use of PVP in the protection of bone-marrow cells (Mazur *et al*, 1969).

O'Connell *et al* (1968) screened 83 chemicals consisting mainly of alcohols, sugars, amides and amines for their ability to protect *Crithidia* species during freezing. They showed that the alcohols were generally most effective and that the sugars showed some protection. Glycerol and DMSO at 10% (v/v) concentrations were best. The classification of cryoprotectants which have been used in preserving protozoa is given in the table below (see Table II).

Vos and Kaalem (1965) investigated the effectiveness of 26 different compounds as protective agents for slow freezing of cells from tissue cultures. The best protection was found in mixtures which included DMSO and glycerol; less protection was found in the other polyhydric alcohols including sorbitol and some other sugars such as glucose and D-ribose. Little protection was afforded by PVP or serum. Primary alcohols (methanol and ethanol), they found, reduced survival below that of controls. Bender *et al* (1960),

Table II

Classification of cryoprotectants used in Protozoology

Type of protectant	Examples
monohydric alcohols	ethanol, methanol
polyhydric alcohols	glycerol, sorbitol
macro-molecular polymers	PVP, dextran
amides	Acetamide
organic sulphur compounds	DMSO
sugars	glucose, sucrose, (dextran), xylose, dextrose, (sorbitol).

reported that up to 85% of bone-marrow cells survived freezing when they were treated with 0.4 M or 0.8 M sorbitol.

The addition of non-penetrating sugars or polymers can increase survival. Strumia *et al* (1958) reported that when human erythrocytes were frozen rapidly with 0.2 M lactose or 0.7 M dextrose there was 95% recovery.

Considering the ability of DMSO to assist the penetration of compounds which are usually biologically impermeant, Robertson and Jacob (1968) suggested that the combination of DMSO with other cryoprotectants might give a cryoprotective synergism. Diamond (1964) reported the recovery of up to 40% of *E. invadens* when frozen with a com-

bination of 4.5% (w/v) glucose and 15 % (v/v) DMSO. Djerassi and Roy (1963) found that rat platelets were better preserved in 5% (w/v) dextrose with 5% (v/v) DMSO. The use of these compounds alone at the given concentrations did not confer any protection. But Karrow *et al* (1965) considered that cryoprotective synergism did not result when they failed to recover rat heart-muscles from freezing in the presence of 12.5% (v/v) DMSO in dextran whereas each individual protectant conferred protection on its own.

Nash (1962) studied a variety of neutral solutes that are cryoprotective for erythrocytes and found that they had the following common properties: the ability to penetrate cells, lack of toxicity and a high affinity for water. These qualities agree with those suggested by Lovelock (1953 , 1954), Lovelock and Bishop (1959) and Bemrick (1961), but they added that a cryoprotectant must be miscible with salt solutions. It is obvious that few cryoprotectants can have all of these properties because, for example, cryoprotectants like PVP, having a molecular weight of 30,000, do not penetrate cell membranes by simple diffusion although they have been shown to penetrate bone-marrow cells when used together with pinocytosis inducers (Persidsky *et al*, 1965). The characteristics that appear to be most important are lack of toxicity and a high affinity for water (Pegg, 1976). The absence of intrinsic toxicity is a self-evident requirement and the ability to produce concentrated solutions with low freezing-point is important because such compounds will be the most effective in reducing the damaging effect of salt concentration due to freezing. However, the fact that many commonly used cryoprotectants penetrate cell membranes actually produces some problems and is certainly not a necessary property for cryoprotection (Pegg, 1976). If cryoprotectants are toxic, the freezing procedure is doomed to failure in spite of careful control of all other factors (Karrow and Webb, 1965).

iv <u>The Mechanisms of cryoprotection</u>

The protection of cells *in vitro* against freezing in-

jury by added cryoprotectants such as glycerol, DMSO, sucrose and PVP can be explained partially by their action in lessening the ionic strength reached in the remaining solution during freezing (Farrant, 1975) by reducing the amount of ice present at any temperature during freezing and thus moderating changes in solute concentrations (Lovelock, 1953; Mazur, 1960; Farrant, 1965, and Farrant and Walter, 1976). In addition, the penetrating cryoprotectants will reduce the osmotic stress during thawing or dilution since they will leave the cell more slowly than water enters. Compounds such as DMSO, even at constant ionic strength, reduce the effect of the electrolyte on the cells so that they are less sensitive to change when a constant osmotic stress is applied (Farrant and McGann, 1975).

Lovelock (1953) was the first to propound a theory on the mechanism of glycerol action during freezing of erythrocytes; today, this theory still holds good. He stated that glycerol lowers the freezing point of the medium and allows movement of water out of the cell, increasing the proportion of bound water within the cell as it does so and limiting the degree of electrolyte concentration by dehydration (Lovelock, 1953; Smith, 1961).

Lusena and Cook (1953) and Rapatz and Luyet (1963) suggested that the addition of glycerol to an aqueous medium results in an increase in its viscosity and a decrease in the number of ice-nucleation centres and the rate of growth of ice. The glycerol which enters a cell forms H-bonds with intracellular water at -80° C (Karrow and Webb, 1965) and its concentration may increase to a toxic level as freezing progresses. When freezing does not take place, in the presence of glycerol, the damage caused by dehydration and salt concentration is reduced. The rate of cooling has to be slow to permit diffusion of glycerol through cell membrane to maintain osmotic equilibrium (Lovelock, 1953). However, the rate of transfer through different types of cell membranes

varies and is related to the solubility of the compound in water, its electrical charge, viscosity and the temperature of administration and such a range of permeability for glycerol therefore explains why different glycerol concentrations are required to obtain protection for different biological materials (Karrow, 1969).

Lovelock (1953 , 1954) assumed that living cells can enjoy protection against freezing damage afforded by glycerol only when fully permeated by it before freezing. Sherman (1963) emphasised the need for an appreciation of variability in cell systems and an emphasis on experimental evaluation of cells of different types and sources rather than application of untested assumptions based on one type and source. He presented evidence which indicated that extracellular glycerol confers protection to non-fertilized mouse eggs or bull sperms while intracellular glycerol is actually toxic to these cells.

DMSO is a dipolar solvent with a high dielectric constant. It has a tendency to accept rather than donate protons. Evidence has been produced that molecules of DMSO (and other penetrating cryoprotectants perhaps) have the capacity to bind to water molecules by H-bonds at low temperatures (Doebbler, 1966; Chang and Simon, 1968). Levitt (1962, 1966) considered that the mechanism of protection results from the stability of the hydrogen bonds established between the cryoprotectants and proteins. However, Chang and Simon (1968) considered that there are no grounds to believe that the protective action of DMSO on living cells at low temperature depends on hydrogen bonding but is related to the colligative properties of the cryoprotectant in solution. DMSO has low toxicity and can penetrate cellular membranes even faster than glycerol. It reduces the changes caused by high solute concentrations at usual freezing rates (Smith, 1961; Karrow, 1969; Farrant, 1970). Cryoprotectants such as DMSO and polyhydroxy compounds not only prevent freeze-induced cross-linking of proteins but also urea-induced hybridization.

It has been reported that urea actually decreases the amount of cryoprotection afforded by cryoprotectants (Doebbler and Rinfret, 1962b, 1965).

PVP is a polymer cryoprotectant, it is a large interface-seeking molecule which has been considered to coat erythrocytes immediately following thawing giving them mechanical protection against haemolysis due to osmotic stresses. It could prevent the seeding of supercooled water inside the cells (Mazur, 1966) or it could coat sensitive membranes so that they are no longer denatured by strong salt solutions. PVP is a pure hydrogen acceptor which readily forms H-bonds (Mazur, 1966; Ashwood-Smith and Warby, 1971).

The mechanism of PVP protection is not really very clear; the colligative theory of protection seems not to apply here (Karrow, 1969) but, to some extent, removal of some water during crenation of red cells following addition of PVP may help in preventing osmotic injury during cooling. It has not been shown to prevent intracellular crystallization of water (Meryman, 1966). Lovelock (1957) considered that the loss of lipoprotein from the red-cell membrane makes it permeable to cations and also sensitive to osmotic lysis on thawing; therefore, a coating of polymer might either prevent the denaturation or stabilize the membrane against subsequent osmotic stress (Meryman, 1956; 1968) and PVP may possibly act in this way. Persidsky and Richards (1962) demonstrated that bone-marrow cells are protected by PVP during freezing and thawing. Although these cells can be induced to absorb PVP, protection seems not to be dependent upon intracellular PVP for the cells were still protected when pinocytosis was inhibited. Meryman *et al* (1962; Meryman, 1966) showed that thawing erythrocytes in a PVP solution improved recovery despite the fact that PVP obviously could not contact the cells until after thawing was completed, suggesting that PVP imparts stability to the cell during the interval immediately following thawing. Woolgar

(1972) considered that reduction in haemolysis by the presence of external PVP when frozen erythrocyte suspensions were thawed was probably due to the balance that the PVP provided for the intracellular colloid once the cell membranes had become leaky to cations.

Meryman (1966) emphasised that protection appears to be afforded to nucleated cells only by penetrating additives; when these are used, at adequate concentration, slow freezing is tolerated. Rapid freezing is rarely tolerated by nucleated cells regardless of additive. Extracellular additives and polymers appear to be of value only for freezing of erythrocytes (Meryman, 1966).

Farrant and Woolgar (1970) have shown that the presence of 15-30% (w/v) PVP depresses the rise in salt concentrations of the system PVP-NaCl-H_2O sufficiently to enable human red cells to survive exposure to -10° C for 20 minutes. Even though these compounds do not penetrate into the cells, they will reduce the build up of salt concentrations inside the cells since intracellular solute concentrations will be in equilibrium with the reduced external salt concentration (Pegg, 1976).

Karrow *et al* (1965) thought that dextran, an extracellular protectant, protects rat heart muscles against freezing damage probably by blocking cell 'pores' to prevent the escape of intracellular water during freezing and by preventing the seeding of intracellular water by extracellular ice. This they considered might also explain the cryoprotective ability of other non-penetrating cryoprotectants to cells. However, this explanation conflicts with that of protection by the holding period method using penetrating cryoprotectants. Walter *et al* (1975) showed that during the holding time at -26° C, lymphocytes in 5% (v/v) DMSO suspension were exposed to a high external osmolality, which caused cell shrinkage, and when they were cooled rapidly no intracellular freezing occurred and a high survival rate was obtained.

Heber and Santarius (1964) suggested that sugars are cryoprotectants by virtue of their abilities to retain water or substitute for water in structures sensitive to dehydration. They proposed that during freezing, a proportion of water bound to disaccharides may be lost and thereby permit disaccharide-protein interaction. Levitt (1962) also suggested that protection by sugars results from the stability of the H-bonds established between the cryoprotectants and protein and that the H-bond does not rupture during the freezing process. According to Karrow (1969) there is no strong evidence to support these theories because they fail to explain the cryoprotective ability of PVP and dextran.

v Methods of cooling living materials

The reliability and reproducibility of cryopreservation procedure has been questioned because workers have experienced inconsistency in their results. Some of these differences can be attributed to the duration of preservation or to performance of different samples of the same isolate. According to Lumsden, a major proportion of these differences results from the differences or irregularities in cryopreservation techniques (Lumsden, 1972). To obtain consistent results, rigorously controlled techniques are desirable. Once stabilates have cooled to the storage temperature they should be maintained at that temperature impeccably otherwise fluctuation of temperature may seriously affect results. For this reason, storage in containers cooled by solid carbon dioxide (CO_2) or liquid nitrogen (N_2) has been recommended (Meryman, 1963; Lumsden, 1972; Pegg *et al*, 1973; Pegg, 1976) in preference to mechanical refrigeration. With solid CO_2 and liquid N_2 systems strict maintenance of storage temperatures can be assumed so long as refrigerant is present (Lumsden, 1972).

Liquid N_2 is cheap, it is non-inflammable, it has a low boiling point (-196^{o} C) and a high heat of vaporiza-

tion of 85.7 British Thermal Units (B.T.U.)/hr (Corriel *et al*, 1964). It is very easy to handle, and it can be stored in small storage containers which makes it more suited to laboratory conditions (Walker, 1966). Solid CO_2, when used with methanol also easily maintains a constant temperature. Unfortunately, the minimum temperature it gives is -79^{o} C and CO_2-methanol mixtures cannot be used with confidence for prolonged storage.

There is much evidence that liquid N_2 storage is preferable to CO_2 storage especially if materials are to be stored for prolonged periods (Polge and Soltys, 1957; Levine *et al*, 1958; Jeffery, 1962; Diamond, 1964; Pegg *et al*, 1973; Pegg, 1976). Several sophisticated controlled-rate cooling machines have been described (Pegg *et al*, 1973). They are convenient to use and they also provide simultaneously a record of the cooling curve actually obtained. Other reasons for preferring liquid N_2 storage will be discussed in detail later.

A.4 Measuring the success of cryopreservation

One of the most important aspects of freezing parasitic protozoa is to be able to recover in a viable condition from the frozen state as large a proportion of the original population as possible. Experiments to study this cannot be done unless a technique is developed whereby the success of cryopreservation can be quantitatively measured. In the past, biological characteristics, such as ability of frozen inocula to grow in culture or to infect susceptible laboratory animals, morphology, motility or ability to exclude dyes or take up stains, have been used as the criteria for assessing the efficiency of cryopreservation of living biological materials after retrieval from the frozen state. These criteria are unsatisfactory because they give no direct information on the percentage of individuals capable of multiplication in a frozen population. It has been shown that the estimation of the viability of a sample cannot be based on visual means

alone since not all the organisms seen to be motile are capable of reproduction nor are those that are non-motile necessarily incapable. For example, Lumsden *et al* (1966) showed that non-motile suspensions of *T. vaginalis* were capable of reproduction when inoculated into cultures, whereas Kouwenhoven (1967) was unable to infect chickens with the oocysts of *Eimeria tenella* after freezing and thawing even though the parasites looked morphologically unaltered (Dalgleish, 1972). Overdulve and Antonisse (1970a) reported that Leeflang was unable to infect mice with *T. congolense* from the blood of a mouse which had previously been treated with a trypanosomicide although their morphology and motility were unaltered.

In virology and bacteriology enumeration of particle numbers using a haemocytometer has not been practicable as an index of viability or infectivity; titration techniques based on serial dilutions of suspension of organisms have been used followed by inoculation of groups of animals or cultures. This technique is based on the observation of the quantal (all or none) response in the group of inoculated animals or cultures using a standard inoculum at several serial dilutions, the end-point of which is determined by the dilution that infects 50% of the recipients (= infective dose 50 or ID_{50}) (Reed and Muench, 1938) or 63% of them (infective dose 63 or ID_{63}) (Lumsden *et al*, 1963).

These methods have been used to measure the infectivity of many protozoa to susceptible animals or cultures. For example, Collins and Jeffery, (1963) used the ID_{50} end point to compare the effect of glycerol and DMSO as cryoprotectants for *Plasmodium* species. Lumsden *et al* (1963) developed a simple method for the titration of the infectivity of *T. brucei* to mice using the ID_{63} end-point. The same method has been adopted for measuring the infectivity of other *Trypanosoma* species after freezing and thawing (Raether and Seidenath, 1972), for the infectivity of *Trichomonas vaginalis* to cultures after cryopreservation (Lumsden *et al*, 1966) and also for evaluating the effect of

freezing on a variety of protozoa including *Leishmania* (Mieth, 1966). Thus, the ID_{63} method allows quantitative measurement of the survival of parasites after cryopreservation.

Simple techniques based on observations of the time elapsing between inoculations and the appearance of an infection in the blood (Overdulve and Antonisse, 1970a) or a defined level of parasitaemia (Warhurst and Folwell, 1968) have also been developed. Warhurst (1966) titrated the infectivity of *P. berghei* frozen and thawed in the presence of DMSO using the period between inoculation and infection of 2% of the erythrocytes (which he called the pre-2% level) as the criterion for measuring success of cryopreservation. Overdulve and Antonisse (1970b) used linear relationship between infective dose and either prepatent period or survival time in mice to measure the effect of freezing *B. rodhaini* in the presence of DMSO.

Dalgliesh (1972), assessing the cryoprotective effect of DMSO in *B. rodhaini*, found that more reliable estimates of survival could be obtained by titration to an end-point and measuring the mean prepatent period for each group. Callow and Farrant (1973) assessed the success of freezing *L. tropica major* promastigotes by the time taken for the flagellates to appear in cultures after thawing and inoculation.

A.5 Stability of biological characteristics after cryopreservation

The objective of cryopreservation is to ensure that the organisms can survive long-term frozen storage. Having established this, it is important to test whether their biological characteristics have been retained by comparing these characteristics before and after cryopreservation. Biological characteristics such as infectivity, virulence, mutation frequency, drug resistance, antigenic pattern, immunizing capacity and drug-induced photosensitivity have all been suggested by Hawking and

Walker (1966). Other characteristics such as chromosomal patterns and enzyme activities have also been suggested by Moline *et al* (1962).

However, only a very few of these characteristics have been tested, and so far no evidence has been produced to suggest their modification as a result of cryopreservation; perhaps Lumsden (1972) was right in thinking that cryopreservation has a selective effect on the population in a way unrelated to its biological variation (except perhaps selection for resistance to cryopreservation).

One of the most important biological characteristics is virulence. Neal *et al* (1974) tested several strains of *E. histolytica* for their virulence to rats after retrieval from frozen storage; they showed that virulence was not changed although some differences in degree were observed. The same workers (Neal *et al*, 1974) tested the response of *E. histolytica* to emetine after storage for different periods in liquid nitrogen. They found that the drug sensitivity was virtually identical to that before freezing. Gordon *et al* (1969) compared antigens prepared from normal axenically derived amoebae and from descendants of frozen amoebae in cross-titration in an indirect haemagglutination test, using known reactive human sera and hyper-immune rabbit sera. They found that the serum antibody titres were identical at comparable antigen dilutions suggesting that no permanent antigenic changes had occurred as a result of freezing.

A.6 Objectives of the present work

The success of cryopreservation has hitherto been assessed by methods based on the exclusion of supravital stains, mobility, morphology and cultivability in culture media or in laboratory animals. Some of these methods are not easy to quantify for viability assays since they depend on subjective visual assessments. The method

based on dye exclusion does not permit precise quantification of the number of organisms that are still viable after freezing. It is therefore unsatisfactory. Its unreliability will be discussed later. The introduction of an assay method based on Lumsden's ID_{63} end-point offers an attractive alternative to other assay methods (except the ID_{50}). This assay gives objective results and is a sensitive method for the measurement of 'viability' of amoebae after cryopreservation.

It has been reported that on the average (using the dye-exclusion method of assessment) about 10-20% of amoebae 'recover' from freezing, but the number could be as low as 0.2% or as high as 45% (Neal *et al* 1974). It is possible that other conditions apart from the method of assessment of viability may influence the number of parasites which are recorded to have survived freezing. In cryopreserving amoebae, only DMSO and glycerol amongst 83 chemicals known to have cryoprotective ability (O'Connell *et al*, 1968) have been useful. DMSO has also been used successfully in combination with glucose. The investigation of the cryoprotective ability of other chemicals for *E. histolytica* is therefore desirable.

Other factors which may have direct bearing on percentage survival are the suspending medium used for freezing and the type of medium used for cultivation after thawing. It is clear that an axenic medium is not a suitable medium for cultivating amoebae immediately after thawing (Neal *et al*, 1974; Raether and Uphoff, 1976). There is still the need to find a medium which will very readily support the growth of amoebae on retrieval from frozen storage.

Other factors such as period of domestication, age of parasites, concentration of organisms in the suspension, volume of suspension, equilibration conditions and post-thaw handling may all play important rôles in the survival of *E. histolytica* after cryopreservation.

It is surprising that the controlled-rate method of cooling has not been fully investigated; most workers have used freezing rates of 1^{o} C/min to freeze amoebae down to between -35^{o} C and -45^{o} C before rapidly cooling them at uncontrolled rate to the storage temperature. Neither is there enough information on survival; generally, samples have been routinely thawed in water baths at about 37^{o} C. Only Diamond (1964) has produced evidence to show that thawing rate has an effect on the survival of cryopreserved amoebae.

In view of all these irregularities, I have set out to investigate empirically the various factors which may be influential in determining the survival of *E. histolytica* in cryopreservation by:

i. setting up a standard method for the cultivation of *E. histolytica* so as to measure survival from cryopreservation;

ii. optimising the growth conditions of *E. histolytica* in the medium developed;

iii. setting up a sensitive and accurate system, based on infectivity titrations, for measuring the success of cryopreservation;

iv. investigating the various conditions which might influence survival, with particular emphasis on the effect of one suspending medium used for freezing, the cryoprotectants (such as PVP, glucose, ethanol, methanol and sorbitol), equilibration conditions, volume of suspension to be frozen, freezing rates, warming temperatures and length of storage.

Having established optimal conditions for cryopreservation of *E. histolytica* it was necessary to test, using sensitive methods such as growth-rate measurement, iso-enzyme characterization, concanavalin A-induced agglutination, reaction in the indirect fluorescent antibody test and toxicity to leucocytes, that the biological characteristics had not been altered by cryopreservation.

B MATERIALS AND METHODS

B1. General Methods

B1.1 Preparation of solutions used

i. Hank's physiological solution - This solution was prepared by dissolving the following ingredients in a litre of distilled water: 0.4 g potassium chloride, 0.152 g disodium hydrogen orthophosphate, 0.06 g potassium dihydrogen orthophosphate, 8.0 g sodium chloride, 0.14 g calcium chloride, 0.1 g magnesium sulphate, 0.1 g magnesium chloride and 1.0 g dextrose. The pH was adjusted to 7.2 or 7.6 as required with Tris (hydroxymethyl) amino methane solution, using phenol red as indicator. The medium was autoclaved and stored at 4° C.

ii. Phosphate buffered saline (PBS) - This was prepared either by dissolving 1 tablet of PBS (Dulbecco A, Oxoid) in 100 ml sterile distilled water or from a mixture of 0.001 M sodium phosphate and 0.145 M sodium chloride. The pH of PBS from Oxoid was 7.2 and the other was 7.1. They were sterilized by autoclaving.

iii. Peptone water - 10.0 g peptone (Oxoid L37) and 5.0 g sodium chloride were dissolved in a litre of distilled water and adjusted to pH 7.2. The mixture was distributed in 4.0 ml quantities in bijou bottles (a bijou bottle is a 5.0 ml glass bottle with screw cap) and autoclaved. It was used for cultivating and maintaining *Escherichia coli-B* in the laboratory.

B1.2 The bacteria

i. Isolation and cultivation - *Es. coli-B* used in the cultivation of *E. histolytica* in all my experiments was obtained from the National Collection of Type Cultures (No. 10537) in lyophilized state in a vacuum ampoule.

To prepare cultures from this sample, the following

procedure was adopted. A file mark was made on the ampoule near the middle of the cotton-wool plug and a red-hot glass rod was applied at the file mark to crack the glass. Time was allowed for air to seep into the vacuum ampoule through the plug to filter atmospheric air. The tip of the ampoule was broken off very cautiously and disposed of aseptically. The cotton wool plug was removed with a pair of sterile forceps. Both forceps and plug were aseptically disposed of. 0.5 ml of 20% sterile peptone was added to the lyophilized bacteria by the use of a sterile pasteur pipette. The suspension was thoroughly mixed by filling and emptying the pipette. Care was taken to avoid frothing. The suspension was then tested for purity by streak-plating it on to agar.

The plates (nutrient agar, blood agar and eosin/methylene-blue agar) were incubated at 37° C for 24 hours to isolate pure colonies of *Es. coli-B* and also to test purity of the sample. A colony from an eosin/methylene-blue agar plate was subsequently used in maintaining the strain in the laboratory. The colony to be passaged was picked up by a cooled, previously flamed loop and was aseptically placed in 4.0 ml peptone water in a 5.0 ml bijou bottle. The bottle was then incubated at 37° C for 24 hours after which the bacterial suspension was left on the bench at room temperature. Subcultures were routinely made at weekly intervals by first streak-plating an existing culture on to agar and then subculturing a colony from it. Contamination can thus be detected and avoided.

ii. Standardization of the bacterial component of cultures - In order to obtain results which could be duplicated precisely during the whole exercise, it was desirable to have a standardized culture medium for *E. histolytica*. Such standardization requires that the bacterial components of the medium should be quantified so that approximately the same number of viable organisms can be used to initiate cultures at all times. For bacteria, such quantification can be done by estimating viable counts of the organisms in the suspensions.

iii. Determination of viable count of bacterial suspensions- The procedure for the determination of viable counts of live vibrios by Ghosh *et al.* (1966) was adopted. A bacterial suspension to be tested for viability was carefully homogenized by repeated pipetting with a sterile pasteur pipette. Ten-fold serial dilutions were then made by carrying over 0.1 ml of the suspension in 0.9 ml of peptone water. 0.1 ml from each dilution was plated on to two nutrient-agar plates. The covered plates were incubated face downwards at 37° C for 24 hours. The colonies on both plates from an appropriate dilution giving between 100-300 colony-forming units per plate were counted. The number of colony-forming units in each millilitre of undiluted suspension was computed from their average.

When viable counts were to be made on frozen samples of *Es. coli - B*, the following procedure was adopted. A capillary tube whose content was to be tested for viability was scored and broken at both ends and weighed. The content was expelled into 1.0 ml of medium on which the bacteria were grown and the tube was reweighed to determine the weight of the stabilate. The volume was then adjusted in such a way that 1.0 ml of medium contained 10.0 mg of stabilate. Ten-fold serial dilutions were made and estimation done as described earlier. Only the suspensions whose viable number of live organisms had been determined were used for cryopreservation.

iv. Cryopreservation of *Es. coli - B* - One of the reasons for cryopreservation of *Es. coli - B* was to be able to obtain a standard suspension of bacteria at all times. Apart from this, it was necessary to cut down on the time spent on its maintenance *in vitro*, to prevent any physiological changes that might occur with continuous serial passaging, to know beforehand its behaviour during and after freezing in the different media, and to test the effect of cryoprotectants on its viability after freezing.

The procedure described by Lumsden *et al.* (1966) for

the cryopreservation of trypanosomes was adopted. *Es. coli-B* suspensions whose number of viable organisms had been estimated and adjusted in such a way that 1.0 ml of the suspension contained 368 x 10^6 organisms were used.

Es. coli - B were suspended in various media with or without cryoprotectants. Several stabilates were laid down for each type of suspension by half filling (25.0 µl) as many capillary tubes as possible with the suspensions using sterile techniques. The filled capillary tubes were flame-sealed on a microburner. Each batch of stabilate was stored in a screw-cap test tube containing methanol and appropriately labelled. Labelling was done by writing boldly in pencil on a strip of white blotting paper which was placed inside the test tube in such a way that it could be easily read.

The test tubes were placed in an insulating jacket which was then sealed with a piece of adhesive tape. It was deposited in a solid-CO_2 cabinet overnight. On the following day, the insulating jacket was removed from its placement in the solid-CO_2 cabinet, the test tubes were removed and quickly placed in a CO_2-methanol bath (-79° C). The tubes were subsequently stored in an electrical freezer at -79° C.

All bacteria used in preparing "BR" were derived from the stabilates (see page 69).

When a stabilate was required for making BR , a capillary tube was withdrawn from deep freeze, thawed rapidly in tap water and wiped clean with 70% ethanol. Both ends of the capillary tube were scored and broken off and the stabilate was expelled on to a sterile glass slide with the aid of a sterile holey blower and was thoroughly mixed using the end of the capillary tube.

10.0 µl of the thawed suspension was transferred into 4.0 ml peptone water using a sterile 10.0 µl microcap and

incubated at 37° C for 24 hours. The culture was left on the bench at room temperature overnight. On the following day, 0.1 ml of this bacterial suspension was used to inoculate 20.0 ml of sterile medium R in 4.0 oz medical flat bottles and incubated at 37° C for 24 hours (see page 68).

B1.3 The amoebae

i. Origin of stocks* used - Thesiger, SN, JEH, Thirer and Bean stocks were isolated from semi-formed to formed stools of apparently asymptomatic cyst-passers. Stocks Rafiq, Mukerjee and Thirer were isolated and supplied by Mr. P.G. Sargeaunt.

Irvine was isolated from the stool of a patient with an amoebic liver abscess. Antibody to *E. histolytica* was present in the serum at a high titre.

Fenn was isolated from a brown formed stool specimen of an asymptomatic cyst-passer. The patient had a history of acute amoebic dysentery a year before this isolation was made.

Fox was isolated from dark-brown formed stool of an asymptomatic cyst-passer who had just returned from Tanzania. At the time of isolation, stool cyst concentration revealed mixed infection with *E. coli* and *E. nana*.

Atkinson (or ST21) was isolated by Dr. D.C. Warhurst as haematophagous trophozoites from rectal scrapings of a 13 year old English patient with bloody diarrhoea. Her serum showed antibody against *E. histolytica* and she was diagnosed as having amoebic dysentery.

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* A stock is a population derived by serial passage *in vitro* and/or *in vivo* from a primary isolation, without any implication of homogeneity or characterization. WHO (1978). This word has replaced the word 'strain' which is more generally used.

Biswas was isolated in 1964 from the formed stool of an apparently asymptomatic seaman at Devonport Laboratory. Serological tests later revealed invasive amoebiasis. CFT was positive at a titre of 1/90 (Sargeaunt, personal communication). The stock was kindly supplied by Dr. R.A. Neal in HSre medium. It has undergone several serial passages and even cryopreservation (Neal *et al.* 1974).

Ali was isolated from a semiformed mucoid but not bloody stool of an Afganistan patient. The stool contained cysts of *E. histolytica* and *E. nana*.

NIH:200 was supplied by Dr. D.C. Warhurst in axenic (Diamond's) medium, and also in association with *Crithidia* species in TTY medium, and in Robinson's medium with mixed bacterial flora.

ii. Isolation of *E. histolytica* stocks - On the receipt of faecal samples or rectal scrapings direct microscopic examinations were made of fresh samples suspended in normal saline and in double strenth Lugol's iodine to observe viable trophozoites or cysts. Whether this revealed cysts or not, a formol-ether concentration technique (see below) (Ridley and Hawgood, 1956) was used to concentrate cysts from faeces. Cyst concentrates were re-examined microscopically, using a x 20 objective and permanent preparations were made using alum-haematoxylin stain. Also, cyst concentrates were mounted in Sargeaunt's stain to differentiate nuclei and chromatoid bodies for correct identification of species. Amoebae were also isolated from faecal samples or rectal scrapings by direct inoculation into Robinson's primary medium.

Preparation of double-strength Lugol's iodine

1.0 g	iodine
2.0 g	potassium iodide
50.0 ml	distilled water

The iodine and potassium iodide were dissolved in

10.0 ml of the distilled water by shaking together in a 25.0 ml stoppered bottle. It was made up to 50.0 ml with the rest of the distilled water.

iii. Methods of identifying amoebic species

Formol-Ether method of concentrating faecal cysts - A piece of faeces about the size of a walnut was emulsified with a pestle and mortar in about 30.0 ml 10% formalin in 0.9% (w/v) saline. It was strained through a wire sieve with 50 mesh to 1 inch into a bowl. The emulsion was used in filling two-thirds of a 25.0 ml centrifuge tube. 3.0 ml ether was added and the tube was shaken vigorously. The emulsion was centrifuged in a bench centrifuge at 500 *g* for 2 minutes. The surface deposit was loosened with an orange-stick and the supernatant and debris were poured away leaving the sedimented concentrate. A drop of this was removed with a pipette on to a clean slide and examined under a x 20 objective after staining with double-strength Lugol's iodine solution or Sargeaunt's stain.

Staining techniques for *E. histolytica*

Sargeaunt's method for staining amoebic cysts - After formol-ether concentrations of cysts, one drop of faecal concentrate was mixed with one drop of stain on a slide and examined under the microscope for the identification of cyst chromatoid bodies and nuclei.

Preparation of Sargeaunt's stain (Sargeaunt, 1962)

0.2 g. malachite green
3.0 ml glacial acetic acid
3.0 ml 95% ethanol
100.0 ml distilled water

The malachite green was dissolved in the alcohol; acetic acid was added and made up to 100 ml with distilled water.

Alum-haematoxylin staining technique (Robinson, 1968a)
Smears were prepared from sediments from formol-ether

concentrates and stained as described below, or were prepared from suspensions of culture material.

To stain fresh amoebal suspensions, a drop was added to 4 drops horse serum on a clean glass slide and thoroughly mixed. A thin smear was then made. The slide was placed in a fixative mordant (overnight) as the edges began to dry. On day two, it was passed through 33% ethanol and stained in haematoxylin for 20 minutes. It was washed in tap water, differentiated in 2% alum solution for 3-5 minutes and washed again in tap water. It was dehydrated in alcohol, cleared in xylene and mounted in canada balsam.

Preparation of haematoxylin

10% alcoholic solution of haematoxylin
0.3% phenol water

One part haematoxylin was diluted with 19 parts phenol water.

Preparation of fixative mordant

40.0 ml 2% alum solution
150.0 ml fixative base made up thus:
20.0 ml glacial acetic acid
130.0 ml water
0.5 g phosphotungstic acid
Ethanol to 900.0 ml.

B1.4 Culture media

1. Robinson's medium (Robinson, 1968a) - The diphasic medium consists of a saline-agar slope and a fluid overlay.

Saline-agar slopes - 15.0 g fine agar powder (British Drug Houses - BDH) and 7.0 g sodium chloride were dissolved by boiling in a litre of distilled water. It was distributed in 2.5 ml quantities in quarter-ounce screw-capped glass bottles ('bijous') or in 10.0 ml quanti-

ties in universal bottles. The bijou bottles had rubber-lined perforated screw caps to facilitate easy inoculation and minimize risk of contamination. The bottles were sloped and the saline-agar allowed to set after autoclaving. They were stored at room temperature.

The overlay consisted of different solutions, the preparation of which is set out below:

Antibiotics - 0.5 g erythromycin powder (Abbot Laboratories) was dissolved in 2.5 ml 70% ethanol in a sterile container and left in the refrigerator at 4^{o} C for 2 hours. 97.5 ml sterile distilled water were added aseptically. The solution was distributed in 4.0 ml volumes in bijou bottles and stored at 4^{o} C.

Bactopeptone - 20.0 g Bactopeptone (Difco) were dissolved in 100.0 ml sterile distilled water. It was dispensed in 4.0 ml volumes in bijou bottles, sterilized by autoclaving and stored at 4^{o} C. 10% Lablemco (Oxoid) was substituted for bactopeptone when stock cultures were failing.

Starch - Rice powder (Oxoid) was distributed in small amounts in bijou bottles, sterilized by autoclaving and stored at room temperature.

Phthalate solution - 204.0 g Potassium hydrogen phthalate were dissolved in 100.0 ml freshly prepared 40% sodium hydroxide solution. This was made up to 2.0 litres with distilled water; the pH was adjusted to 6.5. It was then dispensed in 100.0 ml medical flat bottles, autoclaved and stored at room temperature, When it was required for use, it was diluted 1:10 with sterile distilled water to make a 0.05 M solution.

Medium "R" - The following ingredients were dissolved in a litre of distilled water: 62.5 g sodium chloride, 25.0 g citric acid monohydrate, 6.25 g potassium dihydrogen orthophosphate, 12.5g ammonium sulphate, 0.625 g magnesium sulphate heptahydrate and 100.0 ml lactic acid (BDH, 90.08%). This stock solution, pH 7.0, was stored for at least 4 weeks before use to avoid change of pH. For use, 7.5 ml freshly prepared 40% sodium hydroxide and 2.5 ml 0.04% bromthymol blue were added to 100.0 ml stock solution and made up to 1 litre with distilled water. The medium was dispensed in 20.0 ml quantities in 100.0 ml medical flat bottles, autoclaved and stored at room temperature.

Basal amoebic medium "BR" - 10.0 µl of *Es. coli-B* stabilate was inoculated into 4.0 ml peptone water in a bijou bottle, incubated at 37° C for 24 hours and left on the bench overnight. 0.1 ml of this was added to 20.0 ml medium R in medical flat bottles and incubated at 37° C for 24 hours. The BR was stored at room temperature for 2 days before it was used.

Basal amoebic medium "BRS" - 400.0 ml uninactivated horse serum (Wellcome Laboratories, No. 3) was removed from -20° C storage and allowed to thaw on the bench. It was aseptically distributed in 20.0 ml volumes in universal bottles. The bottles were kept at 4° C. To make BRS, 20.0 ml serum was added to 20.0 ml BR which had been kept on the bench for 2 days, and incubated at 37° C for 24 hours. It was subsequently stored at room temperature for 2 weeks before use.

Initiation and maintenance of cultures in Robinson's medium - All the amoebae used except stocks Atkinson and NIH:200 were derived from the faeces of patients. They were isolated in bijou bottles containing Robinson's primary medium: 3.0 ml BR containing 0.06 ml 0.5% (w/v) erythromycin solution and 10.0 mg rice starch as a liquid overlay on the saline-agar slopes. The cultures were then incubated at 37° C. After 48 hours, the overlay was removed by pipetting without disturbing the sediment, and was replaced by 3.0 ml BRS diluted (1/2 - 1/5) in 0.05 M phthalate and containing 0.06 ml 20% (w/v) bactopeptone or 0.06 ml 10% (w/v) "Lablemco", 0.06 ml 0.5% (w/v) erythromycin solution and 10.0 mg rice starch. This BRS medium diluted with phthalate is referred to here as Robinson's 'secondary' medium or RSM. Isolates from rectal scrapings were inoculated straight into RSM.

Stock cultures were subsequently maintained in bijou bottles at 37° C in RSM at phthalate/BRS proportions which appeared to favour growth best. Four drops of sediment

containing viable trophozoites from a pre-existing culture were routinely transferred to fresh culture bottles under sterile conditions at 48–72 hour intervals. When stock cultures exhibited decline in the growth of amoebae, the ratio of BRS:phthalate was adjusted, antibiotics were left out. Sometimes it was necessary to change from 20% bactopeptone to 10% Lablemco. The presence of *E. histolytica* in primary isolations was suggested in the first place by the abundance and rapidity of amoebic growth (Robinson, 1968b).

For experimental purposes, stock cultures of amoebae were grown in large amounts in universal bottles. The overlay, which was made up in bulk, consisted of: 75.0 ml phthalate/BRS solution, 1.5 ml 0.5% (w/v) erythromycin solution, 1.5 ml 20% (w/v) bactopeptone. This RSM was dispensed in 10.0 ml volumes in universal bottles containing sloped agar, and about 30.0 mg of rice starch was added.

For purposes of infectivity titrations, RSM was prepared in bulk as described above but was dispensed in 3.0 ml volumes in bijou bottles containing sloped agar and 10.0 mg rice starch. They were usually pre-warmed before inoculation of amoebae.

ii. Locke-egg-serum (or LES) medium (Boeck and Drbohlav, 1925) - Like Robinson's medium, it is diphasic, but the solid part consists of inspissated egg made up as follows: 4 standard eggs were washed and cleaned with 70% ethanol. They were broken into a sterile flask containing sterile glass beads. 50.0 ml sterile Locke's physiological solution was added and the egg mixture was homogenized by vigorous shaking. It was aseptically dispensed in 10.0 ml volumes in universal bottles, or in 3.0 ml volumes in bijou bottles.

The tubes were sloped and inspissated at 75°-80° C in a hot-air oven for one hour. The slopes were further

sterilized by heating at this temperature for 1 hour on 3 successive days.

The overlay - This consists of 1 part sterile horse serum (inactivated by heating at 56° C for 45 minutes in a water bath) and 8 parts Locke's physiological solution.

Preparation of Locke's solution - 9.0 g sodium chloride, 0.2 g calcium chloride, 0.4 g potassium chloride, 0.2 g sodium bicarbonate and 2.5 g D-glucose were dissolved in 1 litre distilled water. The pH was adjusted to 7.0. It was distributed in medical flat bottles and sterilized by autoclaving.

Initiation and maintenance of cultures in LES medium - 10.0 ml overlay was used to partly fill an egg slope contained in a universal bottle, and about 30.0 mg rice starch and 0.18 ml 0.5% (w/v) erythromycin solution were added. The complete medium was allowed to warm up to 37° C before it was inoculated with washed amoebal sediment from a 48-hour-old Robinson's medium. 0.2 ml of 2-day-old BR was added at the same time as the amoebal inoculum or, for pre-conditioning, it was added 24 hours before amoebal inoculation and incubated at 37° C.

Cultures were maintained in universal bottles by inoculating a few drops of sediment from a 72-hour-old culture into fresh culture medium and incubating at 37° C. Subcultures were made at 72-hour intervals.

iii. Modified Locke-egg-serum (or M-LES) medium (Harinasuta and Harinasuta, 1955) - This is basically the same medium as described by Boeck and Drbohlav with a difference in the overlay solutions. Instead of Locke's physiological solution, buffered saline, pH 7.4, was used.

Preparation of buffered saline - 3.55 g sodium hydrogen orthophosphate, 0.68 g potassium dihydrogen orthophosphate and 8.0 g sodium chloride were dissolved in a litre of

distilled water. The pH was adjusted to 7.4. It was distributed in medical flat bottles, sterilized by autoclaving and stored at room temperature.

Initiation and maintenance of cultures was carried out as described for LES (Boeck and Drbohlav, 1925).

iv. TTY medium used in maintaining crithidial and amoebic cultures was provided by Dr. D.C. Warhurst. It was prepared as described by Diamond (1968a).

Maintenance of crithidial culture - *Crithidia* species was grown in the completed TTY medium at room temperature (25° C) with the screw-cap loosely screwed on to allow air into the tube. Maintenance was by subculturing 1.0 ml of *Crithidia* into a fresh 15.0 ml TTY tube at 72 hour intervals.

Maintenance of amoebic cultures - Amoebae from a pre-existing culture were released from the side of the culture tube by chilling it in ice for 10 minutes. It was centrifuged at 180 *g* for 2 minutes and decanted leaving 0.5 ml residue of amoebae. A few drops from this residue were used to inoculate fresh tubes of prewarmed TTY medium to which 0.5 ml *Crithidia* culture had been added. Cultures were maintained at 37° C with twice-weekly subcultures.

v. TPS-1 Axenic medium (Diamond, 1968b) - This medium which consists of a nutrient broth supplemented with horse serum and vitamins was also provided by Dr. D.C. Warhurst.

Maintenance of amoebic cultures - Amoebae were prepared from stock cultures as described for amoebae grown in TTY medium. A few drops of the residue were used to inoculate fresh tubes which had been prewarmed at 37° C. Subcultures were made twice weekly.

B1.5 Cloning of amoebae in Robinson's medium (Farri, 1978).

Once isolates from faeces or rectal scraping had been established in RSM, clones were derived from them. At

least 10 trophozoites were individually isolated on every occasion even though only one was subsequently maintained.

Using the liquid phase of Robinson's secondary medium, 10-fold serial dilutions of thoroughly mixed 24 to 48-hour-old amoebic suspensions were made until a dilution was reached at which not more than one trophozoite could be picked up in a drop of suspending medium. A sterile 10 µl capillary tube was placed in thesuspension from which clones were to be prepared, the capillary tube was allowed to take up a small volume of the suspension and, with the help of a holey blower, about 2.0 µl of suspension was dropped on to a sterile coverslip (approximately 7 x 7 mm). This was quickly examined under a low-power microscope to confirm the presence of a healthy-looking, motile trophozoite with a few starch granules within its cytoplasm. The presence of only one organism was confirmed by at least one other observer. A coverslip carrying a single trophozoite was quickly transferred into a 5.0 ml bijou bottle containing the warmed culture medium with added starch. The cultures were incubated at 37° C and were checked for growth of amoebae on days 5, 7 and 10. Cultures which remained negative after day 10 were discarded and recorded as negative.

B1.6 Methods for cultivating *Blastocystis*-free amoebae in Robinson's medium - Among the problems encountered during primary isolation in Robinson's medium was the presence of *Blastocystis hominis*, a fungus, which when present in faecal samples quickly overgrew the cultures and prevented healthy growth of the amoebae. My source of faecal samples positive for *E. histolytica* was limited and I could not afford to discard positive faecal material simply because it was contaminated with this fungus. I therefore performed experiments to eliminate these fungi from my cultures when they were present.

Tap-water washing method - Cyst or trophozoite suspensions positive for *B. hominis* were washed with tap water

twice (trophozoites) or thrice (cysts), centrifuging at 180 *g* for 5 minutes on each occasion. This caused lysis of the *Blastocystis* when present; it also caused lysis of some of the amoebae. Washing cysts before subculture was preferred.

Use of chemicals - Amoebal suspensions with overgrowth of *Blastocystis* were treated with either of the following chemicals: 0.1 N hydrochloric acid, 4.4% sodium bicarbonate or 5000 units/ml Nystatin fungicide. None of these was successful.

B1.7 Methods for restraining bacterial contaminants in amoebic cultures

An overgrowth of faecal bacteria was a problem in completely monoxenising (i.e. growing amoebae with one other species of concomitant organism) amoebal cultures in Robinson's medium. Cultivating amoebae the way Robinson (1968a) suggested, proved wasteful of time since it usually took not less than 7 subcultures (over 2 weeks) with heavy doses of antibiotics before bacteria other than *Es. coli - B* were completely eliminated from the cultures. The whole point of cryopreservation is to eliminate the necessity of continual serial passages and to avoid physiological and genetic changes in the organisms. This point would have been defeated if amoebae were not cryopreserved in as short a time as possible after a successful initial isolation.

The antibiotic method - In order to eliminate as quickly as possible, bacteria from faecal samples and from primary cultures, single antibiotics of varying concentrations or a combination of them were used. The final concentrations of the antibiotics are as follows:

gentamycin; 200, 400, 800 units/ml
penicillin; 1,000, 2,000, 4,000, 6,000, 8,000 units/ml
streptomycin/penicillin; 2 mg/2,000 units/ml and

4 mg/4,000 units/ml
Chloramphenicol; 1.0, 10.0, 100, 1,000 and 10,000 µg/ml.

Amoebal suspensions were treated with equal volumes of double-strength antibiotics so that the final concentration was achieved. The treated suspensions were then incubated at 37° C for varying lengths of time. They were microscopically examined for living amoebae using double-strength Lugol's iodine and tested for contamination with bacteria other than *Es. coli - B* by plating a loopful of each medium on to nutrient-agar plates. The agar plates were treated as described below. The remaining suspensions were then washed thrice with PBS by centrifugation and the sediments subcultured into fresh Robinson's medium to test viability.

The sucrose-gradient method - A sucrose-gradient technique as described by Cox (1970) was adapted to avoid the effect which antibiotics may have on the behaviour of amoebae. Sucrose gradients of 8.0 ml volumes were prepared by layering 2.0 ml quantities of 1.0, 0.8, 0.5 and 0.2 M sucrose in Hank's physiological solution pH 7.2 successively into a 10.0 ml centrifuge tube using pasteur pipettes. 1.0 ml of filtered stool suspension or amoebal suspension was applied to the top of the gradient which was then centrifuged at 1,200 *g* for 1 minute at 4° C.

The different layers were carefully pipetted into sterile Kahn tubes with sterile pasteur pipettes and were examined microscopically for their flora and fauna. The top layers were discarded after examination but the sediment containing the cysts or trophozoites was subcultured in the usual way (Robinson's medium) after washing it twice in PBS to remove excess sucrose.

B1.8 Preparation of amoebal suspension for experimentation

The sediments of several stock cultures were pooled after chilling culture bottles in ice for 5 minutes

and were centrifuged in 10.0 ml centrifuge tubes at 180 *g* for 5 minutes. The supernatant was pipetted off and the sediment was washed with sterile PBS three times to remove as many of the bacteria as possible in case of any contamination. The final sediment was resuspended in freshly made RSM, and the number of "viable" trophozoites was estimated using a haemocytometer and 0.2% trypan-blue solution (trypan-blue solution was made up in a mixture of medium R and 0.05M phthalate solutions 1:2). From this, the number of trophozoites inoculated into each culture bottle was calculated.

0.1 ml of such amoebal suspensions was used to initiate cultures. Each culture bottle was incubated at 37° C for as long as the experiment lasted.

B1.9 Techniques for testing bacterial contamination of cultures

General bacteriological methods as described by Cruickshank *et al* (1975) were strictly followed. Contamination of cultures was tested by streak-plating the culture medium on to nutrient and blood-agar plates. The plates were incubated aerobically at 37° C, and at 28° C and anaerobically in McIntosh and Fildes anaerobic jars at the same temperatures. Plates were discarded as negative when no growth occurred after 72 hours incubation. Bacterial colonies from positive plates were later identified by Gram's staining method.

i. Preparation of nutrient agar - The medium was prepared by boiling 280 g nutrient-agar powder (Oxoid) in a litre of sterile distilled water in a conical flask over moderate heat. A little agar was added at a time with continuous stirring to prevent clumping until all had dissolved. The medium was then dispensed in 4 oz medical flat bottles and sterilized by autoclaving. The sterile agar was stored at room temperature until it was required.

When agar plates were to be poured, the stored agar bottle was placed in a boiling water bath heated over a bunsen until the agar had melted. It was allowed to cool down to about 45° C and then poured.

ii. Preparation of blood agar - The base was prepared by dissolving 40.0 g blood-agar base powder (Oxoid) in a litre of distilled water which was then dispensed in 4 oz medical flat bottles, autoclaved, allowed to cool and stored at room temperature. When it was required for use, the base was melted and cooled to about 45° C when 7% defibrinated fresh horse blood (Wellcome) was added slowly. This was carefully mixed and poured into sterile Petri dishes. The plates were stored upside down at 4° C for not more than 3 weeks.

iii. Preparation of Eosin/methylene-blue agar - The eosin/methylene-blue agar was obtained in tablet form commercially (Oxoid). Two tablets were added to 10.0 ml distilled water and allowed to soak for 15 minutes before autoclaving. When it had cooled toabout 60° C it was shaken and then poured. This was sufficient for one plate only. The plates were used for isolating colonies of *Es. coli - B.*

iv. Techniques for pouring and drying agar plates - Prepared agar medium was aseptically poured into sterile 90.0 mm diameter disposable Petri dishes on a flat surface. The plates were left undisturbed until the agar had solidified.

The condensed steam from the hot liquid agar was removed by drying the poured and set plates in an incubator at 37° C for 1 hour. The lid of the dish was first laid down on the incubator shelf, the medium-containing portion of the Petri dish was then inverted face downwards and placed in the incubator with the free edge resting on the lid.

v. Preparation of Gram stain* - This method was used for diagnostic identification of various contaminants from amoebal and *Es. coli - B* cultures. A colony unit from the agar plate was taken up by means of a sterile wire loop and spread in a drop of saline over a dry, clean slide and heat fixed. Staining was then carried out as follows: The slide was stained with ammonium oxalate crystal violet solution for 30 seconds; it was rinsed briefly with cold water then fixed with one-strength Lugol's iodine solution for 30 seconds to prevent decolorization with 100% acetone with which it was treated for about 3 seconds. The decolorized organisms, when present, were ultimately stained with safranin for 30 seconds. The slide was rinsed with tap water and allowed to drain and dry on the bench. Gram-positive organisms stained violet while Gram-negative ones stained pink. The types of bacteria, e.g. cocci, bacilli, were identified using an oil-immersion lens at x 100 magnification. Identification was confirmed by a trained, experienced bacteriologist.

vi. Use of anaerobic jars - McIntosh and Filde's anaerobic jars used were obtained from B + T (A Searle Company).

The metallic jar (8 x 5 in) is provided with a lid that can be clamped down to make it air-tight. The lid is furnished with two tubes and taps. A capsule containing pellets of palladiumized alumina is suspended under the lid. This acts as a catalyst in the reaction $2H_2 + O_2 = 2H_2O$ during anaerobiosis. The jar itself is connected to a tube near its rim, the bottom of which is filled with an anaerobic indicator (methylene blue in 0.8% agar).

When it was required for use, the jar was cleaned and dried. After putting the plates or culture bottles (with loose caps) in it, the lid was replaced and clamped on. The air inside was then evacuated using a vacuum pump. As air was removed it was necessary to clamp down the lid even tighter.

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* For details of preparation see Cruickshank *et al* (1975).

A gas mixture of 90% hydrogen and 10% CO_2 (BOC) was delivered to the jars via a football bladder attached to the gas cylinder. The jar was then incubated at the required temperature.

vii. Sterilization techniques - All chemicals and materials were sterilized by autoclaving at 121° C for 15 minutes unless otherwise stated.

B2. Assay Methods

B2.1 Haemocytometer estimation of "viability" using trypan-blue stain

When it was required to estimate the number of viable amoebae in a culture using trypan blue, the sediment was first removed from the culture bottle into a clean, graduated centrifuge tube, then the fluid overlay. The mixed suspension was centrifuged at 180 *g* for 5 minutes. The supernatant was discarded and the sediment was resuspended in fresh RSM enough to make 1.0 ml of suspension. The suspension was thoroughly mixed with an equal volume of 0.2% trypan blue in a mixture of phthalate solution and medium R on a clean glass slide. Estimation was done in a haemocytometer (Improved Neubauer, depth 0.1 mm, 1/400 mm^2) (Dacie and Lewis, 1963) using a x 10 eye-piece and a x 40 objective and the number of amoebae per culture was computed from the result using the formula

$$\frac{N.X.10^4}{9}$$

where N = number of trypan-blue excluding amoebae in 9 squares of haemocytometer cell
X = the dilution factor with trypan-blue.

B2.2 Infectivity titration procedure

Forty-eight-hour-old cultures of *E. histolytica* main-

tained in RSM were harvested and resuspended in fresh medium. Several ten-fold dilutions were made from the fresh suspensions by carrying over successively 0.1 ml quantities in 0.9 ml of medium in Kahn tubes. Sterile precautions were taken to prevent contamination of media.

Five-millilitre bijou bottles, each containing 3.0 ml RSM with agar slope and rice starch were set up in sets of 6. A set was allocated to each of the dilutions which were to be tested for infectivity. 0.1 ml of the dilutions to be tested was inoculated into each bottle of the set (pre-warmed) by means of a 1.0 ml syringe and needle (gauge 20G 1½) through the perforated bijou cap. The inoculated culture bottles were incubated at 37° C and examined for growth of *E. histolytica* on days 2, 5, 7 and 10.

From the numbers of cultures showing amoebic growth at each dilution, the infectivity of the original suspension to cultures was computed from the tables given by Lumsden *et al*, (1963) and was expressed as the $\log_{10}$ of the number of 63% infective doses (ID_{63}) per ml.

B2.3 Estimation of the ID_{63} end-point

0/6 and 6/6 are regarded as weightless data and are not considered. The useful data lie between these results. For the ID_{63} table, ratios are:

$$\frac{\text{number of cultures infected}}{\text{number of cultures inoculated (6)}}$$

Proceeding in the dilution series from the $\bar{1}$ end, the first useful dilution is called "x"*. The results of experiments were classified into one, two or three-point data. The table of Lumsden *et al* (1963) was then used to give direct readings of ID_{63} values and their standard errors (see Table IIIa,b and c).

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* Sign changes to positive

Table IIIa. One-Point Series

Ratio	log ID_{63}	SE ±
5/6	x.1	0.5
4/6	x.0	0.3
3/6	(x-1).8	0.3
2/6	(x-1).6	0.3
1/6	(x-1).4	0.5

Table IIIb. Two-Point Series

Ratio	log ID_{63}	SE ±
5/6 4/6	x.8	0.4
5/6 3/6	x.7	0.3
5/6 2/6	x.4	0.3
5/6 1/6	x.3	0.3
4/6 4/6	x.5	0.6
4/6 3/6	x.5	0.3
4/6 2/6	x.3	0.2
4/6 1/6	x.1	0.3
3/6 3/6	x.3	0.5
3/6 2/6	x.2	0.4
3/6 1/6	(x-1).9	0.5
2/6 2/6	x.1	0.6
2/6 1/6	(x-1).8	0.5

Table IIIc. Three-Point Series

Ratio	log ID_{63}	SE ±
4/6 4/6 3/6	(x+1).2	0.6
4/6 4/6 2/6	(x+1).0	0.5
4/6 3/6 3/6	(x+1).2	0.6
4/6 3/6 2/6	x.8	0.4

Note (on Table IIIc). Where one point is 5/6 or 1/6 it is ignored and 0.1 is added to the SE obtained for the two points. Where 2 points are 5/6 or 1/6 and are consecutive, the extreme result is ignored and 0.2 is added to the SE given for the two points. Where two points are 5/6 and 1/6 (on each side of 4/6, 3/6 or 2/6) they are ignored, the value for the single central point is added, and 0.2 is added to the SE. Otherwise the table is used.

Series with no useful points

In these the last 6/6 is treated as x, then the ID_{63} = x.4 with SE = ± 0.5.

B3. Special techniques for amoebae

B3.1 Methods for growth-rate measurement of *E. histolytica*

The growth curve of *E. histolytica* is reported to con-

form to the classical bacterial pattern but with a decrease in lag phase and an increase in log phase (Balamuth and Howard, 1946; Abioye, 1973). There is a short stationary phase, when it is likely that deaths of amoebae are keeping up with population increase. In late log phase, many deaths must already be occurring and measurement of the growth-rate of the organisms using the slope of the population growth curve in the log phase is liable to be inaccurate. A technique for growth-rate measurement based on the time elapsing before the population reaches an arbitrary low number, which was described for *Plasmodium berghei* in mice (Warhurst and Folwell, 1968), was adapted here for cultures of *E. histolytica*. If cultures were started with serial dilutions of an inoculum, the number of days taken for each culture to produce a fixed low number of organisms, say, 20,000, should be linearly related to the log of the inoculum size providing growth-rate remains constant. The slope of the line relating pre-20,000 time to log inoculum gives the 10 times increase time as a proportion of 1 day.

In order to get an accurate pre-20,000 time estimate for each serially diluted inoculum when sampling daily at the same time, two consecutive daily measurements above and below 20,000 were plotted on a log scale against time. The estimate of pre-20,000 time was then interpolated on a straight line drawn between the two values.

The equation used in calculating growth rate and drawing regression lines (Figure 29), was derived from the basic growth equation for infective particles:

$$N' = N\,(2^{\frac{1}{g} \cdot t})$$

where N' = population after time t
N = initial population
g = generation time in days
t = time elapsed in days.

Expressing the equation in a linear form:

$$\log N' = \log N + \log 2.\frac{1}{g}.t$$

$$\therefore \quad g = \frac{\log 2.t}{\log N' - \log N}$$

When t is tenfold increase time, then,

$$g = \log 2.t$$

To convert to hours, the result was multiplied by 24 hence

$$g = \log 2.t.24$$

$$\text{Growth rate} = \frac{1}{g}$$

For statistical analysis of generation time, the method was as follows: The three pre-20,000 times from each inoculum were plotted (see Figure 29) and the common slope (b) of parallel lines (one for each sample) fitted to these points was calculated by regression analysis. The fall in pre-20,000 time associated with an increase in inoculum of log 2 (equal to -b · log 2) was calculated using this slope. This was the estimated generation time. The SE of the slope calculated in the regression analysis was multiplied by log 2 to give the SE of the generation time.

B3.2 Immunological methods

The Indirect Fluorescent Antibody Technique (IFAT) was used in testing each of the *E. histolytica* stocks used for its reaction with human anti-*E. histolytica* serum at a titre of 1/100. All the stocks used gave a similar positive reaction.

1. Procedure for IFAT using culture smears - A thick smear of a 48-hour-old culture of *E. histolytica* was made on a clean glass slide and allowed to dry. It was fixed for 15 minutes in methanol, allowed to dry and then wrapped in tissue paper and polythene and stored at -20° C until required (it was usually tested within a week of preparation).

When required for use, the slide was removed from deep freeze and allowed to dry in front of a hair-dryer. Meanwhile, human anti-*E. histolytica* serum from a patient with a proved amoebic liver abscess, IFAT titre 1/512, was made up 1 in 100 with PBS (pH 7.1). A grease ring was made round the smear to prevent serum from running off the slide. The slide was placed in a moist tray.

The ringed area was covered with the dilute anti-serum and incubated at room temperature for 30 minutes. The slide was washed twice for 10 minutes with PBS. It was drained and allowed to dry on the bench in front of a hair-dryer.

Fluorescein-conjugated anti-human immunoglobulin (sheep) (Wellcome) was prepared 1:50 in PBS while the slide was drying. The smear was re-ringed with vaseline and replaced in the moist tray. The ringed area was covered with diluted conjugated antiserum and incubated at room temperature for 30 minutes. It was then washed twice with PBS for 10 minutes each. Excess PBS was drained off and the slide was allowed to dry as usual.

90% glycerol solution was made in PBS. This was used in mounting the slides. The amoebae were observed for fluorescence under a Zeiss fluorescent microscope (incident illumination) using a x25 objective and a x8 eye-piece. In a positive test, *E. histolytica* normally fluoresces a bright green against a green/black background. Cross-reactions are not found with *E. coli*.

ii. Procedure for IFAT using multispot slides

Forty-eight-hour-old cultures of *E. histolytica* were harvested, washed and resuspended in PBS as before. The volumes of the suspensions were adjusted in such a way that 1.0 ml contained 6,000 amoebae. Using a 5.0 µl automatic pipette, 5.0 µl volumes were placed on each of 12 spots of the teflon-coated multispot slide (Hendley

Ltd). The drops were allowed to dry in warm air in front of a hair-dryer. When dry, they were fixed with methanol in a coplin jar for 20 minutes. They were dried as before, wrapped up back to back in tissue paper and stored as already described.

Before the slides were removed from the deep freeze, the anti-*E. histolytica* antiserum (No. 311) derived from a Madras case of liver abscess (titre positive at 1/1024) and a "normal" serum (HI50) from a patient with *Pneumocystis* infection were separately diluted with PBS. 0.1 ml of serum was added to 3.1 ml PBS to give a dilution of 1/32. Two-fold serial dilutions were made by carrying over 1 ml from the 1/32 dilution until a dilution of 1/1024 was reached.

The slides were removed from deep freeze and dried as before. Using a pasteur pipette and starting from the highest dilution, one drop from each control serum dilution was put on one spot of the dried antigen (6 spots in all); the replicate antigen spots were treated with a drop from each of the diluted positive serum (6 spots in all). The slides were incubated in a moist tray at room temperature for 30 minutes, after which the excess serum and antiserum were shaken off. The slides were washed with PBS for 2 x 10 minutes in a staining jar; they were drained and dried as usual.

Sheep anti-human immunoglobulin conjugated with fluorescein (Wellcome) was prepared (1:30 in PBS). A drop of this was placed on each of the antigen/antibody preparations and incubated at room temperature for 30 minutes. The slides were drained and washed as before but not dried. They were counterstained with Evans blue (diluted 1:10,000 in PBS) for 5 minutes; this was followed by rinsing and drying. Slides were mounted in 90% glycerol in PBS. Fluorescence was observed as before.

B3.3 Procedure for concanavalin A-induced agglutination

The method described by Trissl *et al*(1977) was followed but with some modifications. Stocks of amoebae used were:

Biswas, Irvine, Thirer, JEH and NIH:200 (with bacteria)
NIH:200 (with *Crithidia*)
NIH:200 (axenic)

Amoebae grown with bacteria in Robinson's medium were passaged twice without added starch before they were harvested for these experiments. (Experience showed that fewer rice-starch granules in the culture medium facilitated easy observation and immediate reaction if any.)

All amoebae, whether monoxenically or axenically cultivated were harvested and washed twice by centrifugation at 200 *g* for 5 minutes in PBS (pH 7.2) and were resuspended in fresh PBS so that the final concentration was 5 x 10^5 - 1 x 10^6 amoebae/ml.

0.4 ml of the amoebal suspension was incubated in equal volume of concanavalin A (or con A) (Sigma - from Jack beans) of known concentration in PBS for 30 minutes at 37° C with intermittent shaking in Kahn tubes. Con A was used in the following final concentrations: 10, 25, 50, 100 and 200 µg/ml. A drop of the precipitate was observed on a clean glass slide at x 40 magnification using phase contrast.

The size of the amoebal clumps was measured using a calibrated eye-piece at x 10 magnification when the number of amoebae forming a clump was difficult to estimate otherwise; the degree of clumping was estimated by counting the average number of cells per clump in 3 experiments.

The specificity of the agglutination reaction was tested by pre-incubating an equal volume of double-strength

con A with 0.2M α-methyl mannoside (Sigma) to abolish the agglutination reaction.

A control was set up using PBS alone as spontaneous agglutination had been observed to occur in certain strains with PBS (Trissl *et al*, 1977).

B3.4 Techniques for starch-gel electrophoresis

The techniques of isoenzyme analysis by means of thin-layer starch-gel electrophoresis used were originally developed by Wraxall and Culliford (1968) for enzyme typing of blood stains. The methods described for glucose phosphate isomerase, phosphoglucomutase and malic enzymes were adapted for amoebae from those described by Bagster and Parr (1973) and Miles *et al*(1977) for trypanosomes. The methods for hexokinase were adapted from those described by Harris and Hopkinson (1976) for mammalian red blood cells.

i. Preparation of extracts for electrophoresis - *E. histolytica* was harvested from universal bottles after 48 hours of growth at 37° C by centrifugation at 200 *g* for 5 minutes at room temperature and washed twice in PBS (pH 7.1).

The supernatant was pipetted off as much as possible and the last traces of water were removed using a strip of filter paper. The washed amoebae were lysed by mixing with an equal volume of a hypotonic solution of enzyme stabilizer containing 1.0mM ethylene diamine tetra acetic acid (EDTA), 1.0mM dithiothreitol and 1.0mM E-aminocaproic acid. The mixture was kept on ice for about 30 minutes, then frozen at -20° C for 24 hours (Godfrey and Kilgour, 1976), thawed by holding in the fist and then centrifuged at 11,000 *g* for 30 minutes at 4° C in an MSE high speed centrifuge.

The clean supernatant was removed by means of a drawn-

out pasteur pipette and 'beaded' (frozen in droplets) immediately in liquid nitrogen. The beads were stored in half-dram glass vials with a hole in their caps at -196° C in liquid nitrogen. Vials were appropriately labelled using strips of lassoband (Lasso Ltd.).

ii. The enzymes studied - For details of enzymes studied see Table IV.

Table IV. Details of enzymes studied

Enzyme	Abbreviation	Enzyme Commission Number
Glucosephosphate Isomerase	GPI	5.3.1.9.
Phosphoglucomutase	PGM	2.7.5.1.
L-Malate:$NADP^+$ oxidoreductase (oxaloacetate decarboxylating)	ME	1.1.1.40
Hexokinase	HK	2.7.11

iii. Preparation of buffers - 0.2M sodium phosphate buffer pH 7.0 was prepared for GPI and ME, 0.1M Tris maleate buffer pH 7.4 was prepared for PGM and Tris/boric acid buffer pH 8.6 for HK.

GPI and ME buffers - 17.4 g disodium hydrogen orthophosphate and 12.15 g sodium dihydrogen orthophosphate were dissolved in distilled water and made up to 1 litre. For making gels, this solution was diluted 3 in 46 (0.015M final concentrations).

PGM buffer - 12.14 g Tris, 11.61 g maleic acid, 2.03 g magnesium chloride and 3.72 g EDTA were dissolved in distilled water and made up to 900 ml. The pH was adjusted with 10.0M sodium hydroxide to 7.4 and made up to 1 litre. The solution was diluted 3 in 20 for making gels (final concentration was 0.015M).

HK buffer - The stock solution consisted of 0.9M Tris, 0.2M boric acid, 0.02M EDTA and 0.025M magnesium chloride. The stock solution was diluted 1 in 7 to fill the electrophoretic tank and 1 in 10 to make gels.

iv. Preparation of starch gels - Forty millilitres of starch solution was made up for every plate at 11.9% final concentration. The weighed out starch was poured into a clean, dry, round-bottomed flask; to this was added the appropriate volume of buffer and the mixture was gently swirled to avoid lumping.

The starch solution was warmed on a bunsen flame directly with vigorous swirling. The starch first thickened and then thinned out as the solution approached boiling, but it was not allowed to boil.

The solution was degassed with a vacuum pump until it was clear and free of bubbles. The glass gel-forming plates were placed on paper. The hot starch solution was poured against the bottom edge strip of the plate in a stream until about two thirds of the plate had been covered. With a spreader, the solution was spread over the plate by pushing it away from the lower edge in one quick stroke. The spreader was passed over the upper edge of the plate with the excess gel and the plate was pulled away to avoid back flow of gel. The gel, when poured was 14 x 22 x 0.1 cm. (i.e. 1 mm thick). The plate was covered with a cover plate and left to solidify.

v. Electrophoresis and enzyme staining (Bagster and Parr, 1973) - The electrophoretic tank was filled with

approximately 1 litre of the appropriate buffer. The cooling unit was switched on and adjusted to +8° C. A pair of sponge wicks were placed in the buffer to soak.

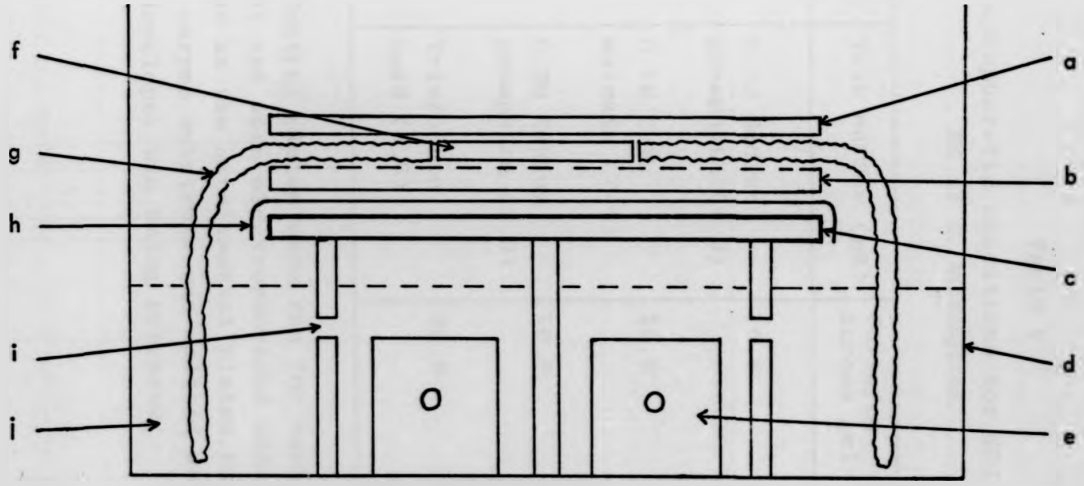
The beaded lysates were removed from the frozen storage into small sample tubes and allowed to thaw on ice.

8-10 (0.8 - 1.0 cm long) slots were made in the gels near the bottom edge strip of the plate using a template. Sample threads were cut a little shorter than the slots by wrapping cotton threads in graph paper and using the marking on the graph paper as a guide. Cut sample threads were soaked in the lysate, one piece was applied to the gel at a time by placing it along the slot and tucking it under the surface with a pair of clean, fine forceps. Forceps were rinsed in distilled water and wiped between samples to prevent cross-contamination.

The prepared gels were transferred to the electrophoretic tank. A glass plate was placed width-wise across the gel making sure that the edge of the plate was parallel to the line of the slots. The soaked wicks were moved close against the thin glass plate placed length-wise (see Figure 4). The tanks were connected and the power pack was turned on and run at constant voltage (See Table V).

Fifteen minutes before the end of each run, the developer was prepared by mixing together all ingredients except agar, PMS and MTT. The prepared agar was left to cool to approximately 50° C but not allowed to set, before the end of the run. When running-time was up, 10.0 ml of the cool agar was added to an equal volume of reaction mixture. The PMS and MTT were added last (see TableVI). The bottle was inverted once and the mixture was poured into an overlay surround (which had been premounted on the gel plate on completion of the run) and

Fig. 4 Front view of an electrophoretic tank in use



Legend
a - Thick cover glass plate placed lengthwise
b - Starch-gel resting on cooling plate
c - Cooling plate
d - Tank containing buffer
e - Electrode in electrode chamber
f - Thin cover glass plate placed widthwise across sandwich
g - Spontex wick
h - Melinex insulating sheet
i - Interconnecting hole between wick and electrode chambers
j - Wick chamber

allowed to spread evenly. When set, the plate was incubated at 37° C for 30 minutes and photographs were taken using a Polaroid camera.

Table V

Electrophoretic conditions for GPI, PGM, ME and HK of *E. histolytica*.

Enzyme	Tank buffer (pH)	Volts/cm across gel	Current in mA	Running time (hrs)
GPI	0.2M Sodium phosphate (7.0)	16.6	25	3
PGM	0.1M Tris maleate (7.4)	16.6	25	3
ME	0.2M Sodium phosphate (7.0)	16.6	25	3
HK	Tris/boric acid (8.6)	20.0	35	3

Control plates were run for each enzyme for every extract and were electrophoresed under the same conditions as the experimental plates, except that the specific enzyme substrate was omitted when the enzyme locating developer was being prepared.

TABLE VI

Enzyme-staining conditions for GPI, PGM, ME and HK of *E. histolytica*

Enzyme	Developing conditions			
	Buffer	Coenzyme	Substrate	Other additions
GPI	7.0 ml 0.3M Tris/HCl pH 8.0	0.5 ml NADP (10 mg/ml)	0.2 ml D-fructose-6-phosphate (10 mg/ml)	2.0 ml 0.1M magnesium chloride ($MgCl_2$), 0.5 ml G6PDH (100 units/ml), 5.0 mg MTT, 1.0 mg PMS, 10.0 ml L28 agar (12 mg/ml).
PGM	7.5 ml 0.3M Tris/HCl pH 8.0	0.5 ml NADP (10 mg/ml)	0.6 ml glucose-1-phosphate (20 mg/ml)	2.0 ml 0.1M $MgCl_2$, 0.1ml G6P DH (100 units/ml) 5.0 mg MTT, 1.0 mg PMS, 10.0 ml L28 Agar (12 mg/ml).
ME	7.0 ml 0.3M Tris/HCl pH 7.4	0.5 ml NADP (10 mg/ml)	0.1 ml 1.0M L-malic acid	2.0 ml 0.1M $MgCl_2$, 5.0 mg MTT 1.0 mg PMS, 10.0 ml L28 agar, (12 mg/ml).
HK*	20.0 ml 0.1M Tris/HCl pH 7.5	1.5 ml NADP (5.0 mg/ml), 40 mg ATP (1.3mM final concentration)	900 mg glucose	0.5 ml 0.02M $MgCl_2$, 40 µl G6PDH (140 units/ml), 5.0 mg MTT, 1.0 mg PMS, 10 ml L28 agar (12 mg/ml).

NADP = Nicotinamide adenine dinucleotide phosphate

MTT = MTT tetrazolium salt

PMS = Phenazine methosulphate

G6PDH = Glucose-6-phosphate dehydrogenase

* 10 ml of the reaction mixture was added to 10 ml agar

B3.5 Techniques for testing leucocytotoxicity of *E. histolytica*

The method described by Jarumilinta and Kradolfer (1964) was followed with very slight modifications.

Preparation of leucocytes-Leucocytes were obtained from a person whose serum had no detectable antibody against *E. histolytica*. About 20.0 ml of blood was taken by venepuncture of the antecubital vein and stored in the refrigerator (4° C) in heparinized tubes for up to 24 hours. When it was required for use, the blood sample was centrifuged at 2000 *g* for 10 minutes at room temperature. The leucocytes were collected by pipetting from the buffy coat.

Two drops of this were put on a clean, grease-free cavity slide and was incubated for 30 minutes in a moist chamber at 37° C in a drop of inactivated horse serum and Hank's solution (pH 7.6). The proportion of serum to Hank's was 1:3. During this time, the leucocytes adhered to the glass surface. The slide was rinsed with Hank's solution to remove any red blood cells that might be present. The slide was examined for migratory leucocytes under phase contrast at x400 magnification (x40 objective and x10 eye-piece).

Preparation of mixtures of leucocytes and amoebae - Amoebae were grown in Robinson's medium to which starch had not been added on 2 consecutive passages with 48-hourly passage. 24-hour-old amoebae from the last passage were used. (This procedure decreased the amount of starch granules present in the amoebal suspension. Large numbers of starch granules tend to obscure the leucocytes and inhibit leucocytotoxic reactions.) The amoebae were concentrated by centrifugation at 200 *g* for 5 minutes. They were subsequently washed twice in Hank's solution in the same way. The amoebae were then resuspended in freshly prepared Hank's/serum mixture and kept at 37° C

(final concentration was 500,000 amoebae/ml).

One drop of amoebal suspension was added to the freshly prepared leucocytes on the cavity slide and the mixture was covered with a clean coverslip.

The excess fluid was soaked up with a filter paper and the preparation was sealed with paraffin wax. The preparation was observed on a warm stage at 37° C with a dry lens (x40 objective x10 eye-piece) under phase contrast.

B3.6 Measurement of oxidation reduction potential in amoebal cultures - This method is essentially as described by Chang (1946) and Jacobs (1950). The electrodes were prepared from bright platinum wire heat-sealed into a glass pipette. The pipette was filled with mercury to make contact with the platinum and connection was made to the potentiometer (pH meter) by dipping an amalgamated copper wire from the instrument into the mercury. The copper wire was sealed into the pipette with pitch. The coiled platinum wire was kept clean by fitting it into an empty disposable needle's plastic tube. The tube was removed and the electrode was washed in 70% alcohol and sterile distilled water before use. The standard half-cell consisted of a saturated calomel electrode inserted into a beaker of saturated potassium chloride solution (saturated at room temperature). Liquid connection between the standard half-cell and the culture whose potential was to be measured was by means of a potassium chloride-agar bridge tube, (1% agar in saturated potassium chloride solution) appropriately bent so as to fit into the universal culture-bottle and the beaker. There was no permanent fixing of any of the electrodes to the culture-bottle.

The readings were made at 1, 6, 12, 24, 48, 72 and 96-hour intervals.

Measurement of pH - Readings of pH were made at the same time-intervals as O-R readings by means of a glass

electrode using a standard calomel half-cell.

Expression of results from O-R and pH readings - The O-R and pH were determined electrometrically with pH meter type PHM 26C (Radiometer, A/S, Copenhagen). The pH values were registered to 1/100 of a unit. The oxidation reduction potentials were expressed as Eh in millivolts calculated from the equation of Chang (1946):

$$Eh = E_1 + Est$$

where Eh = the potential with reference to the hydrogen electrode

E_1 = the potential observed on the potentiometer

Est = the potential of the standard calomel half-cell with saturated potassium chloride which is approximately 243 mv at 25° C

The accuracy of the redox measuring system was tested using a saturated solution of quinhydrone in sodium dihydrogen phosphate solution pH 4.7 and in PBS pH 7.2. The calculated values of O-R potential for these two buffer solutions were 420 mv and 272 mv respectively from the equation:

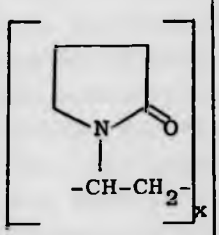
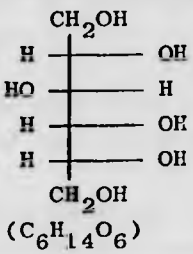
$$Eh = +699 - 59.2 \cdot pH$$

B.4 Cryopreservation techniques

B4.1 The cryoprotectants used - Chemical details of the cryoprotectants used in this work are given in Table VII.

Unless otherwise stated, cryoprotectants were prepared fresh when required by dissolving the required quantities in freshly prepared RSM without antibiotics to make twice the concentration needed. A volume of the double strength cryoprotectant was added to an equal volume of amoebal suspension to give the required final concentration. The content was thoroughly mixed after ampoules had been flame-sealed.

Table VII
Chemical details of cryoprotectants used

Name of cryoprotectant	Chemical formula	Molecular weight	Specific gravity	Freezing Point
Ethanol	C_2H_5OH	46.6	0.788	-117° C
Methanol	CH_3OH	32.04	0.992	-97.8° C
Glycerol	$C_3H_5(OH)_3$	92.09	1.2620	0° C
DMSO (Analar)	$CH_3SO.CH_3$	78.13	1.10	18-18.4° C
PVP K30 (Fluka, Swiss)		40,000	variable	solid
Sorbitol (BDH)		182.17	1.2879	solid
Glucose	$C_6H_{12}O_6$	180.16	1.019	solid

B4.2 Preparation of amoebal suspension for freezing - Amoebae used in the freezing experiments were derived from 48-hour stock-cultures grown in universal bottles, unless otherwise stated. The cultures were harvested in graduated centrifuge tubes aseptically and were washed twice or thrice in PBS, pH 7.2 by centrifugation at 180 *g* for 5 minutes. The supernatant was pipetted off and the amoebae were resuspended in fresh RSM or any other suspending medium as indicated in the experiment. Cryoprotectants were usually added to the amoebal suspension in the ampoules.

B4.3 The freezing container - All amoebae were frozen in sterile 1.0 ml glass ampoules. Before putting amoebal suspension and cryoprotectants in ampoules, the ampoules were flamed, and their necks drawn out in such a way that materials could only be introduced by syringe and needle. When it was time to introduce the freezing material, the long thin neck was aseptically scored and broken off between clean Kleenex tissue papers about 1½ inches from its base. The ampoule was filled and flame-sealed immediately. After the seal had cooled, the suspension was thoroughly mixed. The thinning of the neck of the ampoule helped to reduce the amount of heat required to seal the ampoules and thereby prevented heating of the amoebae, it also helped to minimize contamination.

Each ampoule was clearly labelled by writing with ball pen on a piece of lassoband that had been wrapped round it.

B4.4 The freezing machine - The prototype mini-freezer Unit Type R202 (Dewrance Controls Ltd.) with automatic cooling cycle used for all my freezing experiments was borrowed from Mr. C.D. Kimber (L.S.H.T.M., Winches Farm Field Station). The machine was designed so that it could be programmed to cool steadily at rates between 0.1° C/min to 10° C/min from +20° C to -190° C using liquid nitrogen as the coolant. It could also be adjusted in such a way that freezing could be done at a single sub-zero temperature of choice.

B4.5 Freezing methods - After the ampoules had been filled with the freezing material and flame-sealed, they were left to equilibrate either in an incubator at 37° C or on the bench at 23° - 25° C or they were kept in ice at 0° C (time of equilibration depended on the experiments being performed). Meanwhile the cooling machine was allowed to cool to 0° C and was held at that temperature. At the end of equilibration the rate of cooling was set and the canes (which held the ampoules inside the freezing machine) were loaded.

i. Controlled-rate freezing - When samples were to be frozen at a controlled rate, the dial operating rate-control was set at the required rate and freezing proceeded from 0° C to -60° C unless otherwise stated.

ii. Two-step freezing - The machine was allowed to cool down to the required sub-zero temperature at any rate and was held at that temperature for at least five minutes before the canes were loaded. Materials were kept at this temperature for as long as required.

After the ampoules had cooled to the desired temperature or after they had been held at a temperature long enough, they were immediately transferred into a flask of liquid nitrogen and kept there for at least one hour before they were transferred to a liquid nitrogen refrigerator.

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RESULTS

C.1 Preliminary Investigations

Experiment 1. The effect of cryopreservation on the viability of *Escherichia coli - B*

Forty-eight-hour-old cultures of *Es. coli - B* grown in peptone water were used. They were washed and resuspended in PBS, equally distributed into 4 centrifuge tubes and centrifuged at 300 *g* for 10 minutes. After pipetting off the supernatants, the volume of each tube was made up to 0.5 ml using different suspending media. Tube 1 received 0.5 ml peptone water alone; tube 2, 0.5 ml medium R alone; the volumes of tubes 3 and 4 were made up to 0.25 ml using medium R; in addition tube 3 received 0.25 ml 20% (v/v) DMSO and tube 4, 0.25 ml 15% (v/v) glycerol. DMSO and glycerol were made up in medium R. Equilibration at 25° C was carried out for 15 minutes before freezing.

About 25.0 - 30.0 µl of each suspension was stabilated in glass capillary tubes. As many as fifteen capillaries were laid down for each suspension.

Two stabilate samples from each set were tested for viability after equilibration, before freezing and after 48 hours', 2, 4, 8 and 12-weeks' storage at -79° C. No difference in viable count between frozen and unfrozen samples was observed when DMSO, glycerol and medium R were used. There was a definite reduction in viability using peptone water. See Table VIII.

Experiment 2. Treatment of cysts and trophozoites of *E. histolytica* with antibiotics with a view to modification of concomitant flora

An overgrowth by bacteria was a problem in my attempt to produce monoxenic cultures using Robinson's method. Cultivating amoebae in the way described by Robinson proved wasteful of time since it usually took not less than 8 sub-

cultures with small doses of antibiotics before bacteria other than *Es. coli - B* were completely eliminated from cultures.

Table VIII

On the viability of *Es. coli - B* stabilate before and after freezing.

Suspending medium	Cryoprotectant	*Numbers (in thousands) of colony forming units/ml					
		Before Freezing	After freezing and storage for				
			48 hrs	2 wks	4 wks	8 wks	12 wks
Peptone water	none	19.20	18.25 (96.4)	15.75 (82)	7.0 (36)	13.0 (67)	15.17 (79)
Medium R	none	40.65	44.62 (100)	46.5 (100)	44.87 (100)	39.10 (96)	41.9 (100)
Medium R	10%(v/v) DMSO	26.75	27.58 (100)	28.28 (100)	17.12 (64)	27.3 (100)	28.0 (100)
Medium R	7.5%(v/v) glycerol	33.18	34.8 (100)	36.78 (100)	34.7 (100)	32.95 (99)	35.18 (100)

* results expressed as % of unfrozen samples are given in brackets.

The whole point of cryopreservation is to eliminate the necessity of continual passages and to avoid changes in biological characteristics of the organism by stabilating early. It was also important to avoid resistance of bacteria to antibiotics brought about by repeatedly challenging them with sub-lethal doses. So, experiments were performed to see whether bacteria could be eliminated once and for all with a single heavy dose of antibiotics.

Table IX

Viability of bacteria and of amoebae after treatment of *E. histolytica* trophozoite suspensions with antibiotics

Antibiotics	Final concentration in units/ml or mg/ml	Survival of bacteria after exposure for			Survival of amoebae after 24 hrs exposure	
		2 hrs	8 hrs	24 hrs	Microscopic	Culture
Gentamycin	200*	+	+	+	+	+
	400*	+	+	–	+	±
	800*	+	+	–	+	±
Penicillin	1000*	+	+	+	+	+
	2000*	+	+	+	+	+
	4000*	+	+	–	+	+
	6000*	+	+	–	+	–
	8000*	+	+	–	+	–
Streptomycin/ penicillin	2/2000	+	+	+	+	+
	4/4000*	+	+	–	+	+
Chloramphenicol	0.001	+	+	+	+	+
	0.01	+	+	+	+	+
	0.1	+	+	+	+	+
	1.0	+	+	+	+	+
	10.0	+	+	+	+	±
Control		+	+	+	+	+

+ = good growth, ± = poor growth, – = no growth

Suspensions of amoebae from cultures were treated with single antibiotics of varying concentrations or a combination of them. They were kept at 37° C and examined for viable bacteria (by plating) and viable amoebae (by subculturing) at intervals. See Table IX for summary of results.

Although the antibiotic method is useful, persistent gram-positive bacilli or cocci were not always eliminated from cultures even after 24-hours' incubation. Only repeated treatment with streptomycin/penicillin eventually eliminated these bacteria.

Gentamycin was not satisfactory because it affected the growth of amoebae adversely even when 200 units/ml concentration was used. Penicillin was useful by itself but at concentrations above 4,000 units/ml growth of amoebae was completely inhibited. Streptomycin/penicillin combination was most effective. With 4.0 mg streptomycin and 4,000 units penicillin/ml, all bacteria were eliminated within 24 hours and amoebae remained viable. Chloramphenicol at the concentrations used was not effective.

Because cysts are less vulnerable to chemical treatment, it is suggested that faecal concentrates be treated with antibiotics and washed thoroughly in PBS before they are inoculated into RSM.

Experiment 3. Sucrose-gradient separation of *E. histolytica* cysts and trophozoites from concomitant organisms

When all attempts to free *E. histolytica* cultures from *Blastocystis hominis* using chemicals failed because they were ineffective over limited numbers of subcultures, a sucrose-gradient method was tried. Out of 4 cultures so treated, only one stock, Saigon, was successfully grown without *Blastocystis*. In this case, the trophozoite sediment collected from the sucrose gradient had been washed in tap water instead of the usual PBS. To test this finding, trophozoites from the other three *Blastocystis*-contaminated cultures were washed three times by centrifugation with ordinary tap water before they were inoculated into RSM. This proved very successful as all cultures were freed from *Blastocystis* contamination. However, it was observed that a huge number of the trophozoites were lost through lysis.

The rate at which amoebae grew in subcultures from the treated inocula suggested that the new generation probably developed from mature cysts which had survived the treatment. If so, then *E. histolytica* must have completed its life cycle in RSM. This was later investigated and confirmed.

All *Blastocystis*-contaminated faecal concentrates were subsequently treated with tap water before they were cultivated.

However, the sucrose-gradient method proved very useful. It was possible to separate small cysts from larger ones: for example, *Endolimax nana* from *E. histolytica* and also bacteria from amoebae. Bacteria and *E. nana* cysts when present in the stool preparation were found at the 0.2M layer and *E. histolytica* cysts at the 0.8M and 1.0M layers (see Table X). Cysts so treated were washed in PBS by centrifugation, reconcentrated and subcultured into RSM.

Table X

Result of the sucrose-gradient separation of *E. histolytica* (SN) cysts from *E. nana* cysts and bacteria

Sucrose gradient	Presence of flora and fauna		
	E. nana	*E. histolytica*	Bacteria*
0.2M	+	-	3+
0.5M	-	-	+
0.8M	-	+	±
1.0M	-	3+	-

* by microscopical observations

C2 Optimizing growth conditions of *E. histolytica* in Robinson's medium

Experiment 1. The effect of BRS concentration on the growth of *E. histolytica* in RSM

After initial cultivation of isolates in Robinson's primary medium, isolates were subcultured in RSM of varying BRS/phthalate dilutions. It was observed that amoebae usually grew in all the dilutions used but to a greater or lesser extent.

Experiments were set up to investigate the effect of BRS concentration on the growth of *E. histolytica* stocks. RSM was made up as shown below:

Proportions of BRS/phthalate	% BRS	% serum	RSM group
1:2	33.0	16.6	a
1:3	25.0	12.6	b
1:4	20.0	10.0	c
1:5	16.6	8.3	d

For every stock used in these experiments 96 agar slopes with added starch were set up in bijou bottles. They were divided into 4 groups of 24. Group 1 received 3 ml RSM (a); group 2, 3 ml RSM (b); group 3, 3 ml RSM (c) and group 4, 3 ml RSM (d).

All cultures were inoculated with 0.1 ml of a suspension containing 20,000 amoebae/ml. They were incubated at 37° C. Four cultures from each set were examined separately daily, the numbers of 'viable' (trypan-blue-excluding) organisms were estimated and their average taken as the number of viable organisms/culture. Results for *E. histolytica* stocks JEH and Fenn in different RSM are shown in Table XI. The growth curves are graphically represented in Figures 5a and b.

Table XI

Growth of amoebic populations in RSM with various BRS concentrations

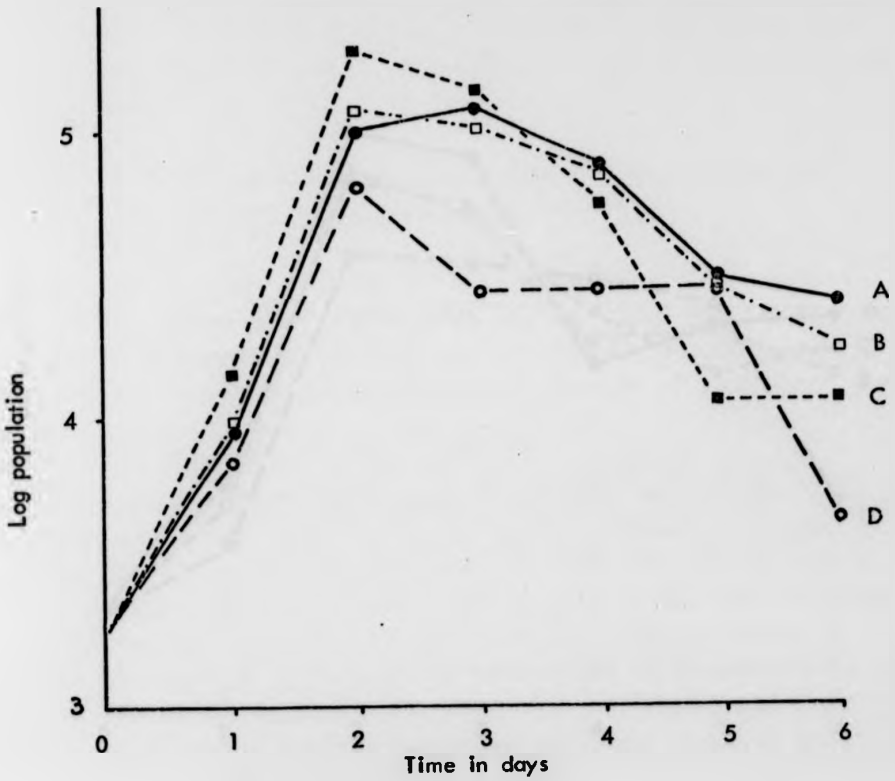
BRS concentration %(v/v)	Serum concentration %(v/v)	Amoebal stock	Population (x 10^4) per culture after incubation at 37° C for						
			0 hr	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
33	16.6	Fenn	0.2	1.0	12.0	14.0	8.3	3.5	2.9
		JEH	0.2	0.8	10.5	9.1	1.58	2.29	2.5
25	12.6	Fenn	0.2	1.0	14.0	12.5	8.3	3.2	2.0
		JEH	0.2	0.5	7.9	5.8	2.0	2.3	1.6
20	10.0	Fenn	0.2	1.5	23.0	16.6	6.3	1.26	1.3
		JEH	0.2	0.6	7.6	6.3	2.5	1.6	2.0
16.6	8.3	Fenn	0.2	0.79	7.6	3.2	3.2	3.2	0.5
		JEH	0.2	0.35	4.0	3.8	3.3	2.6	0.52

Table XI

Growth of amoebic populations in RSM with various BRS concentrations

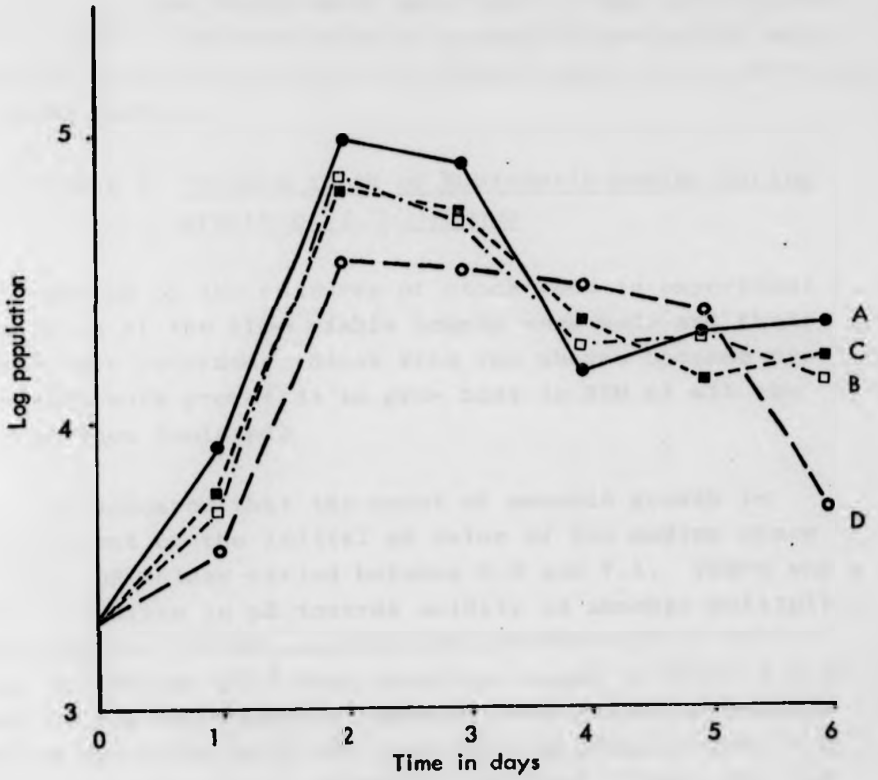
BRS concentration %(v/v)	Serum concentration %(v/v)	Amoebal stock	Population (x 10^4) per culture after incubation at 37° C for						
			0 hr	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
33	16.6	Fenn	0.2	1.0	12.0	14.0	8.3	3.5	2.9
		JEH	0.2	0.8	10.5	9.1	1.58	2.29	2.5
25	12.6	Fenn	0.2	1.0	14.0	12.5	8.3	3.2	2.0
		JEH	0.2	0.5	7.9	5.8	2.0	2.3	1.6
20	10.0	Fenn	0.2	1.5	23.0	16.6	6.3	1.26	1.3
		JEH	0.2	0.6	7.6	6.3	2.5	1.6	2.0
16.6	8.3	Fenn	0.2	0.79	7.6	3.2	3.2	3.2	0.5
		JEH	0.2	0.35	4.0	3.8	3.3	2.6	0.52

Fig 5a
Growth curves of E. histolytica (Stock Fenn)
in Robinson's medium with various BRS concentrations



A = (16.6% serum) 33% BRS
B = (12.6% serum) 25% BRS
C = (10% serum) 20% BRS
D = (8.3% serum) 16.6% BRS

Fig 5b
Growth curves of E. histolytica (stock J.E.H.) in Robinson's medium with various BRS concentrations



A =(16.6% serum) 33% BRS
B =(12.6% serum) 25% BRS
C =(10.0% serum) 20% BRS
D =(8.3% serum) 16.6% BRS

The results show that there is no appreciable difference in the maximum population of those stocks grown in RSM with 20 to 33% BRS (10-16.6% serum). However, all isolates grew best in RSM with 33% BRS (16.6% serum) except stocks Fenn and Rafiq which grew best in RSM with 20% BRS (10% serum). The medium which supported the growth of an isolate best was used when experiments were to be performed on such isolate.

Experiment 2. Changes in pH of Robinson's medium during growth of *E. histolytica*

The pH of the cultures of stock Fenn in experiment 1 was taken at the time viable counts were made and their means were recorded. (Stock Fenn was chosen because preliminary work proved it to grow best in RSM of all the stocks then isolated.)

It appeared that the onset of amoebic growth is independent of the initial pH value of the medium since initial pH values varied between 6.9 and 7.1. There was a steady decline in pH towards acidity as amoebae multiplied, probably due to the soluble waste products of *Es. coli - B* and, by the end of 6 days, the pH had dropped to below 5.5 in each of the experiments. However, the period of maximum growth coincided with the time when the pH fell to about 6.5. Below this pH, growth gradually declined. Table XII and Figure 6 illustrate these points.

Experiment 3. On the growth of *E. histolytica* in Robinson's medium using an oxygen-free gas phase

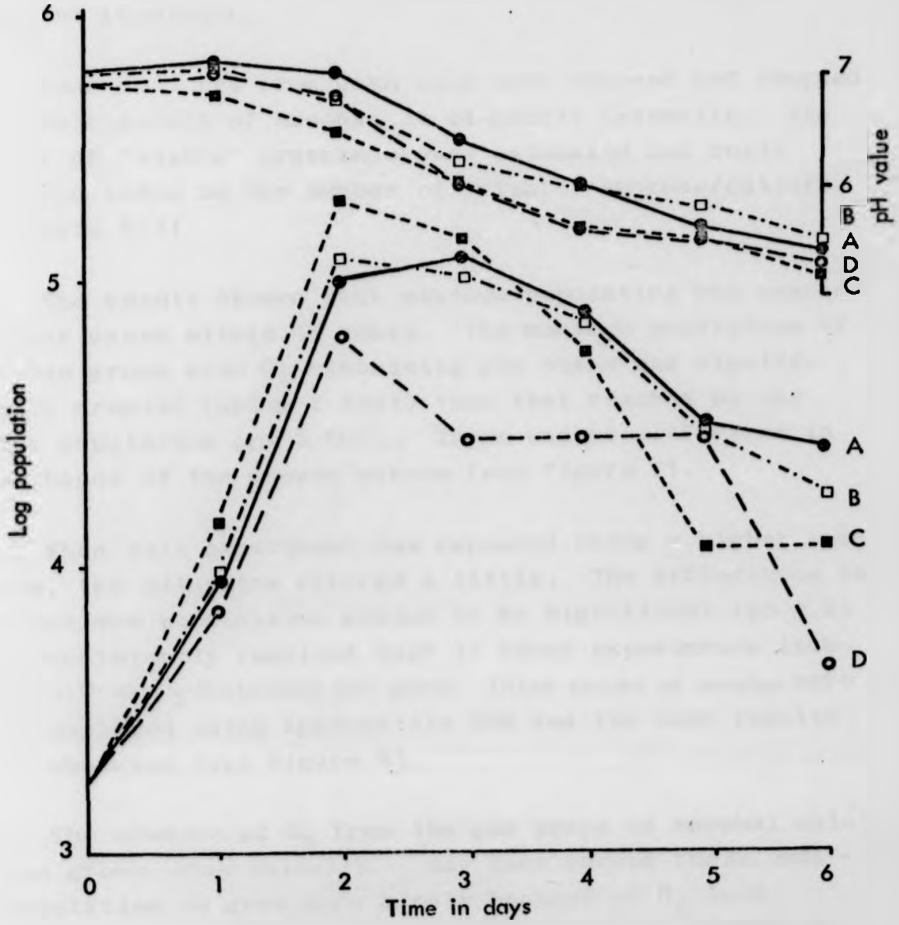
The experiment was performed to see whether the replacement of the gas phase of RSM with 90% Hydrogen and 10% CO_2 would enhance the growth of *E. histolytica* since it is known that *E. histolytica* grows best in an anaerobic environment (Robinson, 1968b). Stock Fenn was used.

Table XII

Changes of pH in relation to growth of *E. histolytica* (stock Fenn) in RSM of varying concentrations

Time in days	Population (x 10^4) per culture % concentration of serum in RSM				pH value of culture medium % concentration of serum in RSM			
	16.6	12.6	10.0	8.3	16.6	12.6	10.0	8.3
0	0.2	0.2	0.2	0.2	7.0	7.0	6.9	6.9
1	1.0	1.0	1.5	0.79	7.1	7.05	6.8	7.0
2	12.0	14.0	23.0	7.6	7.0	6.8	6.5	6.5
3	14.0	12.5	16.6	3.2	6.4	6.2	6.05	6.0
4	8.3	8.3	6.3	3.2	6.0	6.0	5.6	5.6
5	3.5	3.2	1.26	3.2	5.6	5.8	5.5	5.6
6	2.9	2.0	1.3	0.5	5.4	5.5	5.2	5.3

Fig 6
Relationship between pH and growth of an E. histolytica (stock Fenn) population in Rsm



A = (16.6% serum) 33% BRS
B = (12.6% serum) 25% BRS
C = (10% serum) 20% BRS
D = (8.3% serum) 16.6% BRS

Thirty two cultures were set up in bijou bottles using RSM with 10% serum. Each bottle received 0.1 ml inoculum containing 0.2×10^4 amoebae. The cultures were divided into 2 sets. Set a had their caps very loosely screwed on while set b had theirs tightly screwed on. The set a cultures were incubated at 37° C in an atmosphere of 90% H_2 and 10% CO_2 in anaerobic, McIntosh and Filde's jars. The set b cultures were incubated on the racks of the same incubator.

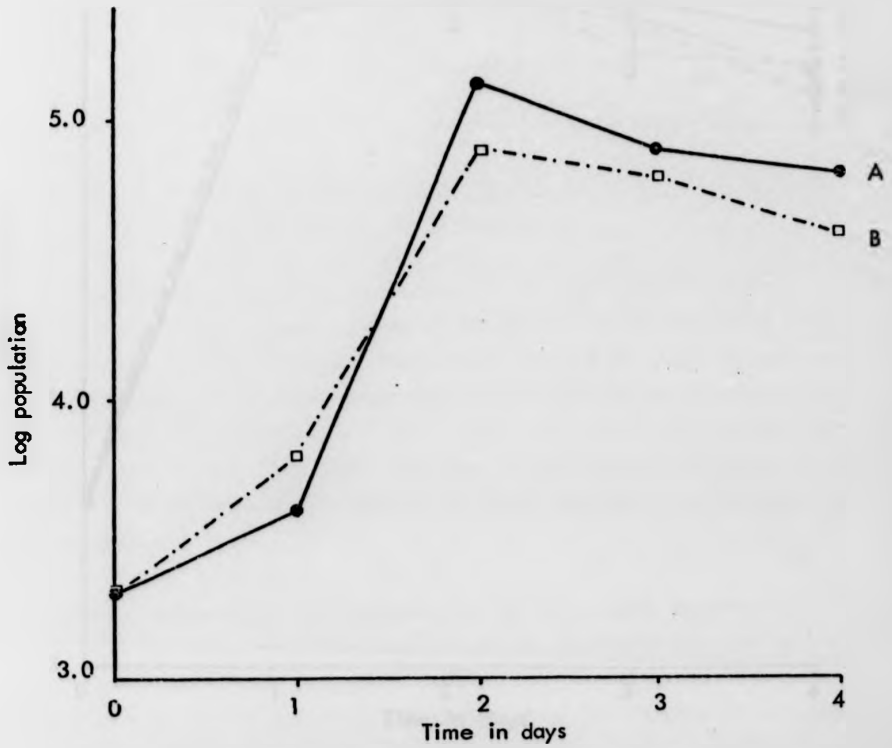
Four cultures from both sets were removed and checked for their growth of amoebae at 24-hourly intervals. The number of 'viable' organisms were estimated and their averages taken as the number of 'viable' amoebae/culture. See Table XIII.

The result showed that maximum population was reached in both cases within 48 hours. The maximum population of amoebae grown with O_2-containing gas phase was significantly greater (using t test) than that reached by the other population ($p < 0.001$). There was no difference in the shapes of the growth curves (see Figure 7).

When this experiment was repeated using a higher inoculum, the situation altered a little. The differences in the maximum populations ceased to be significant ($p > 0.2$) but consistently remained high in those experiments incubated with an O_2-containing gas phase. Other stocks of amoebae were also employed using appropriate RSM and the same results were observed (see Figure 8).

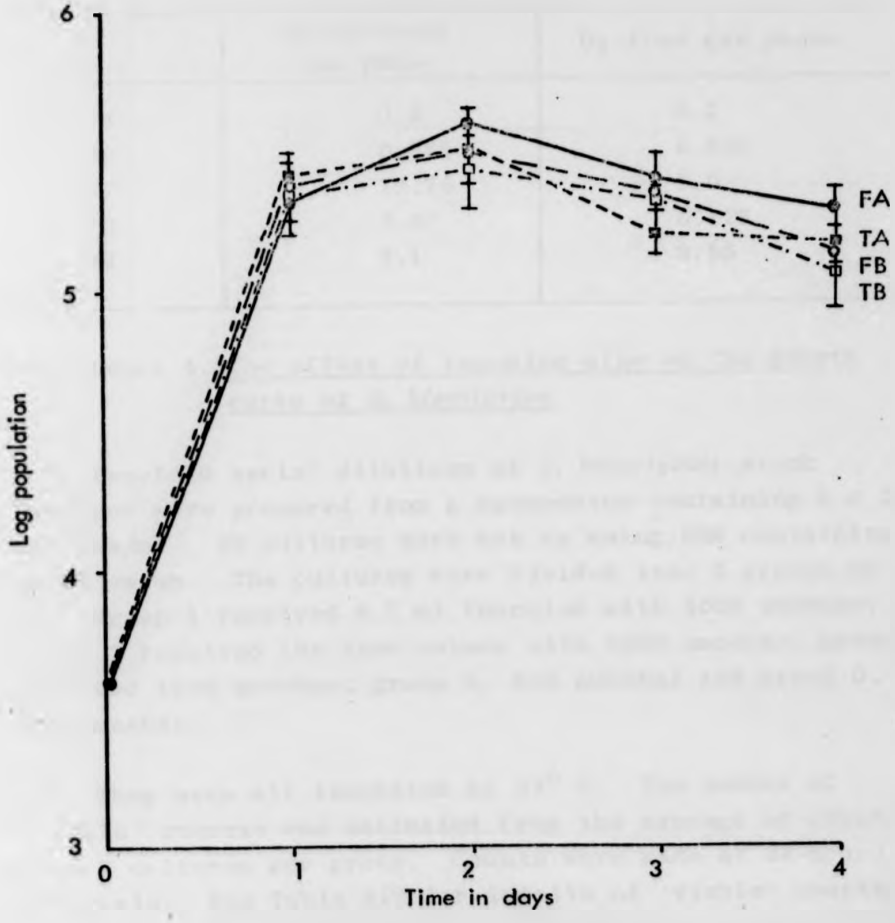
The absence of O_2 from the gas phase of amoebal cultures grown anaerobically may have caused the *Es. coli - B* population to grow more slowly because of O_2 lack (Robinson, 1968b). Bacteria not only serve as source of amoebal nutrients but also provide essential anaerobic conditions for the amoebae. The variations in growth curve with inoculum size were further investigated.

Fig 7
Growth curves of E. histolytica (Stock Fenn) grown in oxygen-containing and oxygen-free gas phases.



A = gas phase - air
B = gas phase - CO_2 and H_2

Fig 8
Growth curves of E. histolytica stocks grown in oxygen-containing and oxygen-free gas phases.



FA = gas phase - air (Stock Fenn)
FB = gas phase - CO_2 and H_2 (Stock Fenn)
TA = gas phase - air (Stock Thesiger)
TB = gas phase - CO_2 and H_2 (Stock Thesiger)

Stock Thesiger was grown in RSM with 16.6% serum

Table XIII

The population of *E. histolytica* (stock Fenn) grown in oxygen-containing and oxygen-free gas phases

Time in hours	Population (x 10^4) of 'viable' amoebae/culture	
	O_2-containing gas phase	O_2-free gas phase
0	0.2	0.2
24	0.375	0.625
48	13.75	7.0
72	7.87	6.625
96	7.1	3.55

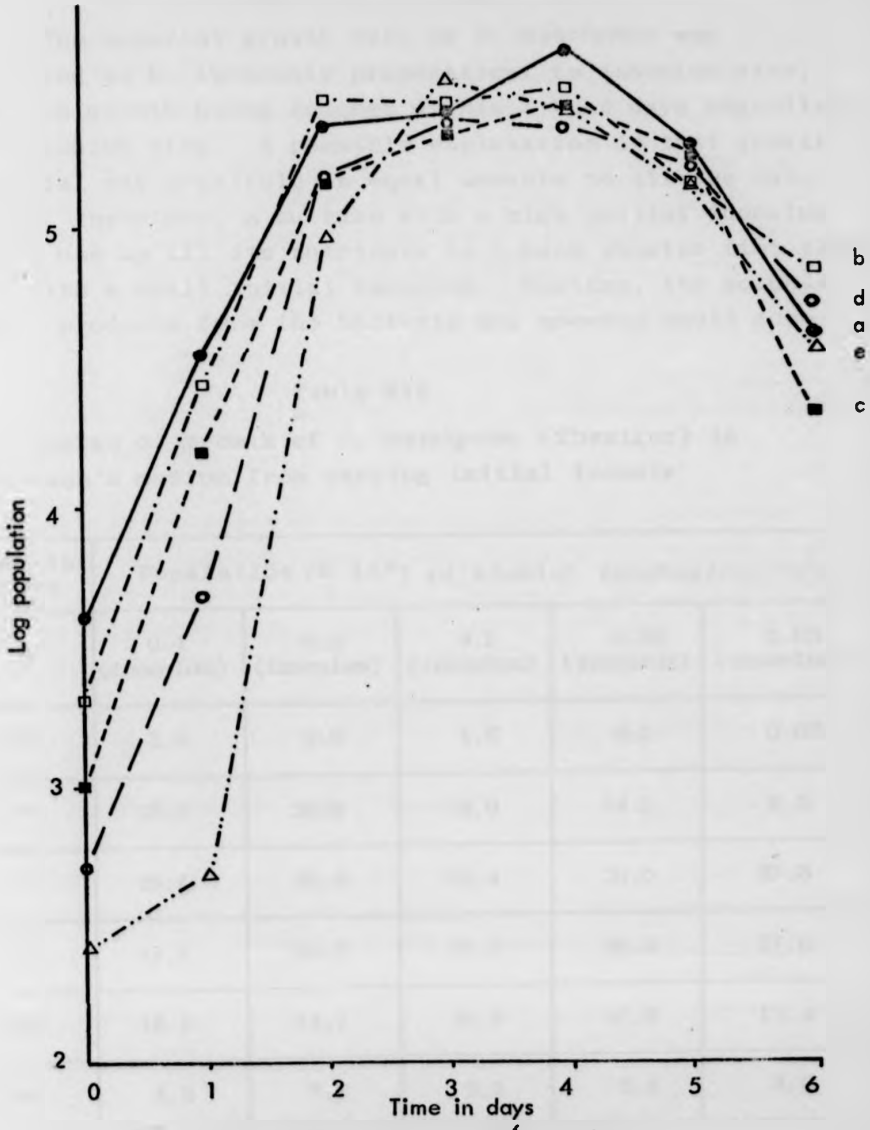
Experiment 4. <u>The effect of inoculum size on the growth curve of *E. histolytica*</u>

Two-fold serial dilutions of *E. histolytica* stock Thesiger were prepared from a suspension containing 8 x 10^4 amoebae/ml. 90 cultures were set up using RSM containing 16.6% serum. The cultures were divided into 5 groups of 18. Group 1 received 0.1 ml inoculum with 4000 amoebae; group 2 received the same volume with 2000 amoebae; group 3 received 1000 amoebae; group 4, 500 amoebae and group 5, 250 amoebae.

They were all incubated at 37° C. The number of viable amoebae was estimated from the average of counts from 3 cultures per group. Counts were made at 24-hourly intervals. See Table XIV for details of 'viable' counts.

It appears from the results that the growth curve of *E. histolytica* is relatively unaffected by inoculum size (see Figure 9) but, as the number of amoebae decreased in the initial inoculum, a slower growth was observed within the first twenty-four hours. It is possible that a lag phase

Fig 9
The growth curves of E. histolytica (Thesiger) in RSM with various inoculum sizes



a = inoculum of 0.4×10^4 amoebae
b = inoculum of 0.2×10^4 amoebae
c = inoculum of 0.1×10^4 amoebae
d = inoculum of 0.05×10^4 amoebae
e = inoculum of 0.025×10^4 amoebae

might even be experienced for 24 hours if the number of amoebae was reduced further to, say, 100.

The apparent growth rate of *E. histolytica* was observed to be inversely proportional to inoculum size, maximum growth being reached within 3 to 4 days regardless of inoculum size. A possible explanation is that growth material was available in equal amounts to all the cultures, therefore, a culture with a high initial inoculum would use up all its nutrients in a much shorter time than one with a small initial inoculum. Besides, the soluble waste products from the bacteria and amoebae would accu-

Table XIV

The course of growth of *E. histolytica* (Thesiger) in Robinson's medium from varying initial inocula

Time in hours	Population ($\times 10^4$) of 'viable' amoebae/culture				
0	0.4 (inoculum)	0.2 (inoculum)	0.1 (inoculum)	0.05 (inoculum)	0.05 (inoculum)
24	3.5	2.8	1.6	0.5	0.05
48	23.4	28.9	16.0	14.8	9.5
72	26.4	28.2	21.4	24.0	33.3
96	47.7	32.2	27.6	23.0	27.6
120	16.6	14.7	19.6	16.6	15.4
144	4.2	7.1	2.2	5.4	3.8

mulate to toxic level much quicker in cultures with a large inoculum. From the point of view of maximizing population it is better to use small inocula.

Experiment 5. The growth curves of *E. histolytica* in Robinson's medium using small inocula

Experiments performed so far had been checked for viable amoebae at 24-hourly intervals. The result of experiment 4 indicated that a lag phase might be detected in the growth curve of *E. histolytica* in Robinson's medium (i) if fewer amoebae were used and (ii) if counts were made more frequently.

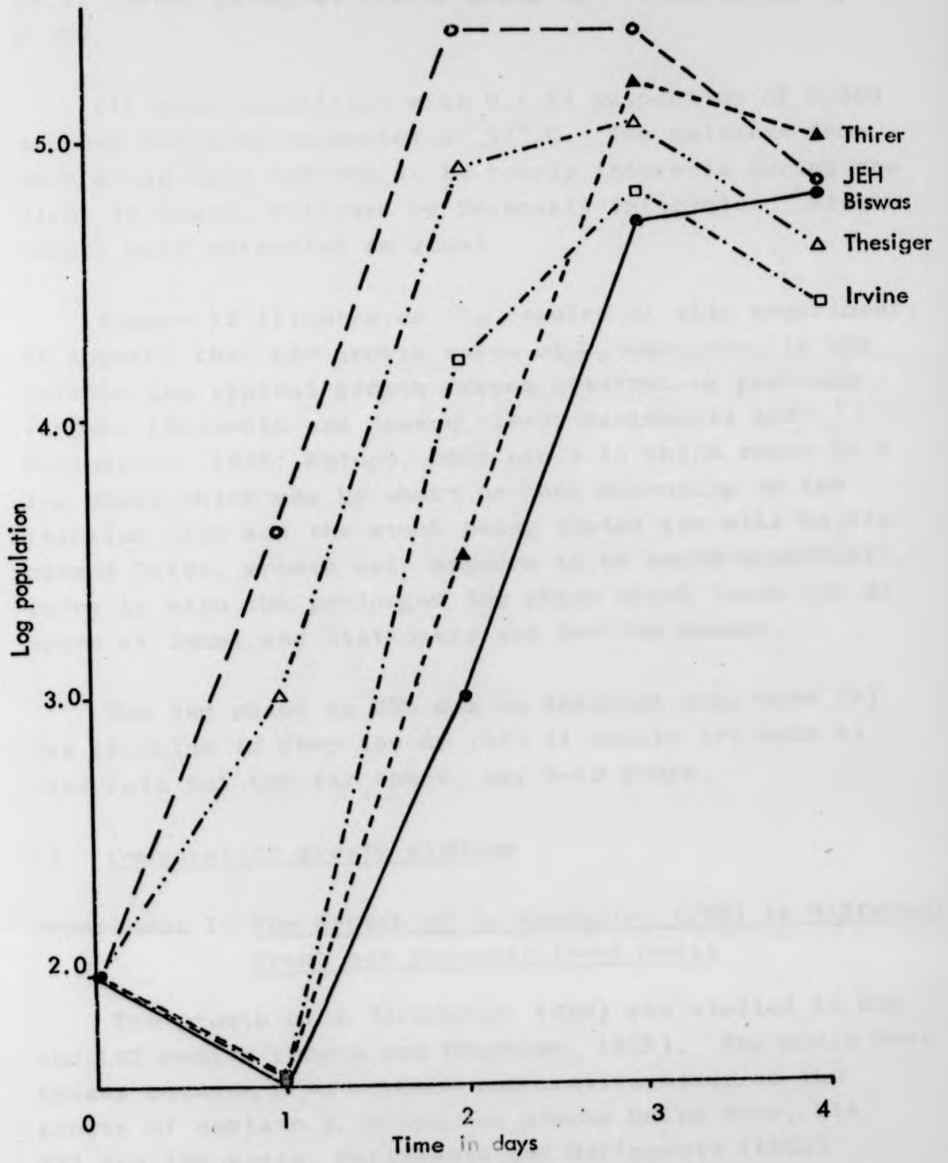
This experiment was set up to see whether a lag phase occurred in RSM.

The first experiment was performed to see whether a decrease in inoculum size brings about a pronounced lag phase. Suspensions of Biswas, Thesiger, JEH, Irvine and Thirer were prepared. Sixty cultures were set up with RSM containing 16.6% serum. They were divided into 5 groups of 12. Each group received 0.1 ml inocula from the same stock. They were incubated at 37^{o} C and population estimates were made at 24-hourly intervals. The average of three culture-counts was taken.

The results partly confirmed that a lag phase certainly exists in the growth curve of *E. histolytica* in RSM, but this was shown by 3 of the 5 stocks tested. See Figure 10. The proportion of organisms in the inoculum which are 'infective' to cultures may also be responsible for the lag phase.

The second experiment was carried out to find out when lag phase occurs and how long it lasts during growth in RSM.

Fig 10
The growth curves of E. histolytica stocks from small inocula in Rsm with 16.6% serum



Again Fenn was used. 56 cultures were set up and divided into 4 groups of 14. Group 1 received RSM with 16.6% serum; group 2, 12.6%; group 3, 10% and group 4, 8.3%.

All were inoculated with 0.1 ml suspension of 2,000 amoebae and were incubated at 37° C. Two cultures from each group were removed at 12-hourly intervals during the first 48 hours, followed by 24-hourly intervals. Viable counts were estimated as usual.

Figure 11 illustrates the results of this experiment. It appears that the growth curve of *E. histolytica* in RSM follows the typical growth curves obtained by previous workers (Balamuth and Howard, 1946; Harinasuta and Harinasuta, 1955; Abioye, 1971; etc.) in which there is a lag phase, which may be short or long depending on the inoculum size and the stock being tested (as will be discussed later, growth rate appears to be stock-dependent), there is also the prolonged log phase which lasts for 24 hours at least, and stationary and decline phases.

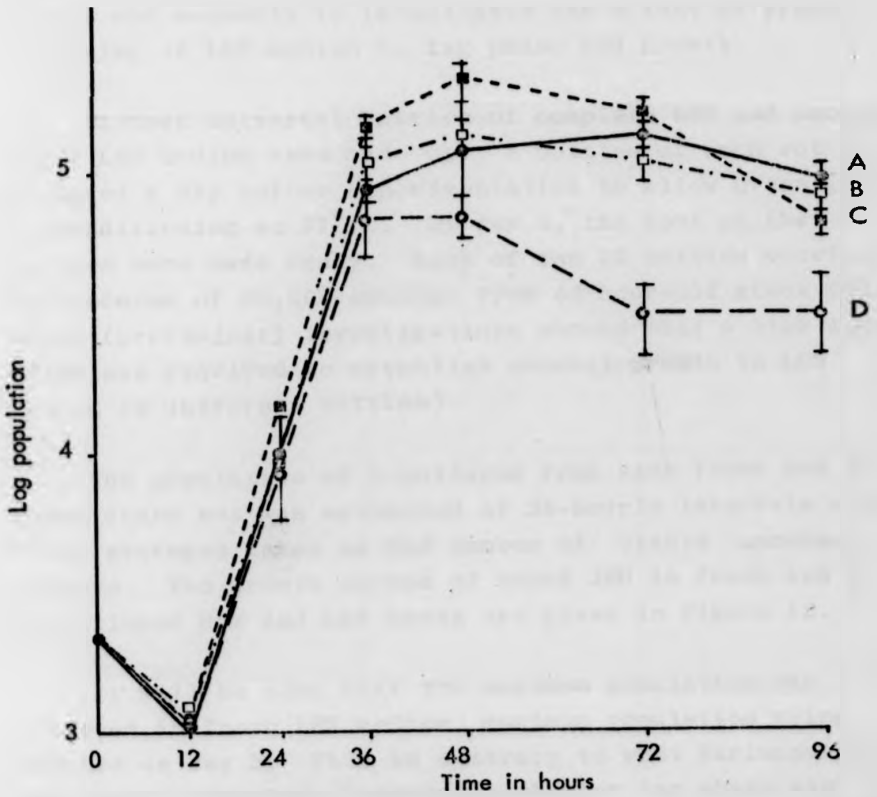
The lag phase in RSM can be detected only when (i) the inoculum is very low or (ii) if counts are made at intervals not too far apart, say 6-12 hours.

C3 Comparative growth studies

Experiment 1. The growth of *E. histolytica* (JEH) in different fresh and preconditioned media

The growth of *E. histolytica* (JEH) was studied in RSM and LES medium (Boeck and Drbohlav, 1925). The media were chosen because, in an extensive comparative study on the growth of certain *E. histolytica* stocks using HSre, LIA, EYI and LES media, Harinasuta and Harinasuta (1955) showed that amoebae grew best in LES medium. They also showed that the lag phase was considerably shortened and

Fig 11
The growth curves of E. histolytica (stock Fenn) in Rsm with varying BRS concentrations



A =(16.6% serum) 33% BRS
B =(12.6% serum) 25% BRS
C =(10.0% serum) 20% BRS
D =(8.3% serum) 16.6% BRS

growth was more prolific when media were preconditioned with bacteria. In another report by Abioye (1971), it was stated that Ibadan strains of *E. histolytica* grew best in Robinson's and LIA media of all the media he employed (these included HSre., LIA, LES and Robinson's media).

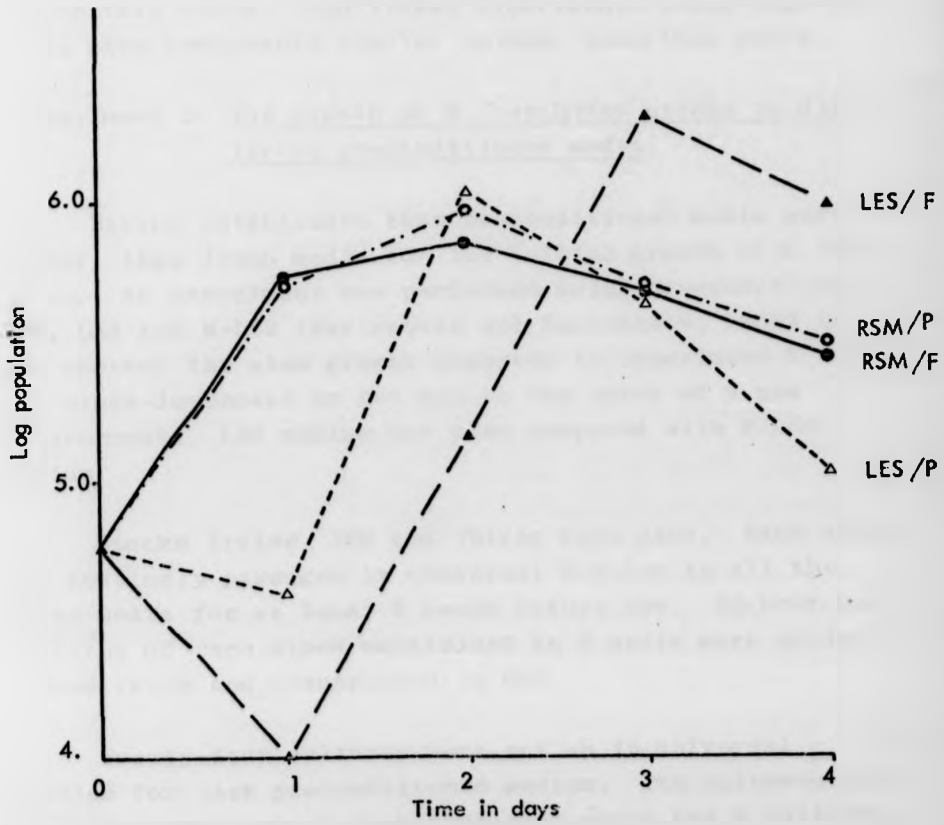
This experiment was designed first to see whether 'preconditioning' of RSM (which already contains bacteria in their stationary phase of growth) improved amoebal growth and secondly to investigate the effect of preconditioning of LES medium on lag phase and growth.

Sixteen universal bottles of complete RSM and another 16 of LES medium were made up. 8 bottles of each set were prepared a day before experimentation to allow overnight preconditioning at 37° C. On day 2, the rest of the bottles were made ready. Each of the 32 bottles received an inoculum of 60,000 amoebae from 48-hour-old stock cultures (preliminary investigations showed that a high inoculum was required to establish amoebal growth in LES medium in universal bottles).

The population of 2 cultures from each fresh and preconditioned set was estimated at 24-hourly intervals and their averages taken as the number of viable amoebae/culture. The growth curves of stock JEH in fresh and preconditioned RSM and LES media are given in Figure 12.

It will be seen that the maximum population was achieved in fresh LES medium, maximum population being reached on day 3. This is contrary to what Harinasuta and Harinasuta reported; however, a shorter lag phase was observed in the preconditioned medium. The prolonged lag phase may be due to the fact that stock JEH found it difficult to establish itself in a new culture environment having been passaged several times in RSM.

Fig 12
The growth curves of E. histolytica (stock JEH) in fresh and preconditioned media



F refers to fresh medium
P refers to preconditioned medium

With RSM, there appeared to be no difference between the growth patterns of amoebae in the fresh and preconditioned media; no lag phase was observed probably because (i) the initial inoculum was rather high and (ii) the fresh medium was already preconditioned.

Experiments performed with lower (2000) inocula made no difference to *E. histolytica* growth in fresh and preconditioned RSM, but it prolonged the lag phase in both fresh and preconditioned LES medium to more than 48 hours in certain cases. Additional experiments using high inocula gave comparable results to those described above.

Experiment 2. The growth of *E. histolytica* stocks in different preconditioned media.

Having established that preconditioned media were better than fresh media for the initial growth of *E. histolytica*, an experiment was performed using preconditioned RSM, LES and M-LES (Harinasuta and Harinasuta, 1955) to see whether the slow growth observed in experiment 1 was (a) stock-dependent or (b) due to the shock of a new environment. LES medium was also compared with M-LES medium.

Stocks Irvine, JEH and Thirer were used. Each stock was routinely passaged in universal bottles in all the three media for at least 3 weeks before use. 48-hour-old cultures of each stock maintained in 3 media were pooled, washed twice and resuspended in PBS.

Twenty-four cultures were set up in universal bottles for each preconditioned medium. The cultures were then regrouped into 3, such that each group had 8 cultures of each medium. Each group received 0.1 ml inoculum of 55,000 amoebae from the same stock. They were all incubated at 37° C and counts were made at 24-hourly intervals as usual.

The population estimates of the amoebic stocks cultured in the 3 media are given in Table XV. It will be seen that the best initial growth occurred in RSM for all the stocks, maximum population being reached within 2 days. In LES and M-LES media, the maximum populations were reached after 2 - 3 days.

No lag phase was detected in RSM for reasons already discussed but lag phase was pronounced in the case of stocks JEH and Thirer in both LES and M-LES media (see Figure 13). The lag phase of stock Irvine was short in LES medium and shorter still in M-LES medium.

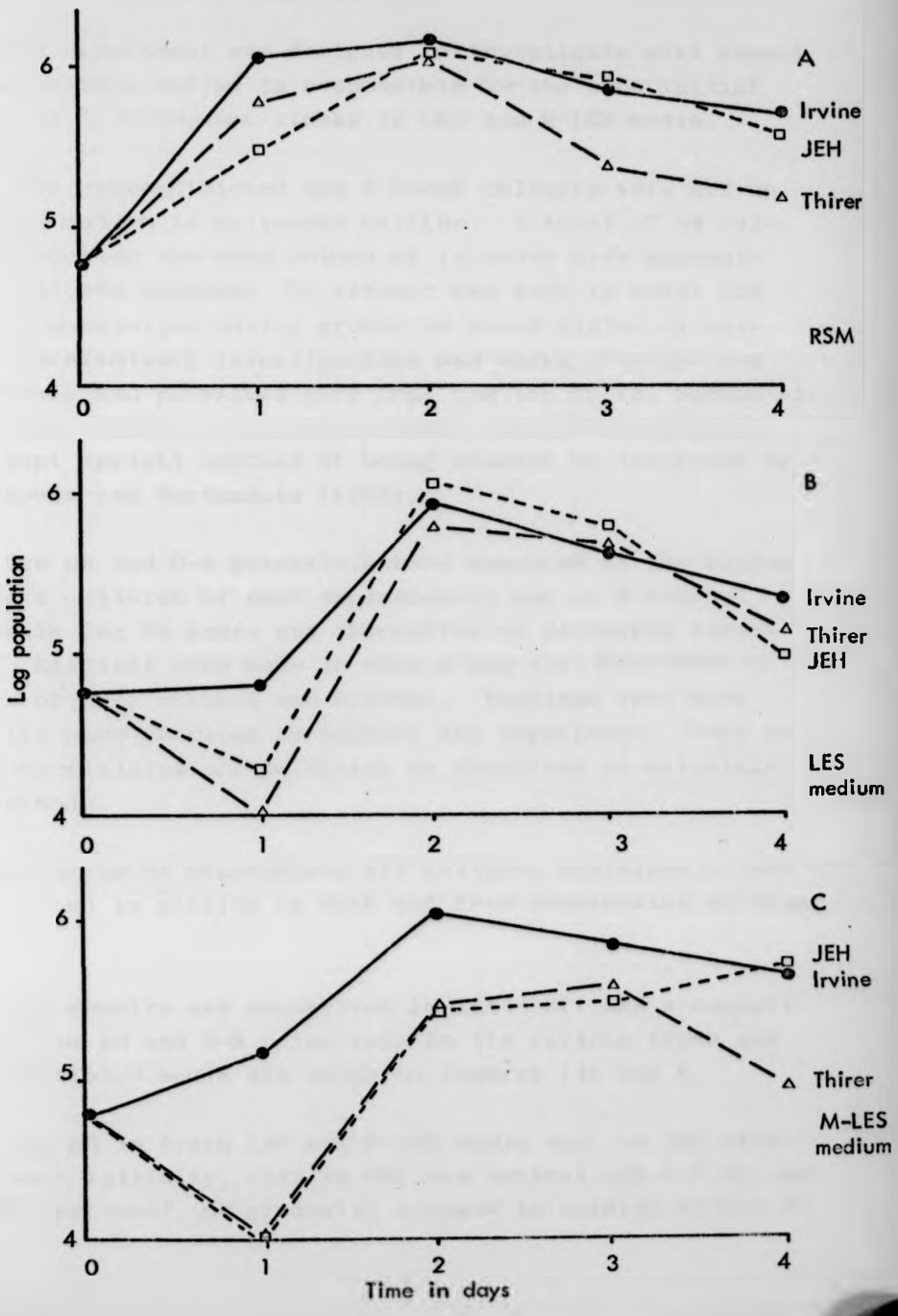
Results confirm that lag phase was not due to shock from a sudden change of environment. Generally, growth was best in RSM, LES medium was better than M-LES in my experience. Growth was more dependent on the culture medium than on the stock of amoeba.

Table XV

The growth of *E. histolytica* stocks in various preconditioned media

Culture medium	Amoebal stock	Population($\times 10^4$) per culture after incubation at 37° C for				
		0 hr	24 hrs	48 hrs	72 hrs	96 hrs
Robinson's (Robinson, 1968a)	Irvine	5.5	113.0	152.0	76.4	54.6
	Thirer	5.5	64.5	110.0	24.6	16.0
	JEH	5.5	30.5	127.0	87.1	40.65
LES (Boeck and Drbohlav, 1925)	Irvine	5.5	5.8	89.7	41.7	23.0
	Thirer	5.5	1.0	63.0	47.3	14.2
	JEH	5.5	2.0	110.0	63.4	47.3
M-LES (Harinasuta and Harinasuta, 1955)	Irvine	5.5	15.3	103.0	70.8	45.0
	Thirer	5.5	1.3	27.0	38.6	9.5
	JEH	5.5	1.0	27.0	30.1	56.65

Fig 13
Growth curves of E. histolytica stocks in various preconditioned media



Experiment 3. Changes in oxidation-reduction potential and pH during amoebic growth in various media

The experiment was designed to investigate what aspect of the culture medium is responsible for the slow initial growth of *E. histolytica* stocks in LES and M-LES media.

Four preconditioned and 4 fresh cultures were set up for each medium in universal bottles. A total of 24 cultures received the same volume of inoculum with approximately 60,000 amoebae. No attempt was made to count the amoebal population during growth to avoid mixing of cultures (preliminary investigations had shown that the O-R potentials and pH values vary from the top to the bottom of a culture bottle). This was another reason why cultures were kept upright instead of being slanted as described by Harinasuta and Harinasuta (1955).

The pH and O-R potentials were measured at the bottom of the 4 cultures of each experimental set at 6-hourly intervals for 24 hours and thereafter at 24-hourly intervals. Readings were made in such a way that disturbance at the bottom of each culture was minimal. Readings were made from the same cultures throughout the experiment. Care was taken to minimize contamination as described in materials and methods.

At the start of experiments all cultures contained *Es. coli - B* as tested by plating on agar and from preparation of Gram stains.

The results are summarized in Table XVI and a comparison of the pH and O-R potentials in the various fresh and preconditioned media are shown in Figures 14a and b.

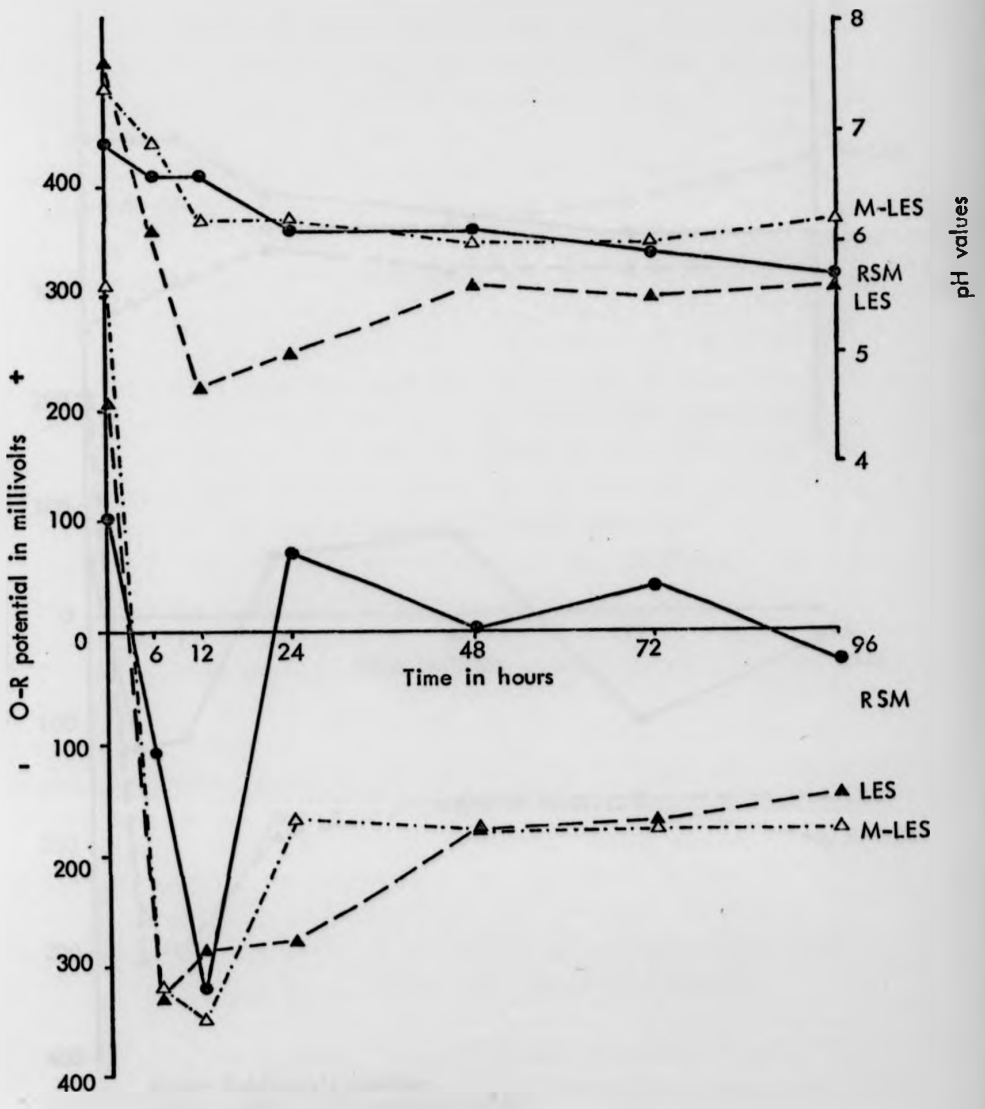
The pH in fresh LES and M-LES media was on the alkaline side initially, that in RSM was neutral (pH = 7.0). As growth continued, pH gradually dropped to acidity within 24

Table XVI

Changes in pH and O-R potentials of *E. histolytica* (stock JEH) cultures growing in fresh and preconditioned media.

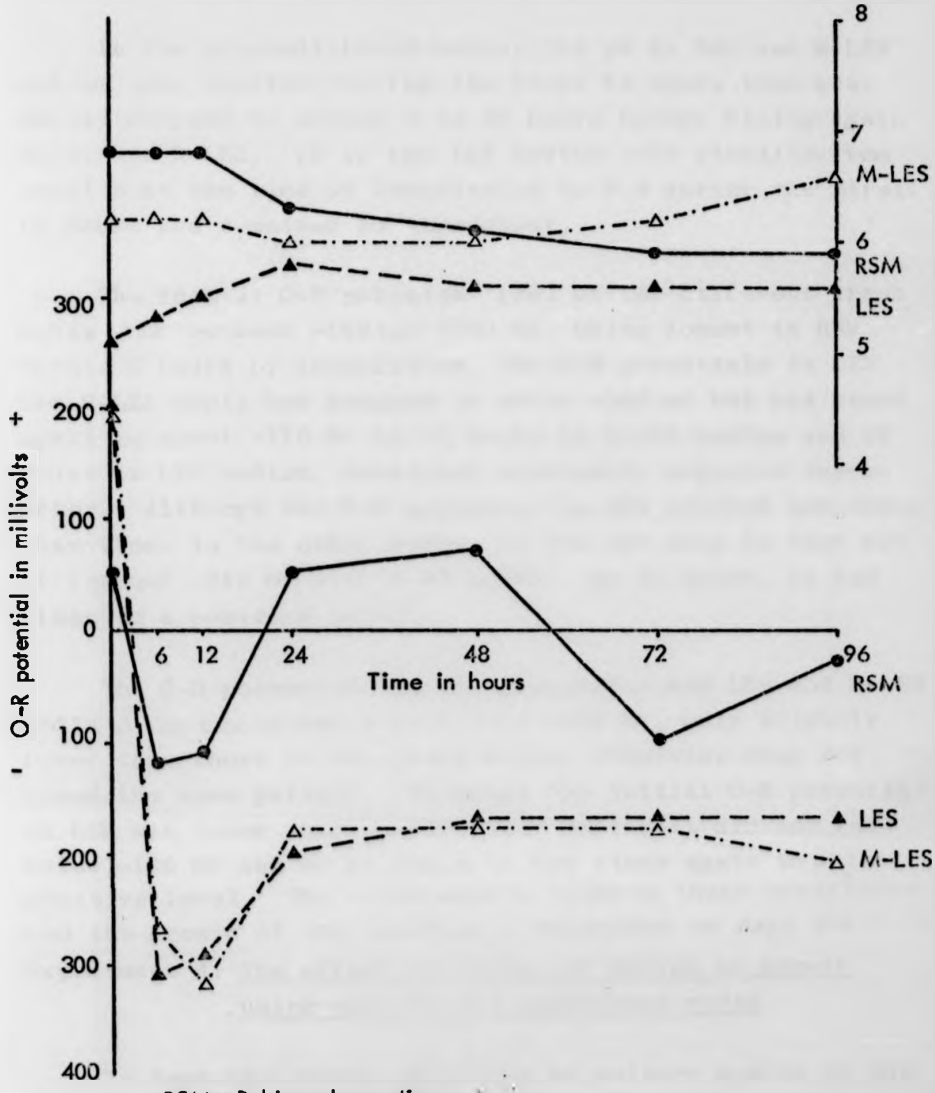
Medium		pH after cultivation for						
		0 hr	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs
RSM	F	6.9	6.6	6.6	6.1	6.1	5.9	5.7
	P	6.8	6.8	6.8	6.3	6.1	5.9	5.9
LES	F	7.6	6.1	4.7	5.0	5.6	5.5	5.6
	P	5.1	5.3	5.5	5.8	5.6	5.6	5.6
M-LES	F	7.4	6.9	6.2	6.2	6.0	6.0	6.2
	P	6.2	6.2	6.2	6.0	6.0	6.2	6.6
		O-R potential in millivolts (corrected)						
RSM	F	(+ 103) -140	(-107) -350	(-317) -560	(+73) -170	(+3) -240	(+43) -200	(-27) (-270)
	P	(+53) -190	(-117) -360	(-107) -350	(+53) -190	(+73) -170	(-97) -340	(-27) (-270)
LES	F	(+ 233) -10	(-327) -570	(-287) -530	(-277) -520	(-177) -420	(-172) -415	(-147) -390
	P	(+193) -50	(-307) -550	(-292) -535	(-197) -440	(-167) -410	(-167) -410	(-167) -410
M-LES	F	(+313) +70	(-317) -560	(-347) -590	(-167) -410	(-177) -420	(-177) -420	(-177) -420
	P	(+203) -40	(-257) -500	(-317) -560	(-177) -420	(-177) -420	(-177) -420	(-207) -450

Fig 14a
Changes in pH and O-R potential of different fresh culture media during amoebic growth



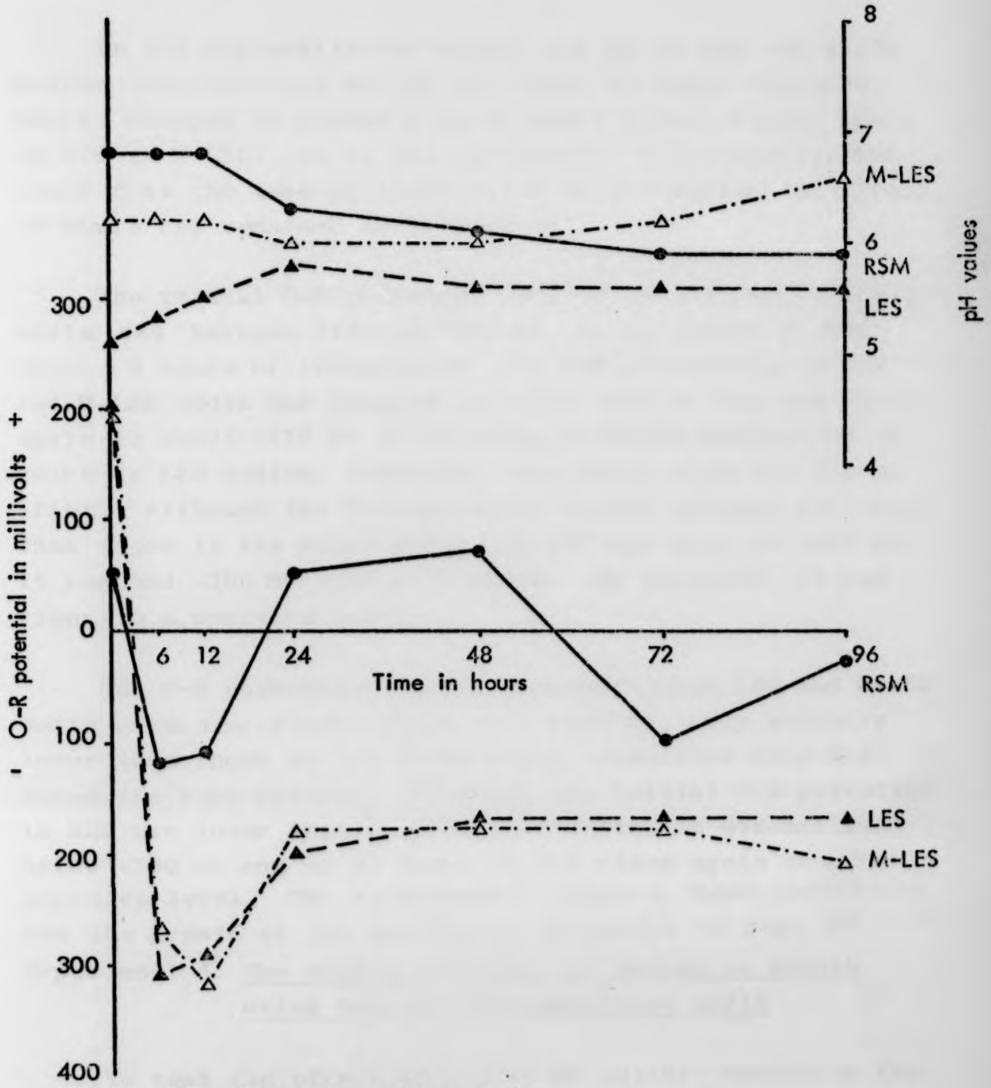
RSM = Robinson's medium
LES = Locke - egg-serum medium
M-LES = modified LES medium

Fig 14b
Changes in pH and O-R potential of different preconditioned culture media during amoebic growth.



RSM= Robinson's medium
LES = Locke - egg-serum medium
M-LES = modified LES medium

Fig 14b
Changes in pH and O-R potential of different preconditioned culture media during amoebic growth.



RSM= Robinson's medium
LES = Locke - egg-serum medium
M-LES = modified LES medium

hours in M-LES and RSM and remained so throughout. However, the pH in LES medium dropped very rapidly to below 5 within 12 hours, but gradually rose again to about 5.5 by the 48th hour, remaining so to the end. The sudden drop in pH in LES medium could be attributed to its inefficient buffer system.

In the preconditioned media, the pH in RSM and M-LES medium was constant during the first 12 hours then gradually dropped to around 6 in 48 hours before rising again to 6.6 in M-LES. pH in the LES medium rose steadily from about 5 at the time of inoculation to 5.8 during the first 12 hours and remained so throughout.

The initial O-R potential (Eh) of the different fresh media was between +100 and +300 mv, being lowest in RSM. Within 6 hours of inoculation, the O-R potentials in LES and M-LES media had dropped to below -300 mv but had risen again to about -170 mv in 24 hours in M-LES medium and 48 hours in LES medium, remaining constantly negative thereafter. Although the O-R potential in RSM started off lower than those in the other media, it did not drop as fast but it reached -300 mv within 12 hours. By 24 hours, it had risen to a positive level.

The O-R potentials in the preconditioned LES and M-LES media at the time of inoculation were +200 mv, only slightly lower than those in the fresh media, otherwise they followed the same pattern. Although the initial O-R potential in RSM was lower than in the other media, it did not fall below -130 mv and by 24 hours it had risen again to a low positive level. The relationship between these conditions and the growth of the amoebae is discussed on page 231.

Experiment 4. <u>The effect of volume of medium on growth using various preconditioned media</u>

To test the effect of volume of culture medium on the growth of *E. histolytica*, preconditioned media were set up in 12 universal and 12 bijou bottles for each type of culture

medium. Each culture bottle received 55,000 *E. histolytica* (JEH) trophozoites per 0.1 ml inoculum. They were incubated at 37° C. Viable counts were made of 3 cultures per group at 24-hourly intervals and their averages recorded. Table XVII summarizes the results of the counts.

It will be seen that using an inoculum of 55,000, the maximum population was reached after 48-hours' incubation in RSM regardless of culture volume. In LES and M-LES media, maximum populations were recorded after 48-hours incubation in the smaller volumes and after 72 hours in the larger volumes.

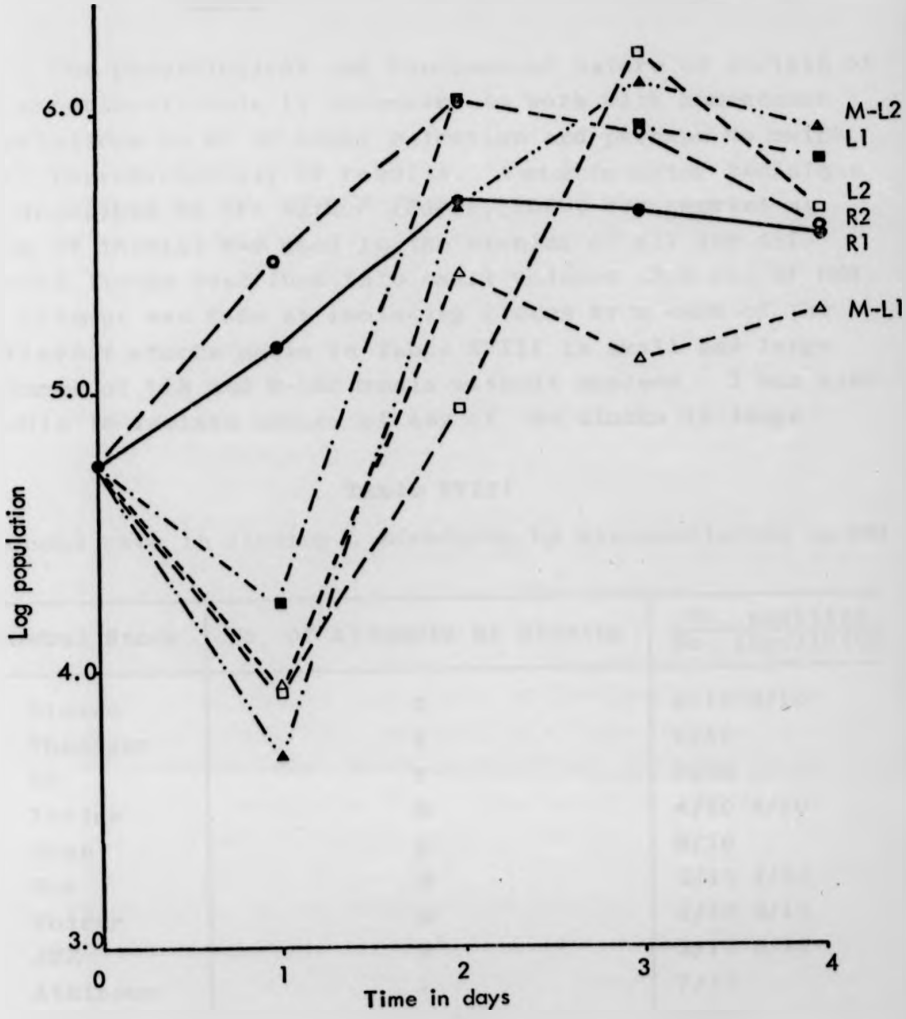
No lag phase was observed in the growth of cultures in large and small volumes of RSM, whereas lag phases were observed in the growth curves of cultures in other media, the lag phase being shorter in the smaller volumes in both cases. See Figure 15. Volume of culture appears to have an effect on the growth of *E. histolytica in vitro*. With an inoculum of 55,000 in RSM, the larger the culture volume,

Table XVII

The growth of *E. histolytica* (JEH) in different volumes of various culture media

Culture medium	Culture volume (ml.)	Population of amoeba (x 10^4) after incubation at 37°C for				
		0 hr	24 hrs	48 hrs	72 hrs	96 hrs
RSM	3	5.5	14.5	49.0	45.6	38.63
	10	5.5	30.5	127.0	87.0	40.65
LES	3	5.5	1.78	127.0	92.2	70.36
	10	5.5	0.9	9.0	170.4	47.3
M-LES	3	5.5	0.9	27.0	13.1	20.05
	10	5.5	0.5	49.0	132.5	93.0

Fig 15
Effect of different volumes of various pre-conditioned culture media on the growth curves of E. histolytica (JEH)



R = RSM
L = LES
M-L = M-LES

1 = 3.0 ml culture volume
2 = 10.0 ml culture volume

the faster the growth rate and the bigger the yield of amoebal population. In the other media with smaller volumes, the initial growth is faster but the maximum yield is less.

Experiment 5. Cloning of *E. histolytica* stocks in various media

The physiological and biochemical nature of certain of my experiments made it necessary to work with homogenous populations so as to avoid selection and perhaps to maintain reproducibility of results. A microisolation technique as described by the author (Farri, 1978, see reprint at back of thesis) was used in the cloning of all the different stocks described into small volumes (3.0 ml) of RSM. An attempt was made at isolating clones from each of the different stocks shown in Table XVIII in small and large volumes of LES and M-LES media without success. I was also unable to isolate clones of any of the stocks in large

Table XVIII

Success rate in cloning *E. histolytica* by microisolation in RSM

Amoebal Stock	No. of attempts at cloning	No. positive / No. inoculated
Biswas	2	0/10 2/10
Thesiger	1	2/10
SN	1	7/10
Irvine	2	4/10 8/10
Bean	1	8/10
Fox	2	2/10 4/10
Thirer	2	0/10 2/10
JEH	2	2/10 6/10
Atkinson	1	7/10
Ali	1	1/10
Saigon	3	0/10 0/10 3/10

volumes of RSM. At each attempt 10 clonal isolations were attempted. Experiments in cloning into large volumes were not repeated because the RSM in small volumes worked very well.

Usually, amoebae were detectable in cultures derived from one individual within 3 to 4 days depending on the stock, except on one occasion when amoebae were detected 5 days after isolation.

It was observed that large, starch-containing trophozoites from 24-hour-old cultures were better for cloning than those derived from 48-hour-old cultures. On no occasion was I able to isolate clones from 72-hour-old cultures.

C4 Assay Method

Experiment 1. Testing the reproducibility of infectivity titrations

Forty-eight-hour-old *E. histolytica* (stock JEH) was harvested and suspended in fresh RSM. 0.5 ml of this suspension was distributed into five Kahn tubes after thorough mixing by repeated pipetting. Using a 0.2% trypan-blue in 1 volume medium R to 2 volumes PBS, and a haemocytometer, four separate estimates were made of numbers of amoebae excluding the stain for each of the five groups. Infectivity titrations were then carried out for each group of suspensions and estimates were computed from the tables of Lumsden *et al* (1963). (Tables IIIa,b and c).

The results on the reproducibility of infectivity titration as an assay for viability of *E. histolytica* are summarized in Table XIXa. The dye-exclusion estimates of numbers of amoebae in the suspensions were concomitant, varying over 0.09 on the log scale, about 1.23-fold arithmetically. The estimates of amoeba number by dye-exclusion were consistently higher than the estimates using ID_{63}. The

statistical sign-test (Armitage, 1971) showed that counts of dye-excluding amoebae obtained were significantly different from the estimates using ID_{63} at the 3% level ($P = 0.03$). Using the ID_{63} method, the two most widely separated estimates (B + D) were not significantly different: $t = 0.86$ and $0.317 > P > 0.05$.

If one considers the five replicates, the average difference (excluding correction for pipette error) between dye-exclusion and infectivity results was 0.612 on the log scale, indicating that only one out of four trypan-blue-excluding amoebae was infective to cultures. When the results from various experiments were compared, it was shown that the number of trypan-blue excluding amoebae greatly exceeded the number of amoebae calculated from the ID_{63}. However, ID_{63} estimates of number of viable amoebae varied in different 48-hour-old cultures from 15.8 - 95.5% and was much less than 15.8% with 72-hour-old cultures (See Table XIXb).

Experiment 2. Using infectivity titration to measure the effect of age of culture on the viability of *E. histolytica*

Twelve cultures were set up with RSM in universal bottles as usual. Each culture received 400 amoebae in 0.1 ml inocula. After 48-hours incubation, 4 cultures were removed and the number of trypan-blue-excluding amoebae estimated from the mixed suspension. Four separate counts were made and their average taken. The procedure was repeated after 72 and 96-hours incubation.

The suspensions were serially diluted and infectivity titrations made. The results of the estimates are summarized in Table XX.

Table XIXa

Estimation of the numbers of amoebae in, and of the 'infectivity' of five samples of *E. histolytica* suspensions

Sample	Log no. of trypan-blue-excluding amoebae/ml & SE	Infectivity			Percentage Viable
		Log dilution	No. infected / No. inoculated	log ID_{63}/ml and SE	
A	5.40 ± 0.027	-2	6/6	4.8 ± 0.5	25.0
		-3	6/6		
		-4	2/6		
		-5	1/6		
		-6	0/6		
B	5.46 ± 0.05	-2	6/6	5.1 ± 0.5	43.7
		-3	6/6		
		-4	5/6		
		-5	0/6		
		-6	0/6		
C	5.37 ± 0.05	-2	6/6	4.9 ± 0.5	31.8
		-3	6/6		
		-4	3/6		
		-5	1/6		
		-6	0/6		
D	5.44 ± 0.057	-2	6/6	4.6 ± 0.3	16.2
		-3	6/6		
		-4	2/6		
		-5	0/6		
		-6	0/6		
E	5.39 ± 0.027	-2	6/6	4.6 ± 0.3	16.2
		-3	6/6		
		-4	2/6		
		-5	0/6		
		-6	0/6		

Table XIXb

Comparison of the numbers of *E. histolytica* populations (by trypan-blue-exclusion) and their "infectivity" to Robinson's medium

Samples	log no. of trypan-blue-excluding amoebae/ml	log ID_{63}/ml and SE	% viability
1	5.35	5.0 ± 0.3	44.67
2	5.40	5.1 ± 0.5	50.12
3*	5.80	4.8 ± 0.3	10.0
4	5.30	5.0 ± 0.3	50.12
5*	5.34	3.6 ± 0.3	1.8
6*	4.84	3.8 ± 0.3	9.1
7*	5.50	4.6 ± 0.3	12.5
8	6.10	5.6 ± 0.3	31.6
9	6.10	5.8 ± 0.3	50.12
10	6.40	6.1 ± 0.5	50.12
11	6.06	6.0 ± 0.3	87.10
12	6.02	6.0 ± 0.3	95.50
13	6.37	5.9 ± 0.5	33.88
14	5.86	5.4 ± 0.5	34.67
15	6.11	5.4 ± 0.5	19.9
16	6.2	5.4 ± 0.5	15.8
17*	6.0	5.0 ± 0.3	10.0
18*	6.0	4.8 ± 0.3	6.3
19	6.2	6.0 ± 0.3	63.1
20*	6.0	5.0 ± 0.3	10.0

* Amoebae were derived from 72-hour-old cultures.

Table XX

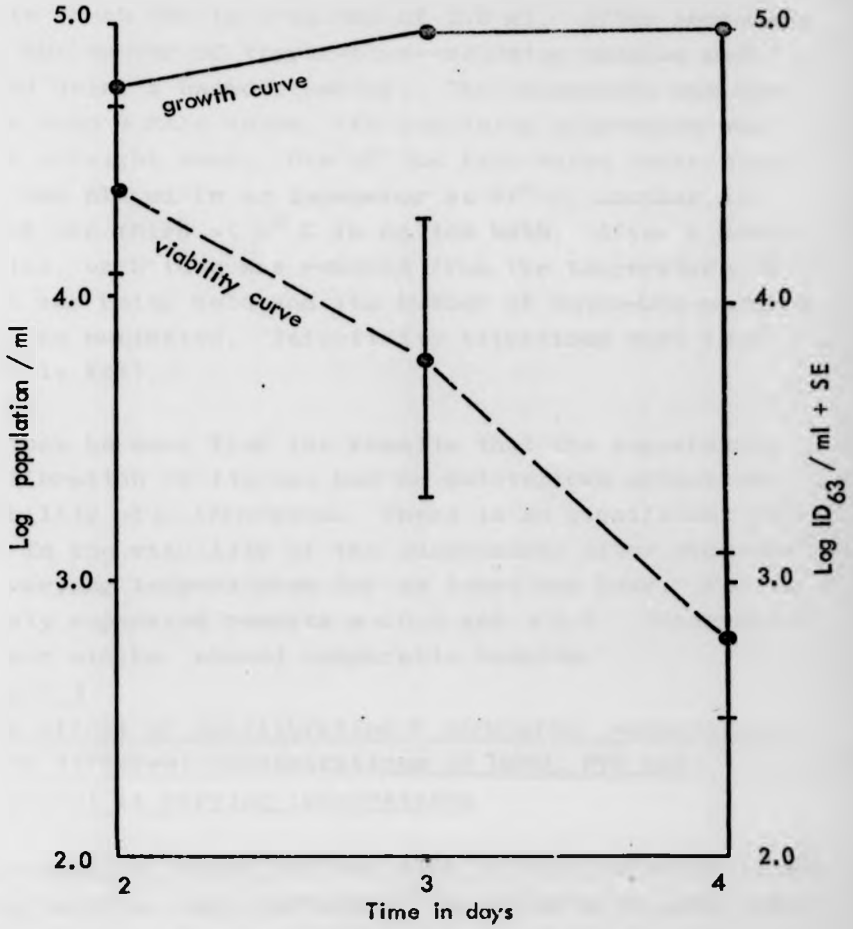
The 'infectivity' of *E. histolytica* (stock Thesiger) suspensions to Robinson's medium at different times during cultivation

Age of culture (hrs)	Log no. of trypan-blue excluding amoebae/ml ± SE	Infectivity			% viability
		log dilution	No. infected / No. inoculated	Log ID_{63}/ml and SE	
48	4.78 ± 0.05	-2	6/6	4.4 ± 0.3	41.17
		-3	5/6		
		-4	2/6		
		-5	0/6		
		-6	0/6		
72	4.98 ± 0.02	-2	6/6	3.8 ± 0.5	8.32
		-3	2/6		
		-4	1/6		
		-5	0/6		
		-6	0/6		
96	5.0 ± 0.05	-1	6/6	2.8 ± 0.3	0.63
		-2	3/6		
		-3	0/6		
		-4	0/6		
		-5	0/6		

The results show that although more trypan-blue-excluding amoebae were counted at 96 hours, the majority of them were not infective. However, 48-hour-old cultures showed higher infectivity and therefore contained more viable organisms. See Figure 16.

Experiment 3. Measurement of the effect of temperature on the infectivity of *E. histolytica* suspensions

Fig 16
The relationship between age of culture and "infectivity" of E. histolytica (stock Thesiger) in Robinson's medium



In cryopreservation, equilibration with the cryoprotectants at varying times and temperatures has been used. In this experiment, the effect of holding at various temperatures in the absence of cryoprotectant was investigated.

Forty-eight-hour-old cultures of *E. histolytica* (stock Thesiger) in RSM were harvested, washed in PBS and resuspended in fresh RSM to a volume of 2.0 ml. After thorough mixing, the number of trypan-blue-excluding amoebae was estimated using a haemocytometer. The suspension was distributed into 3 Kahn tubes, the remaining suspension was titrated straight away. One of the Kahn tubes containing amoebae was placed in an incubator at 37° C, another at 25° C and the third at 0° C in an ice bath. After 1 hour's incubation, each tube was removed from the temperature at which it was being held and its number of trypan-blue-excluding amoebae was estimated. Infectivity titrations were then made (Table XXI).

It can be seen from the results that the temperature of equilibration on its own has no deleterious effect on the viability of *E. histolytica*. There is no significant difference in the viability of the suspensions after exposure to the varying temperatures for at least one hour. For the two widely separated results $p < 0.5$ and > 0.3. Experiments with other stocks showed comparable results.

Experiment 4

The effect of equilibrating *E. histolytica* suspensions with different concentrations of DMSO, PVP and glycerol at varying temperatures

E. histolytica stock JEH was used in this experiment. 48-hour-old cultures were harvested, washed with PBS and resuspended in freshly prepared RSM and kept on the bench.

5.0 ml quantities of the following concentrations of cryoprotectants were prepared using PBS:

10, 15 and 20% (v/v) glycerol

Table XXI

The "infectivity" of *E. histolytica* suspensions to Robinson's medium after incubation at different temperatures for 1 hr

Temperature of incubation	Log no. of trypan-blue excluding amoebae/ml	Infectivity		
		log dilution	No. infected / No. inoculated	Log ID_{63}/ml and SE
0° C	5.09	-3	6/6	4.6 ± 0.3
		-4	2/6	
		-5	0/6	
25° C	5.15	-3	5/6	4.3 ± 0.3
		-4	1/6	
		-5	0/6	
37° C	5.14	-3	6/6	4.4 ± 0.5
		-4	1/6	
		-5	0/6	
Control	5.20	-3	5/6	4.3 ± 0.3
		-4	1/6	
		-5	0/6	

10, 15 and 20% (v/v) DMSO

10, 20 and 30% (w/v) PVP

Nine separate experiments were performed altogether as outlined below:

Expt. No.	Final concentrations of cryoprotectant	Equilibration temperatures	Exposure time(min)
1	5, 7.5 & 10% DMSO	0°C	<2,15 & 30
2	5, 7.5 & 10% DMSO	25°C	<2,15 & 30
3	5, 7.5 & 10% DMSO	37°C	<2,15 & 30
4	5, 7.5 & 10% Glycerol	0°C	<2,15 & 30
5	5, 7.5 & 10% Glycerol	25°C	<2,15 & 30

6	5, 7.5 & 10% Glycerol	37°C	<2,15 & 30
7	5, 10 & 15% PVP	0°C	<2,15 & 30
8	5, 10 & 15% PVP	25°C	<2,15 & 30
9	5, 10 & 15% PVP	37°C	<2,15 & 30

For each experiment, 4 groups of 3 Kahn tubes were set up, each group receiving 0.25 ml of a different concentration of the cryoprotectant being tested; the fourth group (the control group) received 0.25 ml fresh RSM. All tubes received in addition 0.25 ml of the prepared amoebal suspension at different times, the contents of each tube were mixed and were equilibrated at the desired temperature for the required length of time. For 0° C equilibration, tubes were placed in an ice bath; for 25° and 37° C equilibration, tubes were placed in incubators set at the relevant temperature.

After the required time interval, each tube was taken from the equilibrating temperature, its contents were thoroughly mixed and infectivity titrations and inoculations of cultures made. Cultures were immediately incubated at 37° C. The timing of equilibration was so arranged that there was only a 1-minute interval between the end of one set of titration/inoculation and the beginning of the next. Each complete experiment lasted between 45 and 50 minutes.

Tables XXII, XXIII and XXIV summarize the results of infectivity titrations after the various treatments. Figures 17, 18 and 19 illustrate the viability results after various treatments.

It can be concluded from these results that the susceptibility of *E. histolytica* suspensions to the damaging effects of cryoprotectants is dependent not only on the concentration of cryoprotectants but also on the temperature and time of equilibration.

Table XXII

The viability of *E. histolytica* (JEH) after treatment with different concentrations of DMSO at various temperatures and times

Experiment Number	Equilibration temperature	% final DMSO concentration	Equilibration time (minutes)	Log ID[63] ml & SE
1	0° C	5.0	<2	5.0 ± 0.3
			15	5.1 ± 0.3
			30	5.0 ± 0.3
		7.5	<2	5.0 ± 0.3
			15	5.0 ± 0.3
			30	5.1 ± 0.5
		10.0	<2	5.0 ± 0.5
			15	5.0 ± 0.5
			30	5.0 ± 0.5
		Control	<2	5.0 ± 0.3
			15	5.1 ± 0.3
			30	5.1 ± 0.3
2	25° C	5.0	<2	4.0 ± 0.3
			15	3.6 ± 0.3
			30	3.1 ± 0.5
		7.5	<2	4.0 ± 0.3
			15	3.1 ± 0.5
			30	3.1 ± 0.3
		10.0	<2	4.0 ± 0.5
			15	3.4 ± 0.3
			30	3.4 ± 0.3
		Control	<2	4.0 ± 0.3
			15	4.0 ± 0.3
			30	4.1 ± 0.3
3	37° C	5.0	<2	4.4 ± 0.3
			15	4.1 ± 0.3
			30	3.5 ± 0.5
		7.5	<2	4.0 ± 0.5
			15	3.5 ± 0.5
			30	3.0 ± 0.3
		10.0	<2	2.5 ± 0.7
			15	2.1 ± 0.5
			30	1.6 ± 0.3
		Control	<2	4.5 ± 0.5
			15	4.4 ± 0.3
			30	4.4 ± 0.5

Fig 17
The viability of E. histolytica (JEH) after treatment with different concentrations of DMSO at various equilibration temperatures and times

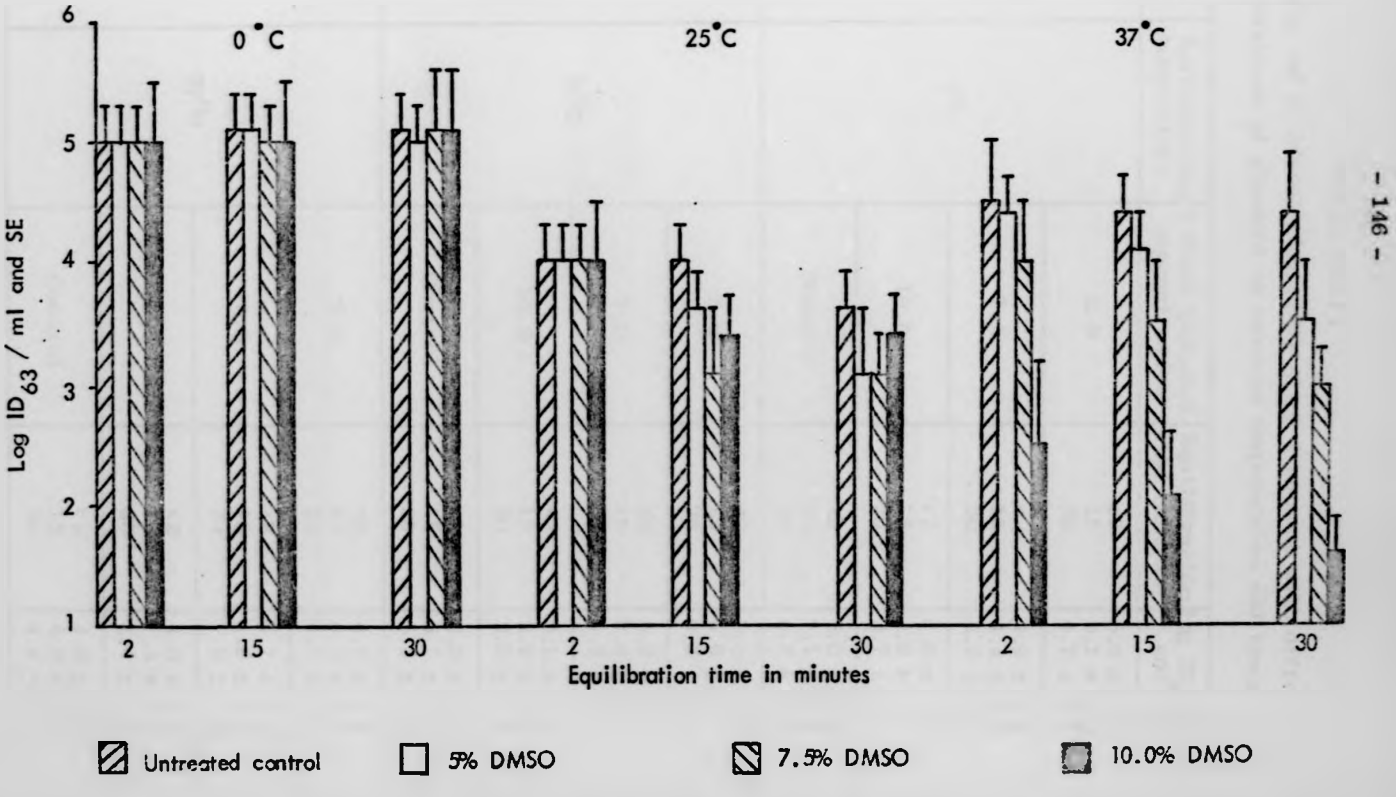


Table XXIII

The viability of *E. histolytica* (JEH) after treatment with different concentrations of glycerol at various temperatures and times

Experiment number	Equilibration temperature	% final glycerol concentration	Equilibration time (minutes)	log ID_{63}/ml and SE
4	0°C	5.0	<2	5.0 ± 0.3
			15	5.1 ± 0.3
			30	4.7 ± 0.3
		7.5	<2	4.9 ± 0.4
			15	4.4 ± 0.3
			30	4.2 ± 0.5
		10.0	<2	4.8 ± 0.3
			15	4.3 ± 0.3
			30	3.7 ± 0.3
		Control	<2	5.0 ± 0.5
			15	5.1 ± 0.3
			30	5.1 ± 0.3
5	25°C	5.0	<2	5.3 ± 0.4
			15	4.6 ± 0.4
			30	4.6 ± 0.5
		7.5	<2	5.2 ± 0.3
			15	3.6 ± 0.3
			30	3.4 ± 0.3
		10.0	<2	5.1 ± 0.3
			15	2.9 ± 0.3
			30	2.3 ± 0.3
		Control	<2	5.3 ± 0.5
			15	5.1 ± 0.3
			30	5.3 ± 0.3
6	37°C	5.0	<2	4.9 ± 0.4
			15	4.2 ± 0.4
			30	4.1 ± 0.5
		7.5	<2	4.7 ± 0.3
			15	1.9 ± 0.5
			30	1.9 ± 0.5
		10.0	<2	4.6 ± 0.3
			15	1.4 ± 0.3
			30	1.4 ± 0.5
		Control	<2	4.8 ± 0.3
			15	4.8 ± 0.3
			30	4.8 ± 0.5

Fig 18
The viability of E. histolytica (JEH) after treatment with different concentrations of glycerol at various equilibration temperatures and times

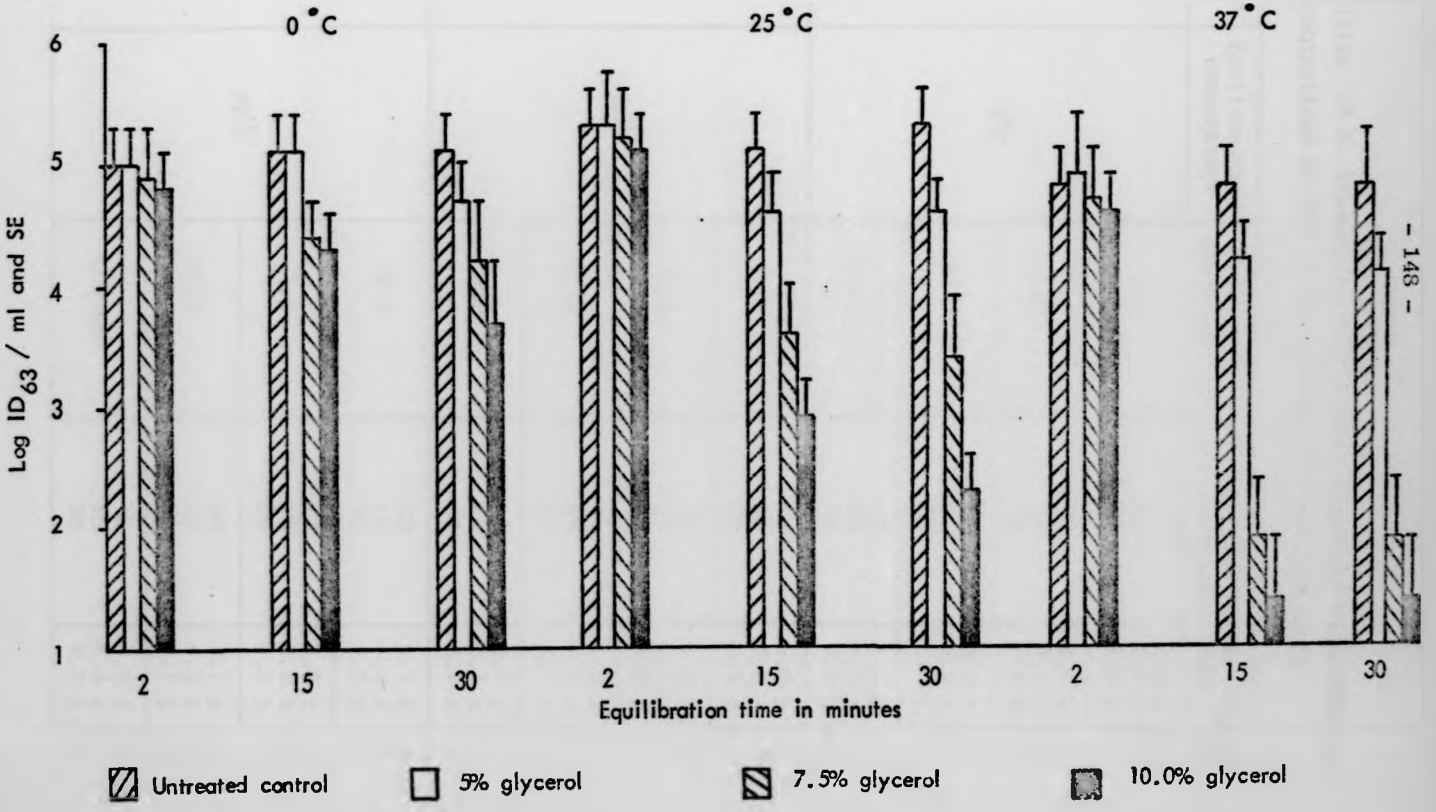
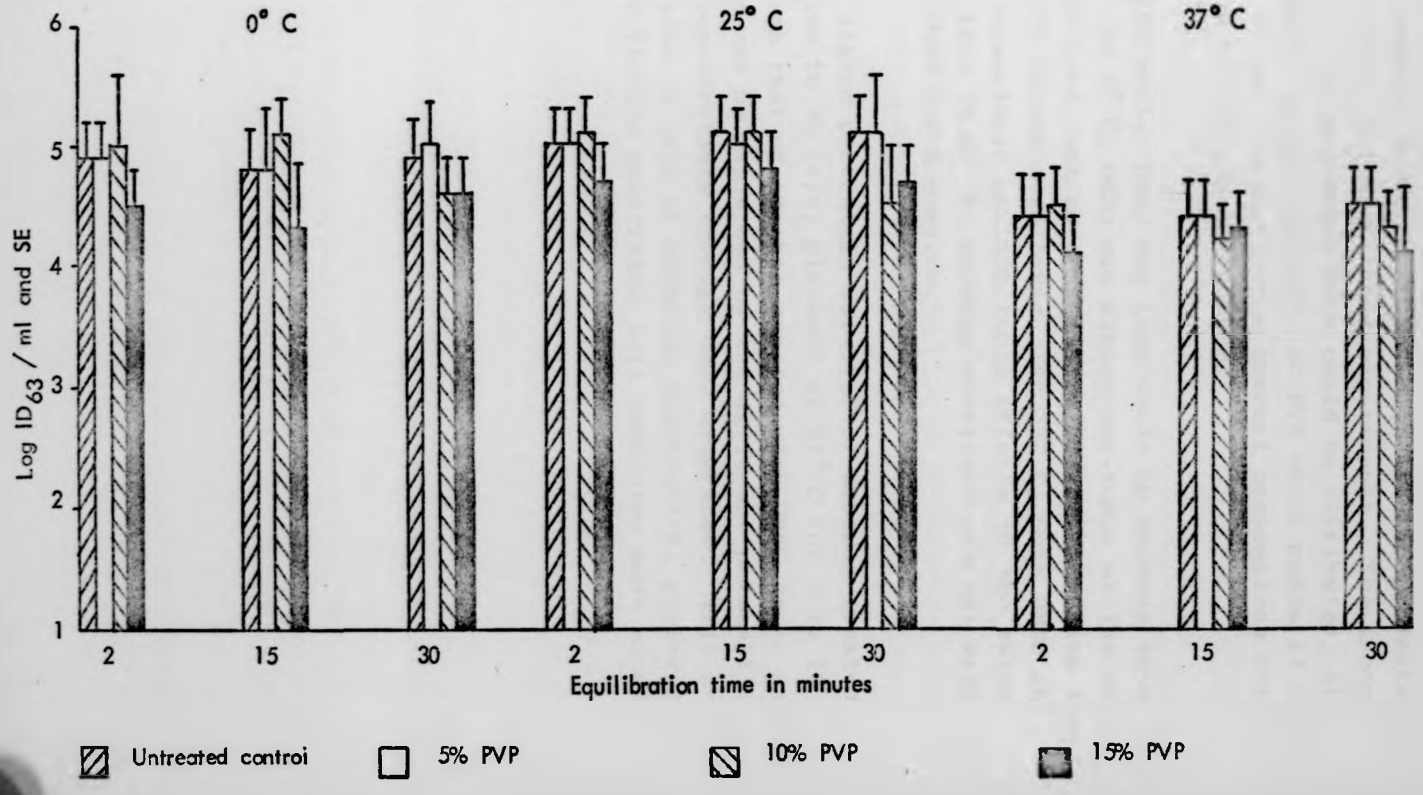


Table XXIV

The viability of *E. histolytica* (JEH) after treatment with different concentrations of PVP at various temperatures and times

Experiment number	Equilibration temperature	% final PVP concentration	Equilibration time (minutes)	log ID_{63}/ml and SE
7	0°C	5.0	<2	4.8 ± 0.3
			15	4.8 ± 0.5
			30	5.0 ± 0.3
		7.5	<2	4.9 ± 0.6
			15	5.1 ± 0.3
			30	4.6 ± 0.3
		10.0	<2	4.5 ± 0.3
			15	4.3 ± 0.5
			30	4.6 ± 0.5
		Control	<2	4.8 ± 0.3
			15	4.8 ± 0.3
			30	4.9 ± 0.3
8	25°C	5.0	<2	5.0 ± 0.3
			15	5.0 ± 0.3
			30	5.1 ± 0.5
		7.5	<2	5.1 ± 0.3
			15	5.1 ± 0.3
			30	4.5 ± 0.5
		10.0	<2	4.7 ± 0.3
			15	4.8 ± 0.3
			30	4.7 ± 0.3
		Control	<2	5.0 ± 0.3
			15	5.1 ± 0.3
			30	5.1 ± 0.3
9	37°C	5.0	<2	4.4 ± 0.3
			15	4.4 ± 0.3
			30	4.5 ± 0.3
		7.5	<2	4.5 ± 0.6
			15	4.2 ± 0.3
			30	4.3 ± 0.3
		10.0	<2	4.1 ± 0.3
			15	4.3 ± 0.3
			30	4.1 ± 0.6
		Control	<2	4.4 ± 0.3
			15	4.4 ± 0.3
			30	4.5 ± 0.3

Fig 19
The viability of E. histolytica (JEH) after treatment with different concentrations of PVP at various equilibration temperatures and times



PVP appears to be least toxic of all the protectants used within the limits of these experiments. The inconsistency of ID_{63} estimates here could be attributed, at least in part, to the viscosity of PVP which makes it very difficult to mix thoroughly the amoebal suspensions before inoculation and during titrations.

On the whole, DMSO was less toxic to amoebae than glycerol. At 0^{o} C, DMSO was almost non-toxic at the concentrations used, but, as temperature of equilibration increased, DMSO became more toxic. At 37^{o} C, both DMSO and glycerol exerted their maximum toxic effects to the extent that less than 1% of the amoebae survived treatment with 10% (v/v) DMSO for 2 minutes.

The higher percentage survival of amoebae treated with 7.5 and 10.0% (v/v) glycerol at 37^{o} C for 5 to 15 minutes than that of amoebae treated with DMSO under similar conditions can be explained by the different permeabilities of the cryoprotectants through cell membranes. This probably relates to rate of membrane penetration; glycerol being more viscous penetrates cell membranes more slowly.

C5 Cryopreservation

The effect of equilibration time and temperature on the viability of *E. histolytica* after freezing in different concentrations of DMSO, glycerol and PVP

Preliminary experiment - With the results of experiment C4.4 in mind, this experiment was set up to see whether the least harmful of the cryoprotectant treatments would give the best freezing protection. The equilibration conditions giving the highest survival were chosen for each of the three cryoprotectants studied.

E. histolytica (JEH) suspension was prepared from stock cultures as usual and resuspended in RSM to a total volume of 3.0 ml. The suspension contained 1.5×10^6 trypan-blue-excluding amoebae per ml.

Double concentrations of DMSO, PVP and glycerol were prepared with RSM. Nine ampoules were set up and divided into 3 groups of 3. Each ampoule received 0.25 ml amoebal suspension and an equal volume of cryoprotectant. Ampoules of the same group received the same cryoprotectant but each varied in its concentration. A control ampoule received 0.25 ml amoebal suspension plus an equal volume of RSM.

Immediately the ampoules were filled, they were flame-sealed and kept on ice at 0° C. After 15 minutes equilibration with glycerol and 30 minutes with DMSO and PVP, they were removed from ice and cooled at 1° C/min to -60° C and immediately transferred into liquid nitrogen for 1 hour. Each ampoule was individually thawed in a water bath at 37° C and its content inoculated into two 3.0 ml RSM (pre-warmed). Cultures were incubated at 37° C and were checked for amoebal growth daily.

All the cultures inoculated with thawed DMSO-treated amoebal suspensions showed amoebae after 48-hours' incubation.

PVP and glycerol-treated amoebae did not survive freezing, neither did the untreated control. The negative cultures were discarded after 7 days.

The results show that the least harmful of the cryoprotectants did not necessarily give the best freezing protection.

Experiment 1

A suspension of *E. histolytica* (JEH) containing 2.5 x 10^6 trypan-blue-excluding amoebae/ml was prepared in fresh RSM. Double-strength concentrations of DMSO, PVP and glycerol were prepared using RSM. 27 ampoules were set up altogether, they were divided into 9 groups of 3 ampoules as outlined below:

group A1 received 0.25 ml 10% (v/v) DMSO
group A2 received 0.25 ml 15% (v/v) DMSO
group A3 received 0.25 ml 20% (v/v) DMSO
group B1 received 0.25 ml 10% (v/v) glycerol
group B2 received 0.25 ml 15% (v/v) glycerol
group B3 received 0.25 ml 20% (v/v) glycerol
group C1 received 0.25 ml 10% (w/v) PVP
group C2 received 0.25 ml 20% (w/v) PVP
group C3 received 0.25 ml 30% (w/v) PVP

A control group received 0.25 ml RSM. Each of the thirty ampoules received 0.25 ml of the amoebal suspensions at varying times such that for each set there was an equilibration period of 2, 15 and 30 minutes.

All the ampoules were flame-sealed and were cooled from their ambient temperatures at 1° C/min to -60° C, they were then transferred into liquid nitrogen for at least one hour before thawing them one at a time at 37° C and inoculating RSM as before.

Three separate experiments were performed using the method described except that in experiment 1, the equilibration

temperature was 0° C, in experiment 2, it was 25° C and in experiment 3 it was 37° C. Each separate experiment lasted between 50 and 60 minutes.

The results are summarized in Table XXV. It can be seen that DMSO is by far the best cryoprotectant used under these experimental conditions. DMSO-treated suspensions gave positive cultures within 2 days, it took at least 4 days before PVP or glycerol-treated suspensions became positive in cultures.

Positive results were obtained with PVP treatment only when suspensions were equilibrated in 15% (w/v) PVP at 37° C for 30 minutes (provided that the number of trypan-blue-excluding amoebae did not fall below 2×10^6/ml).

The results with glycerol were variable depending on the equilibration period and the concentration of amoebal suspension. Amoebae grew from suspensions which had been treated with 5% (v/v) glycerol at 25° C for 15 minutes at least before freezing but better results were obtained when equilibration time was extended to 30 minutes.

Experiment 2

The last experiment showed that DMSO, PVP and glycerol could protect *E. histolytica* from freezing damage. It was desirable to know the percentage of amoebae protected under the various conditions; to do this, infectivity titrations had to be made and ID_{63} estimated for thawed suspensions.

The procedure outlined in the last experiment was adopted but only the conditions which had given positive culture results were studied. Cultures of stock JEH were used, freezing was carried out in exactly the same way. Infectivity titrations of untreated, unfrozen samples were made. Ampoules containing thawed suspensions were aseptically scored and opened. Infectivity titrations were made

Table XXV

Viabilities of *E. histolytica* suspensions treated with various cryoprotectants at 0°, 25° and 37° C, after freezing and thawing

Equilibration temperature	Equilibration period (min)	Culture results (viability) after freezing and thawing									control
		Final % cryoprotectant concentration									
		DMSO			glycerol			PVP			
		5	7.5	10	5	7.5	10	5	10	15	
0°C	2	+	+	+	-	-	-	-	-	-	-
	15	+	+	+	-	-	-	-	-	-	-
	30	+	+	+	-	-	-	-	-	-	-
25°C	2	+	+	+	-	-	-	-	-	-	-
	15	+	+	+	+	-	-	-	-	-	-
	30	+	+	+	+	-	-	-	-	-	-
37°C	2	+	+	+	-	-	-	-	-	-	-
	15	+	+	+	-	-	-	-	-	-	-
	30	+	+	+	-	-	-	-	-	+	-

and the remainder of each thawed suspension was inoculated into cultures. 0.1 ml of the serially diluted samples were inoculated into cultures as described earlier. The ID_{63} estimates were obtained using Lumsden's tables. Table XXVI summarizes the results of infectivity estimates of DMSO-treated amoebal suspensions.

Table XXVI

Infectivity estimates of frozen DMSO-treated amoebal suspensions

Equilibration temperature	Equilibration period (min)	Infectivity: Log ID_{63}/ml + SE (% survival)			Viability of untreated frozen control
		Final DMSO concentrations			
		5%	7.5%	10%	
0° C	2	*Not measurable (0)	1.6 ± 0.5 (0.005)	*Not measurable (0)	Nil
	15	1.4 ± 0.5 (0.003)	2.1 ± 0.5 (0.015)	2.4 ± 0.5 (0.03)	Nil
	30	1.6 ± 0.5 (0.005)	3.1 ± 0.3 (0.16)	2.4 ± 0.5 (0.03)	Nil
	untreated unfrozen control	5.9 ± 0.5 (assumed to be 100)			—
25° C	2	2.4 ± 0.5 (0.01)	2.6 ± 0.3 (0.015)	2.4 ± 0.5 (0.01)	Nil
	15	2.6 ± 0.5 (0.015)	3.4 ± 0.5 (0.1)	2.4 ± 0.5 (0.01)	Nil

Table XXVI cont.

	30	3.0 ± 0.3 (0.04)	3.4 ± 0.5 (0.1)	2.6 ± 0.3 (0.015)	Nil
	untreated unfrozen control	5.4 ± 0.3 (assumed to be 100)			—
37° C	2	2.4 ± 0.5 (0.4)	2.4 ± 0.5 (0.4)	2.1 ± 0.5 (0.2)	Nil
	15	2.4 ± 0.5 (0.4)	3.8 ± 0.3 (10.0)	3.8 ± 0.3 (10.0)	Nil
	30	2.4 ± 0.5 (0.4)	3.6 ± 0.3 (6.3)	2.4 ± 0.5 (0.4)	Nil
	untreated unfrozen control	4.8 ± 0.3 (assumed to be 100)			—

* direct inoculations of thawed samples gave positive culture results within 2 days
percentage viability given in brackets

The results with PVP and glycerol showed that the numbers of amoebae which survived freezing were so small that infectivity was lost at the lowest dilutions. Cultures inoculated with undiluted samples were found to contain amoebae on day 4.

It can be seen from Figure 20 that, under the various experimental conditions, DMSO, at 7.5% (v/v) concentration, offered *E. histolytica* the best protection. The highest survival was achieved when amoebae were equilibrated at 37° C for 15 minutes.

There was no appreciable difference between the results obtained from suspensions equilibrated at 0° and 25° C for 15 and 30 minutes, but at 37° C, especially with 10% (v/v) DMSO, viability was lower after 30 minutes' equilibration.

It was concluded that 7.5 and 10% (v/v) DMSO protected *E. histolytica* best from freezing damage when equilibrated at 37° C for 15 minutes. Under these experimental conditions about 10% of the amoebae survived.

Experiment 3

The effects of DMSO concentrations, equilibration time and temperature on survival after freezing were tested using stock JEH suspended in RSM.

As in the previous experiments, viability was measured after freezing-down suspensions to -60° C at 1° C/min, then direct into liquid nitrogen followed by 2-hours' storage. A thawing temperature of 37° C was used in all the experiments.

The results of 15 separate experiments performed using the different combinations of treatments and changing variables are summarized in Table XXVII. In some of the experiments the temperature of equilibration was constant while the concentration of DMSO and equilibration time

varied. In others, the concentration of DMSO was constant while the equilibration temperature and time varied. Percentage survival was measured as a percentage of untreated, unfrozen control.

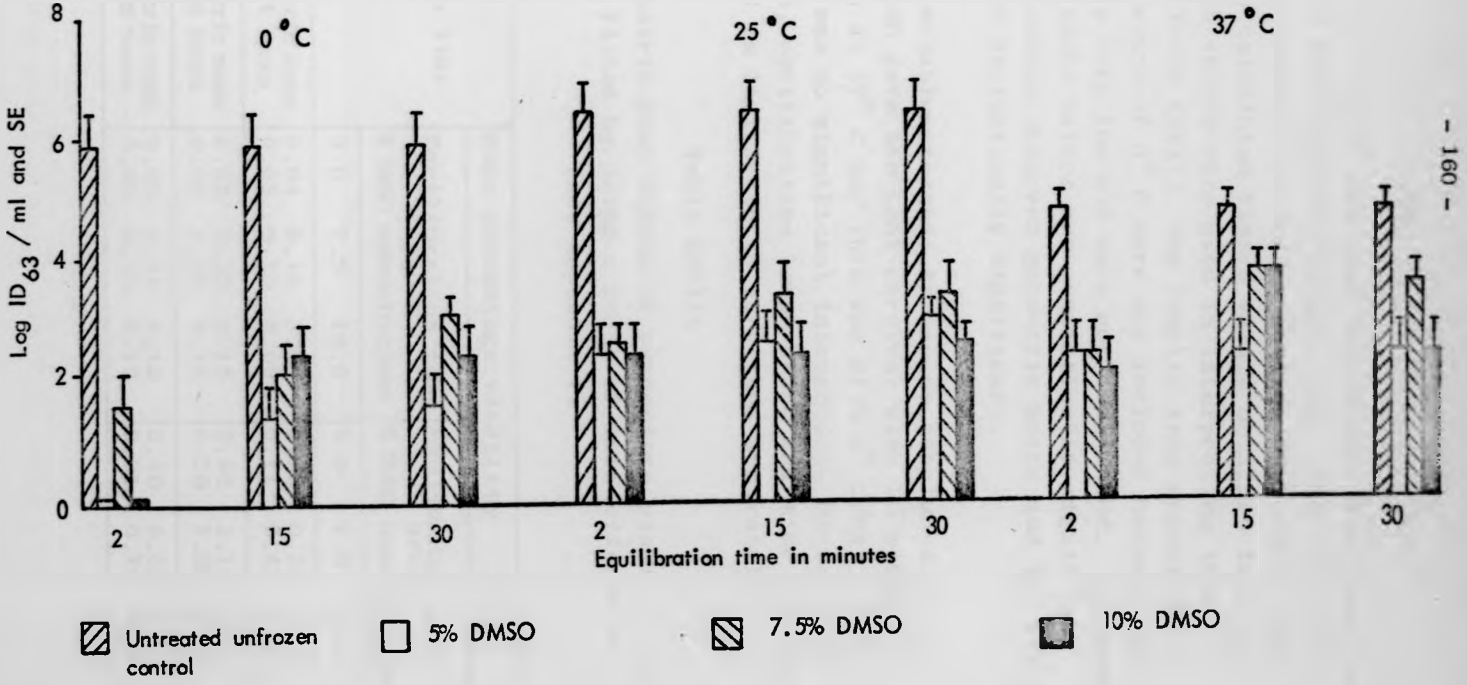
Table XXVII

The percentage survival of *E. histolytica* variably treated in the presence of DMSO after freezing and thawing

Equilibration time	Percentage survival after treatment with								
	5% (v/v) DMSO for			7.5% (v/v) DMSO for			10% (v/v) DMSO for		
	2 min	15 min	30 min	2 min	15 min	30 min	2 min	15 min	30 min
0°C	0	0.003	0.005	0.005	0.018	0.158	0	0.03	0.03
25° C	0.03	0.28	0.06	0.28	0.28	0.63	0.05	0.3	0.63
	0.01	0.015	0.04	0.015	0.1	0.01	0.01	0.01	0.015
	0.04	0.025	0.04	0.1	0.4	0.6	0.05	0.3	0.2
	0.16	0.01	0.06	0.25	1.0	0.16	0.2	0.6	0.3
						1.0			
37° C	0.06	2.5	2.5	0.25	12.5	2.5	0.25	2.5	0.25
	0.4	0.45	0.45	0.4	10.0	6.3	0.23	10.0	0.4
	0.13	0.4	0.4	0.35	0.4	2.5	0.2	1.6	0.3
	0.1	0.1	0.1	0.4	0.3	0.4	0.2	0.01	0.2
					5.0	0.02			
					2.5	0.1			
						0.4			
						0.3			
						0.125			
						0.3			
						0.3			
						0.6			
						1.5			
						3.2			
						0.8			

The results were analysed using a computer programme (see Appendix). After the arithmetic means of the figures shown

Fig 20
The viability of frozen E. histolytica (JEH) suspensions after pre-freeze treatments with DMSO at various temperatures



in the table of results had been transformed into logs, a variance-ratio plot was made (Figure 21). The log transform produces geometric means (= antilog of means of log values). The calculated fitted values obtained from the geometric mean values were used in interpreting these results, see Table XXVIII. The results from experiments with a temperature of 0^{o} C were not included because they were obviously very low and were not replicated. Contrasts between the fitted values are statistically significant. Differences between observed geometric means and the fitted values are not statistically significant.

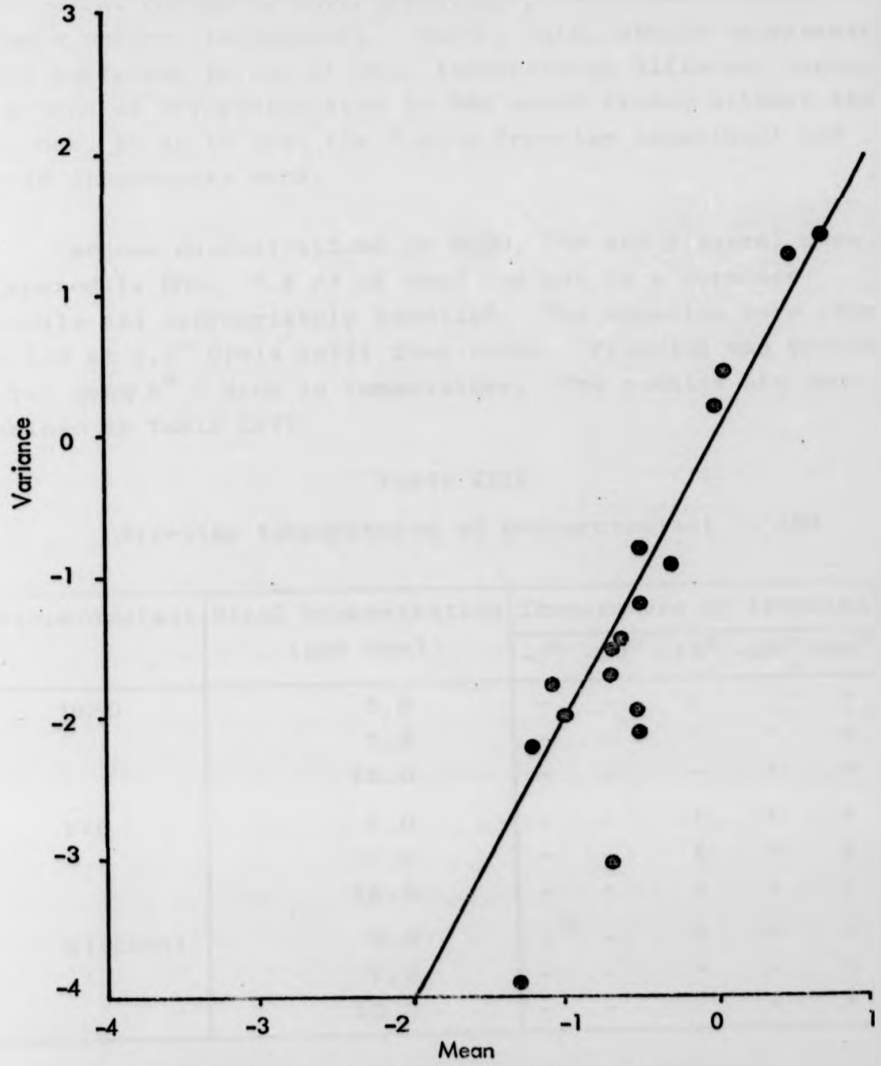
Of the variables tested, it can be concluded that 7.5% (v/v) DMSO gave the best survival with 15 minutes equilibration at 37^{o} C and this was with 1^{o} C/min cooling rate. There was no significant interactions between DMSO concentration, equilibration temperature and time and this means that it is legitimate to vary them separately.

Table XXVIII

Observed geometric mean values of percentage viability and values fitted by using a model of main effects only (see Appendix I)

Equilibration time		Mean percentage viability					
		Equilibration temp 25^{o} C % DMSO concentrations			Equilibration temp. 37^{o} C % DMSO concentrations		
		5.0	7.5	10.0	5.0	7.5	10.0
2 min	geometric mean	0.04	0.10	0.05	0.13	0.34	0.22
	fitted mean	0.03	0.10	0.06	0.11	0.41	0.22
15 min	geometric mean	0.03	0.33	0.15	0.46	2.11	0.80
	fitted mean	0.09	0.33	0.18	0.36	1.29	0.69
30 min	geometric mean	0.05	0.36	0.15	0.46	0.51	0.27
	fitted mean	0.05	0.19	0.10	0.21	0.74	0.40

Fig 21
Mean / variance plot (scatter diagram)
using a log transform which produces the geometric mean *



* geometric mean = antilog of mean of log values

The effect of 2-step freezing on the viability of *E. histolytica* suspensions using DMSO, glycerol and PVP as cryoprotectants

Experiment 1

Preliminary studies were carried out to see whether *E. histolytica* suspensions would survive freezing better if they were first cooled to their freezing point and held at a single subzero temperature. Before this, simple experiments were performed to see at what temperatures different concentrations of cryoprotectants in RSM would freeze without the amoebae, so as to plan the 2-step freezing experiment and avoid unnecessary work.

Various concentrations of DMSO, PVP and glycerol were prepared in RSM. 0.5 ml of each was put in a separate ampoule and appropriately labelled. The ampoules were then cooled at 0.5° C/min until they froze. Freezing was checked after every 5° C drop in temperature. The results are summarized in Table XXIX.

Table XXIX

Freezing temperatures of cryoprotectant in RSM

Cryoprotectant	Final concentration (per cent)	Temperature of freezing °C				
		-5°	-10°	-15°	-20°	-25°
DMSO	5.0	-	-	+	+	+
	7.5	-	-	+	+	+
	10.0	-	-	-	+	+
PVP	5.0	-	-	+	+	+
	10.0	-	-	+	+	+
	15.0	-	-	+	+	+
glycerol	5.0	-	-	+	+	+
	7.5	-	-	+	+	+
	10.0	-	-	+	+	+

Experiment 2a

A suspension of *E. histolytica* (stock JEH) was prepared in RSM. Log ID_{63} of this suspension was estimated as 5.4 ± 0.5.

Double-strength concentrations of DMSO were prepared. 12 ampoules were set up and divided into 4 groups of 3.
Group 1 received 0.2 ml 10% (v/v) DMSO
Group 2 received 0.2 ml 15% (v/v) DMSO
Group 3 received 0.2 ml 20% (v/v) DMSO
Group 4 received 0.2 ml RSM (control group)

0.2 ml amoebal suspension was added to each ampoule at varying times such that equilibration period of ampoules from a set varied between 2 minutes and 30 minutes. Equilibration was done at 0^{o}, 25^{o} and 37^{o} C. Ampoules were cooled from their ambient temperatures at -10^{o} C for 20 minutes. They were then transferred into liquid nitrogen for at least 1 hour before they were individually thawed and inoculated into cultures. One thawed suspension was inoculated into 2 culture bottles. Cultures were incubated at 37^{o} C and checked for amoebal growth daily.

It usually took less than 45 minutes between preparation of suspension and the beginning of freezing. The whole procedure was repeated several times using a different single sub-zero temperature on each occasion. See Table XXX for results.

Under these experimental conditions, it appears that the best protection was conferred by 5.0 or 7.5% (v/v) concentrations of DMSO equilibrated at 37^{o} C for a period of 30 minutes. The single sub-zero temperature of -25^{o} C appears to be the best used.

Experiment 2b

In order to determine the best single sub-zero tempera-

Table XXX

The effect of 2-step freezing on the viability of *E. histolytica* suspended in DMSO

Final concentration of cryoprotectant	Equilibration time and temperature	Culture results of samples frozen at -10°C	-15°C	-20°C	-25°C	-30°C	Day culture became positive -10°C	-15°C	-20°C	-25°C	-30°C
5% (v/v) DMSO	2 min at 0°C	-	+	+	+	+	-	4	4	3	3
	15 min at 25°C	-	-	+	+	+	-	-	2	2	2
	30 min at 37°C	+	+	++	+++	+++	4	2	2	1-2	2
7.5% (v/v) DMSO	2 min at 0°C	-	+	+	+	+	-	4	4	3	3
	15 min at 25°C	-	-	+	++	+	-	-	2	1-2	2
	30 min at 37°C	+	+	++	+++	+++	4	2	2	1-2	2
10% (v/v) DMSO	2 min at 0°C	-	+	+	+	+	-	4	4	2	3
	15 min at 25°C	+	+	++	++	+	4	2	2	1-2	2
	30 min at 37°C	+	+	++	+	+	4	2	2	2	2
untreated	2 min at 0°C	-	-	-	-	-	-	-	-	-	-
frozen	15 min at 25°C	-	-	-	-	-	-	-	-	-	-
control	30 min at 37°C	-	-	-	-	-	-	-	-	-	-

\- no amoebae seen

\+ few amoebae seen

++ less than 10 trypan-blue-excluding amoebae/field (using a x20 objective)

+++ more than 10 trypan-blue-excluding amoebae/field (using a x20 objective)

ture for 2-step freezing, it was necessary to measure infectivity after freezing treatments.

A suspension of JEH was prepared as usual and infectivity titrations made. $^{Log}/ID_{63}$ was estimated as 6.1 ± 0.5.

Four ampoules were set up; each received 0.2 ml 15% (v/v) DMSO in RSM. Three control ampoules received 0.2 ml RSM. All received 0.2 ml amoebal suspension, they were sealed and equilibrated at 37° C for 15 minutes at varying times. One experimental ampoule was opened and its content serially diluted immediately without freezing.

One ampoule and a control were frozen at a single sub-zero temperature of -15° C, another set at -20° C and the third at -25° C for a period of 20 minutes/set.

The ampoules were then transferred to liquid nitrogen. They were thawed, titrated and inoculated into cultures as usual. Undiluted suspensions were inoculated into cultures. The results are summarized in Table XXXI.

This experiment and 2 others carried out under the same conditions showed that -25° C was the best single sub-zero temperature tested for use during 2-step freezing of *E. histolytica* suspended in DMSO solution.

Experiment 2c

Having established the best sub-zero temperature for 2-step freezing, experiments were performed to measure the survival of *E. histolytica* suspensions after treatment with various concentrations of DMSO using the best equilibration conditions.

Two sets of experiments were performed; they differed only in that the first set was equilibrated at 25° C and the other at 37° C.

Table XXXI

The viability of *E. histolytica* suspensions after freezing at single sub-zero temperatures

Freezing treatment (sub-zero temperature)	log ID_{63}/ml and SE	Percentage viability*	Culture results of undilulted samples
Untreated, unfrozen suspension	6.1 ± 0.5	100	+ day 1
Treated, unfrozen suspension	5.4 ± 0.5	20	+ day 1
Suspension treated with 7.5% DMSO (-15° C)	1.4 ± 0.5	0.002	+ day 2
Control untreated (-15° C)	not measurable	0	-
Suspension treated with 7.5% DMSO (-20° C)	2.4 ± 0.5	0.02	+ day 1
Control untreated (-20° C)	not measurable	0	-
Suspension treated with 7.5% DMSO (-25° C)	2.6 ± 0.3	0.03	+ day 1
Control untreated (-25° C)	not measurable	0	-

* expressed as % of unfrozen, untreated control.

Amoebal suspensions were separately prepared for each and ID_{63} estimates made. Eight ampoules were set up and divided into 4 groups of 2. Group 1 received 0.2 ml 10% (v/v) DMSO, group 2 received 0.2 ml 15% (v/v) DMSO, group 3 received 0.2 ml 20% (v/v) DMSO, group 4 received 0.2 ml RSM (control). To each ampoule was added 0.2 ml amoebal suspension (JEH) at varying times such that equilibration took between 15 and 20 minutes. The ampoules were sealed and cooled at -25^{o} C for 20 minutes and were subsequently treated as described in experiment 2b. Table XXXII summarizes the results typically obtained from experiments of this nature.

The results show that there was no significant difference in the viabilities of *E. histolytica* subjected to the different treatments when frozen at a single sub-zero temperature of -25^{o} C. The best viable recovery obtained was 1.0% It wasconcluded that the controlled-rate cooling method at 1^{o} C/min gave better survival than the 2-step freezing method. Maximal viable recovery for the slow freezing method being 12.5% and for the 2-step freezing method used here being 1.0%.

Experiment 3a

Using glycerol

It was established earlier that 5% (v/v) solution protected *E. histolytica* best during cryopreservation when controlled-rate freezing method was used. Experiments were therefore performed to see whether viability would be improved when the two-step freezing method was employed.

Twelve ampoules were set up; 9 received 0.2 ml 10% (v/v) glycerol and 3 received the same volume of RSM. Each tube then received an addition of 0.2 ml amoebal suspension (stock JEH) at different times. The sealed ampoules were allowed 2-30 minutes equilibration at various temperatures. The ampoules were frozen at -10^{o} C. Other sets were also prepared and frozen at the following sub-zero temperatures -15^{o}, -20^{o}, -25^{o} and -30^{o} C. They were put into liquid

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Table XXXII

The viability of DMSO-treated *E. histolytica* suspensions after freezing at -25° C for 20 minutes

Treatment			Infectivity	
Temperature of equilibration	Final DMSO concentration (%)	Equilibration time (minutes)	log ID_{63}/ml and SE	Percentage viability*
25°C*	5.0	15	1.6 ± 0.3	0.1
		30	1.4 ± 0.5	0.06
	7.5	15	2.6 ± 0.3	1.0
		30	2.4 ± 0.5	0.6
	10.0	15	2.4 ± 0.5	0.6
		30	2.1 ± 0.5	0.3
	Control	15	Not measurable	0
		30		0
37°C**	5.0	15	3.1 ± 0.3	0.06
		30	2.8 ± 0.3	0.03
	7.5	15	2.6 ± 0.3	0.02
		30	3.1 ± 0.5	0.06
	10.0	15	3.5 ± 0.3	0.15
		30	2.6 ± 0.3	0.02
	Control	15	Not measurable	0
		30		0

*expressed as percentage of the untreated unfrozen control. * ID_{63} estimate of untreated control = antilog 4.6 ± 0.3. ** ID_{63} estimate of untreated control = antilog 6.3 ± 0.2

nitrogen for at least 1 hour, thawed and inoculated into cultures as before. Cultures were incubated and checked for amoebal growth as usual. See Table XXXIII.

Table XXXIII

The effect of 2-step freezing on the viability of *E. histolytica* suspended in glycerol

Equilibration temperature	Equilibration time (minutes)	Viability of samples frozen at				
		-10°C	-15°C	-20°C	-25°C	-30°C
0°C	2	-	-	-	-	-
	15	-	-	-	-	-
	30	-	-	-	-	-
Control (untreated)	30	-	-	-	-	-
*25°C	2	-	-	-	-	-
	15	-	-	+	-	-
	30	-	-	+	-	-
Control (untreated)	30	-	-	-	-	-
37°C	2	-	-	-	-	-
	15	-	-	-	-	-
	30	-	-	-	-	-
Control (untreated)	30	-	-	-	-	-

* Positive cultures became positive for amoebae between 2-3 days after incubation.

This experiment was repeated 4 times with comparable results except on one occasion when cultures were negative after freezing following treatment with 5% (v/v) glycerol at 25° C for 30 minutes.

The results indicate that in the 2-step freezing technique survival of *E. histolytica* in glycerol was achieved only when held at a temperature of -20° C.

Experiment 3b

Following the result of experiment 3a, it was necessary

to measure the degree of protection by infectivity estimates. Suspensions of stock JEH were used. They were equilibrated at 25° C for 15 and 30 minutes and frozen at -20° C for 20 minutes. After 1 hour in liquid nitrogen, they were thawed, titrated and inoculated into cultures as usual. The remainders of the undiluted, thawed suspensions were inoculated into cultures. Cultures were incubated and checked for amoebal growth as usual.

It was expected that better viability would be obtained using this method since it took 2-3 days for cultures to become positive for amoebae in the last experiment, but this was not the case. Infectivity was lost at the lowest dilution although cultures from undiluted samples became positive for amoebae on days 2 and 3.

Experiment 4a

The results of earlier experiments using the controlled-rate cooling method showed that PVP protects *E. histolytica* after equilibration at 37° C for 30 minutes. This may possibly be due to the fact that PVP only protects after entry of the cell by pinocytosis.

This experiment was performed to see whether the 2-step freezing method would improve the survival of *E. histolytica* after thawing.

Six ampoules were set up, each received 0.2 ml 30% (w/v) PVP. 0.2 ml amoebal suspension (JEH) was added to each at varying times so that 2 ampoules were equilibrated for 30 minutes and another 2 for 15 minutes all at 37° C. The last two ampoules had no time to equilibrate and PVP uptake by pinocytosis would be minimal. Controls were set up as usual without PVP. The ampoules were frozen at -20° C for 20 minutes. The experiment was repeated using various sub-zero temperatures (see Table XXXIV). After 1 hour in liquid nitrogen, they were thawed, inoculated into

cultures and incubated as usual. Cultures were checked daily for amoebal growth.

The results indicate that PVP does not protect *E. histolytica* after minimal equilibration under the conditions of this experiment. Protection is conferred only after 30-minutes' equilibration when PVP has presumably had time to penetrate cell walls.

Cultures made from suspensions held at -10° and -15°C became positive within 2 days. The samples from suspensions held at -20° C took between 3 and 4 days to grow. Holding temperatures below -20° C failed to confer any protection.

Table XXXIV

The effect of 2-step freezing on the viability of *E. histolytica* suspended in PVP

Equilibration		Culture results after freezing at				
Temperature	Time (minutes)	-10°C	-15°C	-20°C	-25°C	-30°C
37°C	0	-	-	-	-	-
	15	-	-	-	-	-
	30	+(2)	+(2)	+(3+4)	-	-
Untreated frozen controls		-	-	-	-	-

Days when cultures became positive are given in brackets.

Experiment 4b

The extent of PVP protection was measured by infectivity estimates. A suspension of *E. histolytica* (JEH) was used, log ID_{63} estimate of this suspension before treatment was 5.8 ± 0.3

Three ampoules were prepared: each received 0.2 ml 30% (w/v) PVP. 0.2 ml of amoebal suspension was added to each ampoule at different times. Suspensions were allowed to equilibrate at 37° C for 30 minutes. There

were three control ampoules which received amoebal suspensions without PVP. The ampoules with treated suspensions were paired with the controls. The first pair was cooled at -10^{o} C for 20 minutes, the second pair was cooled at -15^{o} C for 20 minutes and the third at -20^{o} C for the same period. The experiment was planned in such a way that there was only a 1-minute interval between the completion of one freezing and the beginning of the next, time being allowed for fall in temperature. Thawed suspensions were serially diluted and inoculated into cultures. Undiluted thawed suspensions were also inoculated into cultures. Incubation and checking of cultures were as usual.

Cultures from the undiluted suspensions showed amoebae on day 2 but none of the cultures inoculated with diluted suspensions became positive. It was concluded that the 2-step freezing method had no advantage over the controlled-rate cooling method for the viable preservation of *E. histolytica* with PVP.

The effect of different freezing rates on survival

Using optimal equilibration conditions and optimal cryoprotectant concentrations, the effect of different freezing rates on survival was studied using the controlled-rate freezing technique.

For technical reasons, it was not possible to test the different freezing rates simultaneously, neither was it practicable to use the same amoebal suspensions for the different experiments. Each freezing rate studied therefore constituted an experiment in its own right. Each was appropriately controlled and ID_{63} estimates of viability were made of each amoebal suspension before freezing.

Freezing rates used were 0.2°, 0.5°, 1.0°, 2.0°, 5.0° and 10.0^{o} C/min. Ultra-rapid freezing was by direct immersion

of ampoulesin liquid nitrogen after equilibration. Suspensions of stock JEII were used.

For each of the slower rates of freezing, ampoules were cooled at the specified rate to -60° C before they were transferred to liquid nitrogen and were subsequently treated the standard way. For each of the freezing rates 3 experiments were performed using the conditions found in earlier experiments to be optimal for PVP, glycerol and DMSO treatments. The mean percentage viability for each group of experiments is given in Table XXXV.

The results obtained from DMSO treatment are expressed in graphical form in Figure 22. The means of percentage viability and their standard errors were plotted.

Freezing rates higher than 5° C/min resulted in total loss of *E. histolytica* viability. None of the samples frozen at this rate and at 10° C/min survived. No recovery was obtained from samples directly plunged into liquid nitrogen neither was there any in all the different untreated, frozen controls. PVP appeared to be protective only when freezing rate was 1° C/min. Glycerol too protected at 0.5° and 1° C/min but viability was lost at the first tenfold dilution indicating very small percentage survival.

E. histolytica in DMSO tolerated freezing rates between 0.2° C and 2° C/min although the best survival was obtained at 1.0° C/min. The differences between 0.5° C and 1.0° C/min, and between 0.2° C and 0.5° C/min freezing rates were significant ($t = 4.98$, $p < 0.01$ and $t = 3.19$ and $p < 0.05$ respectively).

The effect of freezing amoebae in suspensions containing various combinations of cryoprotectants.

Diamond (1964) found that the viability of frozen *E. invadens* suspension was improved when a combination of DMSO and glycerol was used.

Table XXXV

The viability of frozen *E. histolytica* suspensions cooled at different freezing rates

Expt	Final protectant concentration (Equilibration condition)	Freezing rate	Mean $\log ID_{63}/ml$ & SE of untreated unfrozen samples	Mean % viability (Mean log ID_{63}/ml + SE)
1	a b c d	0.2°C/min	5.0 ± 0.5	0 0 0.004 ± 0.001 (0.6 ± 0.4) 0
2	a b c d	0.5°C/min	5.4 ± 0.5	0 + (n.m) 0.65 ± 0.202 (3.2 ± 0.5) 0
3	a b c d	1.0°C/min	5.8 ± 0.3	+ (n.m) + (n.m) 2.72 ± 0.363 (5.2 ± 0.3) 0
4	a b c d	2.0°C/min	5.4 ± 0.5	0 0 + (n.m) 0
5	a b c d	5.0°C/min	5.6 ± 0.5	0 0 0 0
6	a b c d	10.0°C/min	5.4 ± 0.3	0 0 0 0

a - 15% (w/v) PVP (37° C for 30 min)

b - 5% (v/v) glycerol (25° C for 30 min)

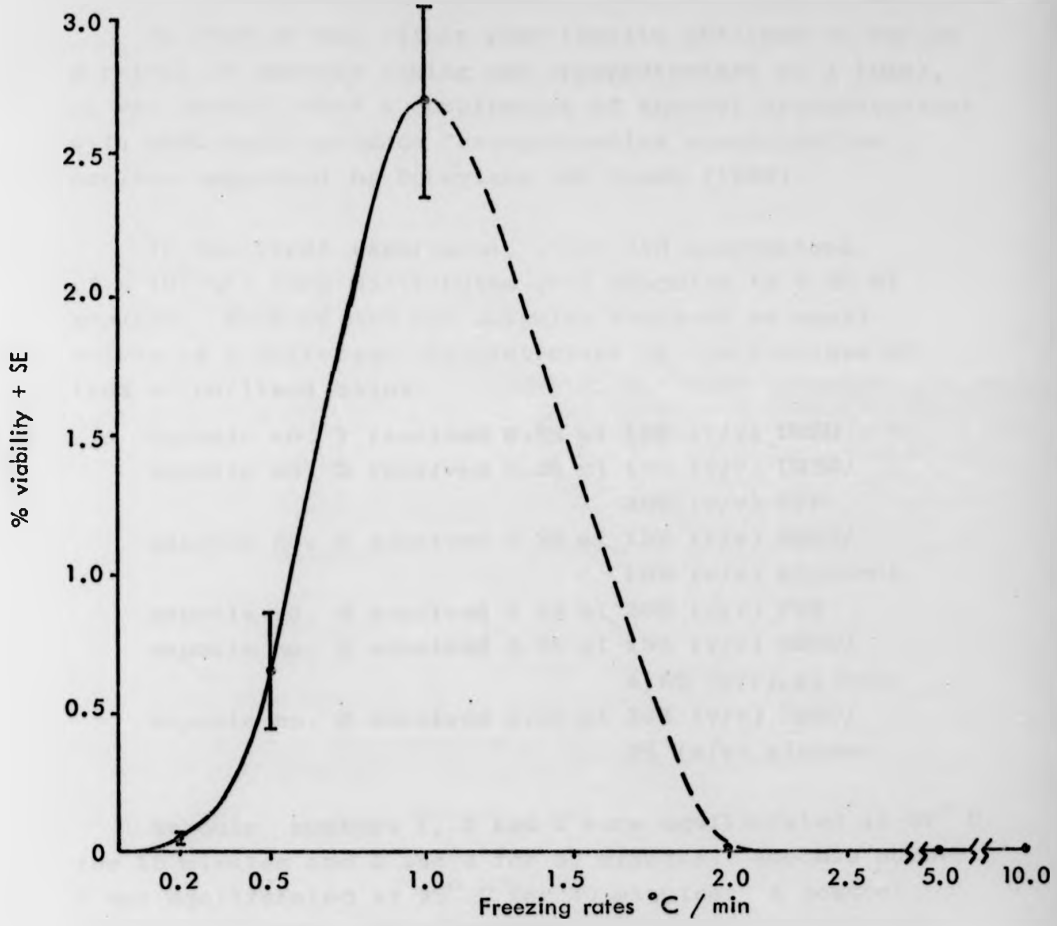
c - 7.5% (v/v) DMSO (37° C for 15 min)

d - Untreated frozen control

(n.m) = ID_{63} not measurable

\+ = untitrated thawed suspensions were cultivable but infectivity was lost at the first ten-fold dilution.

Fig 22
The viability of frozen E. histolytica (JEH) suspensions cooled at various freezing rates



Mean percentage viability and SE

0.2° C / min = 0.004 ± 0.001
0.5° C / min = 0.65 ± 0.202
1.0° C / min = 2.72 ± 0.363
2.0° C / min = 0 (untitrated sample grew in culture)
5.0° C / min = 0 (negative, no growth)
10.0° C / min = 0 (negative, no growth)

In view of the rather poor results obtained so far on survival of amoebae (using one cryoprotectant at a time), it was thought that a combination of another cryoprotectant with DMSO might produce "cryoprotective synergism", as earlier suggested by Robertson and Jacob (1968).

In the first experiment, stock JEH suspensions (2×10^6/ml) were distributed into ampoules in 0.25 ml amounts. Each of the six ampoules received an equal volume of a different cryoprotectant or combinations of them as outlined below:

ampoule no. 1 received 0.25 ml 15% (v/v) DMSO
ampoule no. 2 received 0.25 ml 15% (v/v) DMSO/ 30% (w/v) PVP
ampoule no. 3 received 0.25 ml 15% (v/v) DMSO/ 10% (v/v) glycerol
ampoule no. 4 received 0.25 ml 30% (w/v) PVP
ampoule no. 5 received 0.25 ml 15% (v/v) DMSO/ 4.5% (w/v) glucose
ampoule no. 6 received 0.25 ml 30% (v/v) DMSO/ 9% (w/v) glucose

Ampoule numbers 1, 5 and 6 were equilibrated at 37° C for 15 minutes and 2 and 4 for 30 minutes. Ampoule number 3 was equilibrated at 25° C for 30 minutes. A control ampoule received 0.25 ml amoebal suspension and 0.25 ml RSM and no cryoprotectant. They were frozen at 1° C/min as usual. After 2 hours at -196° C, they were thawed and titrated. The rest of the untitrated samples were inoculated into cultures as before.

Experiment 2 was a repeat of experiment 1.

Experiment 3 was exactly the same as described for experiment 1 except that the freezing rate was 0.5° C/min.

The results (Table XXXVI) show that DMSO on its own was more effective as a cryoprotectant under these circumstances than when it was used in combination with other cryoprotectants. The use of 15% (v/v) DMSO and 4.5% (w/v) glucose did not enhance survival as reported by Diamond; in fact the viability of samples so treated was lost at the first ten-fold dilution although cultures inoculated

Table XXXVI

The viability of *E. histolytica* suspended in various cryoprotectant combinations after freezing and thawing

Final cryoprotectant concentration (Equilibration conditions)	% viability after freezing at		
	1° C/min		0.5°C/min
	Expt. 1	Expt. 2	Expt. 3
7.5% (v/v) DMSO (37° C for 15 minutes)	2.0 (3.4 ± 0.5)	3.16 (3.8 ± 0.3)	1.0 (3.4 ± 0.5)
7.5% (v/v) DMSO/15% (w/v) PVP (37° C for 30 minutes)	0.3 (2.6 ± 0.3)	1.4 (3.4 ± 0.5)	1.0 (3.4 ± 0.5)
15% (w/v) PVP (37° C for 30 minutes)	0.02 (1.4 ± 0.5)	0.01 (1.3 ± 0.3)	0.01* (1.4 ± 0.5)
7.5% (v/v) DMSO/10% (v/v) glycerol (25° C for 30 minutes)	0.005 (0.8 ± 0.3)	0.01 (1.3 ± 0.3)	0.01 (1.4 ± 0.5)
15% (v/v) DMSO/4.5% (w/v) glucose (37° C for 15 minutes)	(n.m)	(n.m)	(n.m)
7.5% (v/v) DMSO/2.25% (w/v) glucose (37° C for 15 minutes)	0.2 (2.4 ± 0.5)	1.2 (3.4 ± 0.5)	0.1 (2.4 ±0.5)

Log ID_{63} + SE are shown in brackets

(n.m) = ID_{63} not measurable

* Untitrated thawed sample was negative for amoebal growth in culture

Log ID_{63} estimates of viability of unfrozen, untreated suspensions of experiment 1, 2 & 3 were 5.1 ± 0.5, 5.3 ± 0.3 and 5.4 ± 0.5 respectively.

from the untitrated sample grew amoebae within 2 days. The untreated, frozen control did not produce any amoebic growth in culture.

The effect of other cryoprotectants on survival

The sensitivity of *E. histolytica* to various concentrations of ethanol, methanol and sorbitol was tested by equilibrating amoebae in the following final concentrations for the various times and temperatures normally used:

Sorbitol 0.4, 0.8 and 2.0M (2M Sorbitol = 3% (w/v))
Ethanol and methanol 5 and 10% (v/v)

After equilibration, the suspensions were inoculated into cultures without freezing. Results showed that, at the concentrations used, these chemicals had no detrimental effect on the cultivability of *E. histolytica*, cultures becoming positive in 2 days.

Suspensions of stock JEH were then prepared (2 x 10^6/ml) with the various concentrations of these substances and equilibrated at various temperatures and times as before. They were distributed into ampoules and frozen at different rates in 3 separate experiments.

In experiment 1, they were frozen at 1^{o} C/min, in experiment 2 at 0.5^{o} C/min and in experiment 3 at a holding temperature of -25^{o} C. After storage at -196^{o} C for 2 hours, they were inoculated into cultures untitrated. None of the cultures inoculated from the thawed samples showed amoebic growth.

The experiments were controlled on each occasion with a 7.5% (v/v) DMSO-treated suspension and another without a protectant. Controls with DMSO were viable on every occasion, cultures becoming positive for amoebae within 2 days.

Samples which had been plunged direct into liquid nitrogen were also non-viable.

The effect of suspending medium on survival

During preliminary investigations on the cryoprotective activity of glycerol for *E. histolytica*, it was observed that, when amoebae were suspended in PBS and frozen at 1^{o} C/min in 5% (v/v) glycerol, no amoebae survived freezing. This was a clear indication that PBS was an unsuitable suspending medium and other media such as 1-3% serum, BR, RSM and Hanks physiological solutions were tried.

The results showed that glycerol only protected *E. histolytica* during freezing when RSM was used as the suspending medium. With DMSO, viable amoebae were obtained using RSM, PBS or BR, but not using Hank's or serum. However, these results were not based on quantitative work.

After the optimum equilibration conditions and freezing rates using RSM had been established, it was necessary to find other extrinsic factors which would enhance survival rate. The use of other suspending media was tested.

Stock JEH was harvested from RSM cultures and distributed in equal amounts to 4 centrifuge tubes. The suspensions, containing 5×10^{5} amoebae/ml, were washed twice in PBS and the supernatant pipetted off and treated as follows:

Tube 1 was made up to 1 ml with PBS
Tube 2 was made up to 1 ml with RSM (freshly prepared)
Tube 3 was made up to 1 ml with RSM (in which amoebae had grown)
Tube 4 was made up to 1 ml with BR.
15% (v/v) DMSO concentrations were made up using the different suspending media.

Eight ampoules were prepared and paired. One of each pair received 0.2 ml different amoebal suspension and was then made up to 0.4 ml with the corresponding medium without protectant. The other set received 0.2 ml amoebal suspension and 0.2 ml of the cryoprotectant solutions. The

experiment was controlled using the various media without protectant. The ampoules were sealed, equilibrated at 37° C for 15 minutes then cooled at 1° C/min to -60° C before transferring them to liquid nitrogen where they remained for 2 hours. They were thawed and titrated as usual. Table XXXVII illustrates the result typically obtained.

It can be concluded that RSM is the best suspending medium for freezing *E. histolytica* being at least 20 times more effective than PBS or BR. Neither of the untreated frozen controls survived freezing treatment.

Table XXXVII

The viability of DMSO-treated *E. histolytica* after freezing in various suspending media

Suspending medium	Log ID_{63}/ml & SE	% viability
PBS	1.9 ± 0.5	0.04
RSM (fresh)	3.2 ± 0.3	0.8
RSM (used)	3.2 ± 0.3	0.8
BR	2.0 ± 0.3	0.05
Untreated frozen controls	(n.m)	0
Original suspension	5.3 ± 0.3	assumed to be 100

(n.m) = ID_{63} not measurable.

The effect of age of culture on survival

Hitherto, 48-hour-old cultures have been used in the freezing experiments because it was shown in earlier work that 48-hour cultures contained more viable organisms than did suspensions of older cultures. On this basis, it was assumed that such young cultures would survive freezing better than older ones. An experiment was performed with 48, 72 and 96-hour-old cultures of stock JEH. The suspen-

experiment was controlled using the various media without protectant. The ampoules were sealed, equilibrated at 37° C for 15 minutes then cooled at 1° C/min to -60° C before transferring them to liquid nitrogen where they remained for 2 hours. They were thawed and titrated as usual. Table XXXVII illustrates the result typically obtained.

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BR	2.0 ± 0.3	0.05
Untreated frozen controls	(n.m)	0
Original suspension	5.3 ± 0.3	assumed to be 100

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sions (1.5×10^6 amoebae/ml) were treated similarly using 7.5% (v/v) DMSO in RSM. Controls received no cryoprotectant, equilibration was at 37° C for 15 minutes. Freezing was done at 1° C/min. Thawing and titration were done as usual.

Table XXXVIII shows that there was no significant difference in survival rate between the 48 and 72-hour-old cultures but the results were not consistent enough to draw a firm conclusion on which of the two ages was better. 96-hour-old cultures did not survive freezing well as infectivity was lost at the first dilution and cultures inoculated from the untitrated samples became positive after 3 days. Controls without cryoprotectant were negative.

Table XXXVIII

Showing the viability of young and old cultures of *E. histolytica* after freezing in DMSO

Age of culture (hrs)	log ID_{63}/ml and SE of unfrozen sample*		% viability after freezing (log ID_{63} /ml and SE)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
48	5.1 ± 0.5	5.3 ± 0.3	1.0 (3.1 ± 0.5)	0.4 (2.9 ± 0.5)
72	5.1 ± 0.3	6.3 ± 0.3	2.0 (3.4 ± 0.5)	0.3 (3.8 ± 0.3)
96	5.7 ± 0.5	6.1 ± 0.3	+ (n.m)	+ (n.m)
Untreated frozen controls	as in the experiment	as in the experiment	0	0

* The viabilities of unfrozen samples were assumed to be 100%.

(n.m) = ID_{63} not measurable.

The effect of volume of suspension on infectivity

Farrant *et al*(1974) reported that when the volume of lymphocyte suspension was increased from 0.2 ml to 1 ml, recovery rate was lowered. This experiment was performed to see whether the volume of suspension has an effect on the survival of frozen *E. histolytica*.

Eight ampoules were set up and paired, each pair received different volumes of stock JEH suspension. Each pair was diluted with an equal volume of 15% (v/v) DMSO solution. They all contained approximately the same number of amoebae (5×10^5). ID_{63} estimates were made of the original suspensions as usual. Ampoules were cooled at 1° C/min after equilibrating for 15 minutes at 37° C.

Table XXXIX shows the average survival rates of the amoebae in the different volumes after 2 hours in liquid nitrogen. The results show that there is no appreciable difference between the four volumes used.

Table XXXIX

The viability of *E. histolytica* suspensions frozen in different volumes of suspending media

Volume of frozen suspension (ml)	% viability (log ID_{63}/ml ± SE)
0.4	2.0 (3.6 ± 0.3)
0.6	1.25 (3.4 ± 0.5)
0.8	3.0 (3.8 ± 0.3)
1.0	3.0 (3.8 ± 0.3)

The ID_{63} and SE of original suspension was antilog 5.3 ± 0.2 (assumed 100% viable).

The effect of warming of cultures on survival

It is generally assumed that subculturing of amoebae into pre-warmed culture media enhances initial growth. It was necessary to test the effect of prewarmed cultures on the survival of DMSO-treated suspensions after freezing and thawing.

Equal volumes of amoebal suspensions (Stock JEH) were placed in 2 ampoules; they were equilibrated with 7.5% (v/v) DMSO at 37° C for 15 minutes and cooled at 1° C/min as usual. Titrated samples of the original unfrozen suspensions and of the thawed suspensions were inoculated into 2 sets of cultures - prewarmed, and cold (23° C). The experiment was controlled with untreated suspension.

The results obtained from 3 such experiments are summarized in Table XL. It can be seen that frozen suspensions of *E. histolytica* are less sensitive to temperature effects than the unfrozen suspensions. The unfrozen controls consistently showed better infectivity when inoculated into prewarmed cultures than when they were not.

The effect of repeated cryopreservation procedures on viability after cryopreservation

It has been suggested that cryopreservation may select freeze-resistant individuals within a population. If this is true, then, with repeated cryopreservations, percentage survival should increase.

Suspensions of stocks Irvine, JEH and Thirer were distributed into 3 separate ampoules. To each was added an equal volume of 15% (v/v) DMSO. The samples were equilibrated and frozen in the usual way. All the samples were inoculated into cultures. When amoebae had grown for 48 hours, they were harvested frozen and thawed again and their ID_{63} estimates made. The process was repeated for

Table XL

The viability of frozen and unfrozen samples of amoebal suspensions inoculated into cold and prewarmed cultures

Experiment number	Culture condition	log ID_{63}/ml + SE of original suspension	*% viability of frozen samples (log ID_{63}/ml + SE)
1	Cold	5.6 ± 0.3	1.5 (3.8 ± 0.3)
	Warm	5.8 ± 0.3	0.63 (3.6 ± 0.3)
2	Cold	5.4 ± 0.3	3.0 (3.9 ± 0.3)
	Warm	5.6 ± 0.3	2.0 (3.9 ± 0.5)
3	Cold	5.4 ± 0.3	1.6 (3.6 ± 0.5)
	Warm	6.1 ± 0.5	2.0 (4.4 ± 0.3)

* Viability of frozen samples expressed as a percentage of the untreated, unfrozen original suspensions (shown in column 3 of table).

each stock thrice more. Table XLI shows the results obtained. There is no evidence from these results that survivors showed any increased resistance to freezing. All attempts to cultivate amoebae from repeatedly frozen and thawed samples without intermediate growth in culture failed.

Table XLI

The viabilities of repeatedly frozen *E. histolytica* suspensions

No. of times frozen	*% viability after thawing		
	stock Irvine	stock JEH	stock Thirer
1	5.0	1.5	0.25
2	1.0	3.2	0.4
3	2.5	0.8	1.0
4	0.6	1.2	0.2

* Viability expressed as a percentage of untreated, unfrozen original suspensions (assumed 100% viable).

The effect of thawing temperature on survival

Diamond (1964) observed that thawing temperature affects the viability of frozen *E. histolytica* suspensions. This experiment was carried out to confirm this observation under the conditions used here.

A suspension of stock JEH (2×10^6 amoebae/ml) was distributed to 6 ampoules, equal volumes of 15% (v/v) DMSO were added and equilibration was carried out the usual way. After 2 hours at -196° C, 2 samples were thawed at 25° C (on bench), another 2 at 0° C (in ice) and the last 2 at 37° C (in water bath). Thawing took between <2 minutes (at 37° C) to 15 minutes (at 0° C). Each thawed sample was separately titrated and the ID_{63} estimated.

The results of 2 experiments are summarized in Table XLII. It can be seen that viability was consistently better at the higher thawing temperature than at 0° C. The difference between samples thawed at 37° C and at 25° C was marked although not significant, but that between 37° C and 0° C was significant ($p < 0.01$).

Table XLII

The viability of frozen suspensions of *E. histolytica* thawed at different temperatures

Expt. number	Thawing temperature	Log ID_{63}/ml + SE unfrozen samples	% viability of thawed samples (log ID_{63}/ml + SE)
1	0° C	5.3 ± 0.3 (assumed 100% viable)	0.5 (3.1 ± 0.5)
	25° C		1.2 (3.4 ± 0.5)
	37° C		8.0 (4.2 ± 0.3)
2	0° C	4.6 ± 0.3 (assumed 100% viable)	0.3 (2.1 ± 0.5)
	25° C		0.6 (2.4 ± 0.5)
	37° C		3.2 (3.1 ± 0.5)

The effect of a period of 'structural reconstitution'

Lumsden *et al* (1968) found that frozen trypanosome suspensions require a period of 'structural reconstitution' following thawing and before animals were inoculated. It was necessary therefore to see whether frozen *E. histolytica* suspensions also require this period.

Eight ampoules received 0.2 ml 15% (v/v) DMSO solution and an equal volume of stock JEH suspension (1.5×10^6 amoebae/ml). They were equilibrated and frozen in the usual way.

Thawing was done at 37° C in less than 2 minutes. 4 thawed samples were left in a water bath at 37° C, the rest were put on a rack at room temperature (25° C). At varying times, the ampoules were opened and titrated. Untitrated samples that remained were inoculated into medium. Table XLIII summarizes the results of the titrations. A repeat experiment showed comparable results.

It can be seen that when frozen *E. histolytica* suspension is thawed and left for a period before inoculation into cultures, its viability decreases. It was not possible to show that *E. histolytica* required a period of structural reconstitution after thawing. Inoculation should be done as soon as possible after titration.

Table XLIII

The viability of frozen *E. histolytica* suspensions after periods of 'structural reconstitution'

Reconstitution		% viability*	Culture results of untitrated samples
Temperature	Time after thawing (min)		
25° C	2	0.98	Positive (1)
	5	(n.m)	Positive (2)
	15	(n.m)	Positive (2)
	30	(n.m)	Positive (2)
37° C	2	1.2	Positive (1)
	5	0.05	Positive (1)
	15	(n.m)	Positive (2)
	30	(n.m)	Positive (2)

( ) day on which amoebae were found in cultures inoculated with untitrated samples. (n.m) = ID_{63} not measurable.
*Viability expressed as a percentage of untreated, unfrozen original suspensions (assumed 100% viable).

The viability of *E. histolytica* grown axenically and monoxenically with *Crithidia* species after freezing

Suspensions of stock NIH:200 axenically and monoxenically grown with *Crithidia* and *Es. coli* - B were separately prepared. After centrifugation, they were resuspended in fresh TPS-1 medium, TTY medium with *Crithidia* and RSM respectively.

Earlier experiments had shown that estimation of viable numbers of *E. histolytica* by titration technique gave inconsistent results with axenic and crithidia-grown cultures. Haemocytometer estimates were therefore made using the trypan-blue-exclusion method.

The volumes of the suspensions were adjusted so that each suspension contained 2×10^6 amoebae/ml. 0.25 ml of each suspension was mixed with an equal volume of 15% (v/v) DMSO solution. After equilibration at 37° C for 15 minutes, they were frozen by the standard method and stored at -196° C for one week. The samples were then thawed and the numbers of trypan-blue-excluding amoebae were estimated before inoculation into various pre-warmed cultures. In this experiment and in many others of its kind, it was always impossible to cultivate amoebae from frozen NIH:200 derived from axenic or *Crithidia* cultures in their normal culture media after thawing, although on one occasion it was possible to grow them in RSM to which human faecal bacteria had been added provided the whole content of an ampoule (0.5 ml) was inoculated after washing with PBS. Table XLIV summarizes the results obtained.

The effect of length of storage on survival

During the course of this work several stabilates of stock JEH were laid down. Such stabilates had been subjected to various prefreeze treatments with various concentrations of DMSO. The final volumes of the stabilate were

Table XLIV

Survival of NIH:200 grown axenically and with *Crithidia* after frozen storage

Source of cryopreserved amoebae	% excluding trypan blue	Cultivability in		
		Robinson's plus faecal flora	TTY medium	TPS-1 medium
Robinson's (monoxenic)	10.0	positive	-	-
TTY - *Crithidia* (monoxenic)	15.0	positive*	negative	-
TPS-1 (axenic)	50.0	positive*	negative	negative

* occasionally positive.

0.5 ml. All were cooled at 1° C/min to -60° C; they were subsequently stored at -196° C for various lengths of time. After 1 hour, 7 days and up to 7 months of storage, they were thawed and ID_{63} estimates were made. The results are given in Table XLV. Stabilates of other stocks were made using 7.5% (v/v) DMSO and after equilibration at 37° C for 15 minutes; they were cooled at 1° C/min to -60° C and stored at -196° C. The ID_{63} estimates for stocks other than JEH were not made but their viability was tested by inoculating thawed samples into culture medium.

The results with JEH showed that there was a consistent loss of viability over six-month's storage at -196° C. Using the statistical sign test, this loss was shown to be highly significant. In the sample that survived frozen storage best, 80% of the viable organisms had been lost within 3 months.

Although the ID_{63} estimates of other stocks were not estimated, inoculation of cultures showed that they all

Table XLV

The viability of *E. histolytica* (stock JEH) after storage in liquid nitrogen for various lengths of time

% concentration of DMSO	Equilibration temp. (° C)	Equilibration time (min)	log ID_{63}/ml & SE of fresh sample	% viability after storage at -196° C for		
				1 hour	1 week	Up to 6 months
7.5	0	30	5.9 ± 0.5	0.03 (2.4 ± 0.5)	0.015 (2.2 ± 0.3)	0.008 (1.8 ± 0.3)
7.5	25	15	5.6 ± 0.3	0.25 (3.0 ± 0.3)	0.32 (3.1 ± 0.5)	0.1 (2.6 ± 0.3)
7.5	25	30	5.6 ± 0.3	0.63 (3.4 ± 0.5)	0.63 (3.4 ± 0.5)	0.32 (3.1 ± 0.3)
7.5	37	15	6.0 ± 0.3	12.5 (5.1 ± 0.5)	6.3 (4.8 ± 0.5)	2.5 (4.4 ± 0.5)
7.5	37	15	6.1 ± 0.5	0.8 (4.0 ± 0.3)	-	0.5 (3.8 ± 0.3)
7.5	37	30	6.0 ± 0.3	2.5 (4.4 ± 0.5)	1.5 (4.2 ± 0.3)	0.8 (3.9 ± 0.5)

Table XLV cont.

7.5	37	30	6.1 ± 0.5	1.0 (4.1 ± 0.5)	-	0.8 (4.0 ± 0.3)
10.0	25	15	5.6 ± 0.3	0.35 (3.1 ± 0.5)	-	0.25 (3.0 ± 0.3)*
10.0	37	2	5.6 ± 0.3	0.63 (3.4 ± 0.5)	0.4 (3.2 ± 0.5)	0.35 (3.1 ± 0.3)
10.0	37	15	5.8 ± 0.3	3.9 (4.4 ± 0.5)	-	2.0 (4.1 ± 0.5)*

* period of storage was 7 months

$\log ID_{63}$ & SE of frozen samples shown in brackets

% viability expressed as a percentage of fresh, unfrozen sample.

still contained viable organisms after 8 months' storage. It took 1-3 days for cultures to show amoebae following inoculation and incubation of thawed samples; this suggests that the number of viable amoebae present could still be measured by the ID_{63} method (from previous experience, a culture which took only 1-3 days to show amoebae usually had originally a measurable number of viable organisms using the ID_{63} method).

C6 The effect of cryopreservation on the biological characteristics of *E. histolytica*

Experiment 1. The effect of cryopreservation on encystation

It was observed during the early part of this work that profuse growth was obtained within 48 hours from tap-water-washed suspensions having very low numbers of trypan-blue-excluding amoebae in them.

It was not clear whether the profuse growth was due to the presence of mature cysts or was derived from surviving trophozoites.

An experiment was performed to see whether encystation took place in Robinson's medium in which *Es. coli - B* had grown. Various stocks of *E. histolytica* were harvested, washed and kept at 4° C for one week. They were then suspended in 0.1N HCl and incubated at 25° C for 1½ hours (Dobell and Neal, 1952). The suspensions were then washed in PBS, examined for the presence of cysts and then subcultured into pre-warmed Robinson's medium. It was always difficult to find cysts in the suspensions because the numbers were probably very low. Cultures were examined after 48 hours at 37° C. After this treatment growth was found from stocks Fenn, Rafiq, Fox, SN, Biswas, JEH and Irvine.

In order to test whether the 'encysting ability' was retained after cryopreservation, cryopreserved samples were

grown in Robinson's medium and treated the same way as the unfrozen samples. It was found that cryopreservation did not affect encystation, neither did it induce encystation in the normally non-encysting stocks such as Ali, Thirer and Atkinson.

Experiment 2. Iso-enzyme characterization of *E. histolytica* using thin-layer starch-gel electrophoresis

Soluble extracts from *E. histolytica* stocks were subjected to horizontal thin-layer starch-gel electrophoresis and were specifically stained for GPI, PGM, ME and HK (= GK) as described earlier. The following results were obtained.

ME - a single band was common to all the *E. histolytica* stocks. No band was observed for *Es. coli - B* (Figs 23 - 26 for ME). A slow migrating band was observed for *E. invadens* (Figs 23 & 24 for ME).

GPI - in interpreting the results of GPI and PGM the fast migrating bands shown above the lines (Figs. 24, 26, 27B) were ignored as they were probably bands associated with the bacterial population (Sargeaunt and Williams, 1978). All the stocks except Thirer and Atkinson had single common GPI bands (Figs 23 & 24, GPI). Thirer had two bands while Atkinson had 3 (Figs 25 & 26, GPI). The slow band of Thirer was common to the single bands of other stocks. The three bands of Atkinson were equidistant from each other, with the slowest band moving slightly ahead of the single bands of other stocks, and the fastest band moving not as fast as the faster band of Thirer. It is not uncommon to find this type of 'laddering' with GPI. It may be a result of oxidation of this enzyme during storage in liquid nitrogen (Kilgour, personal communication).

PGM - all stocks had a single band except Thirer which had a double band (Figs 25 & 26, PGM and 27). The mobility of the

slow band of Thirer was the same as the single band of JEH. The single bands of all other stocks were ahead of the slow band of Thirer, and the single band of JEH, but not as fast as the fast band of Thirer. In Figure 24 (PGM), bands of Biswas had a higher mobility than those of Thesiger and SN. The band of *E. invadens* was running slightly ahead of that of Biswas.

HK - all stocks had 2 distinct bands. The pair of bands of Biswas were running faster than the pairs of bands for Thesiger and SN. Although the control *T. vaginalis** (axenic) also had two bands, which were running differently from the bands of *E. histolytica* (Fig. 24, HK). In Figure 28B, it can be seen that *Es. coli - B* had a single faint band which was similar to the fast moving bands of NIH:200, Irvine and Atkinson and the slow running band of *E. coli*. The bands of *E. coli* and that of *Es. coli - B* were faint bands which appeared only after a prolonged incubation. Stocks Bean, JEH and Thirer (clone) had 2 prominent bands. Stocks AM25 and Thirer (parent) had 3 bands, 2 of which were prominent and were similar to those of Bean, JEH and Thirer (clone); the third bands of AM25 and Thirer were faint and they all appeared between the pair of prominent bands. These faint bands were similar to the slow migrating bands of NIH:200, Irvine and Atkinson. *E. coli* had 2 faint bands (suggesting weakness of enzyme activity); the slower moving band was common to the fast moving bands of NIH:200, Irvine and Atkinson, and the fast band was moving ahead of every other band.

Sargeaunt and Williams (1978) showed that the enzyme variants of 14 stocks of *E. histolytica* could be sorted into three groups using the enzyme patterns stained for GPI, PGM and ME. Using their methods, it was possible to group my stocks into three and to confirm the groupings with an addi-

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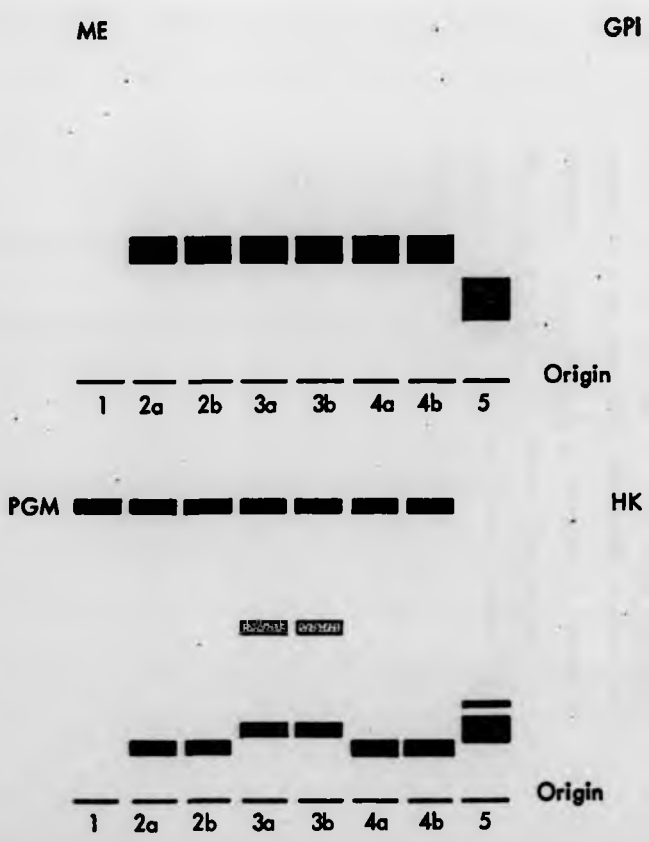
* lysate of *T. vaginalis* was kindly supplied by Dr Atef Soliman (L.S.H. & T.M.).

Fig 23


ME
GPI
Origin
1 2a 2b 3a 3b 4a 4b 5
PGM
HK
Origin
1 2a 2b 3a 3b 4a 4b 5

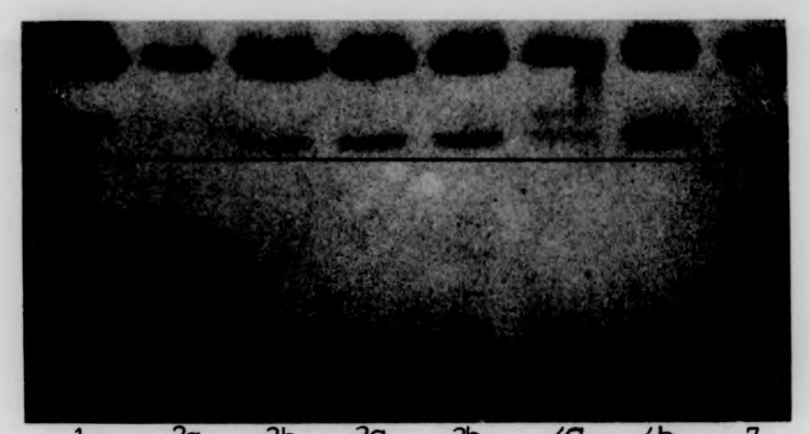
+
1
2a
2b
3a
3b
4a
4b
7
−
+
2a
2b
3a
3b
4a
4b
6
6
−

Fig 23

Diagrammatic representation of starch-gel electrophoretic patterns of soluble extracts of E. histolytica stained for 4 enzymes GPI, PGM, ME and HK (=GK) Position: 1, Es. coli-B; 2, Thesiger; 3, Biswas; 4, SN; 5, E. invadens; 6, Axenic Trichomonas vaginalis, 7, JEH. Positions 2-4and 7 refer to E.histolytica stocks. a, parent; b, clone.

Fig 24

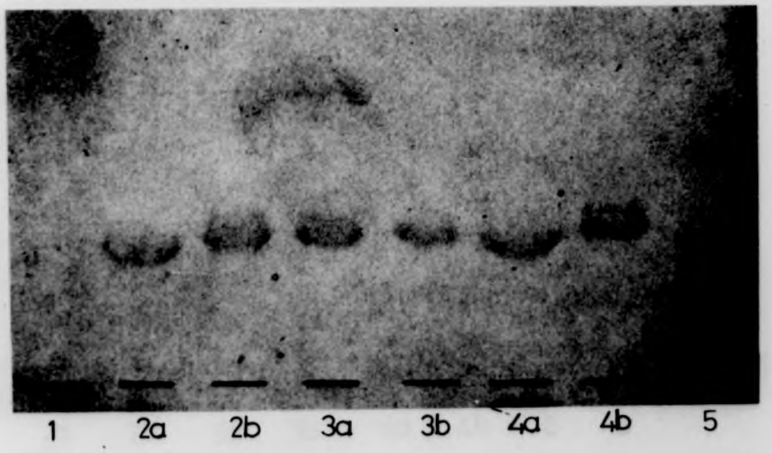
anode



cathode

Photograph of electrophoretic patterns stained for GPI.

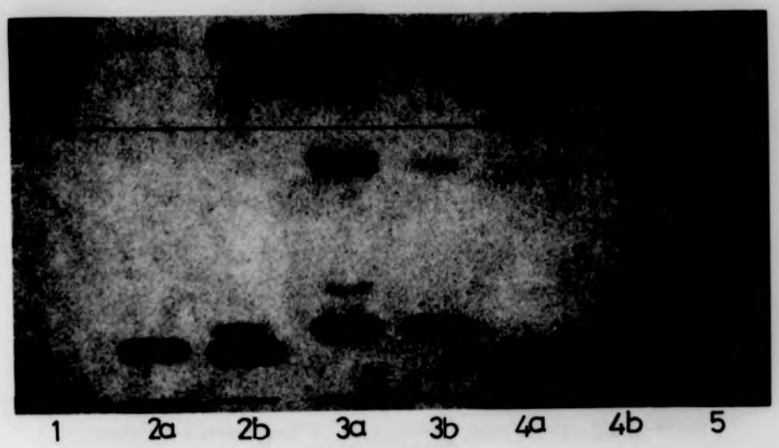
anode



cathode

Photograph of electrophoretic patterns stained for ME

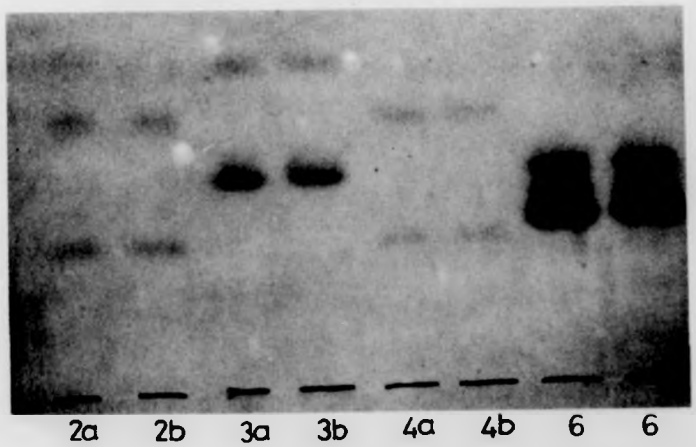
anode



cathode

C - Photograph of electrophoretic pattern stained for PGM

anode



cathode

D - Photograph of electrophoretic pattern stained for HK

Fig 24

Photographs showing starch-gel electrophoretic patterns of soluble extracts of E. histolytica stained for GPI, PGM, ME and HK (=GK).

Position: 1, Es. coli - B; 2, Thesiger; 3, Biswas; 4, SN; 5, E. invadens; 6, axenic T. vaginalis; 7, JEH; a, parent; b, clone. Positions 2-4 refer to E. histolytica stocks.

Fig 25

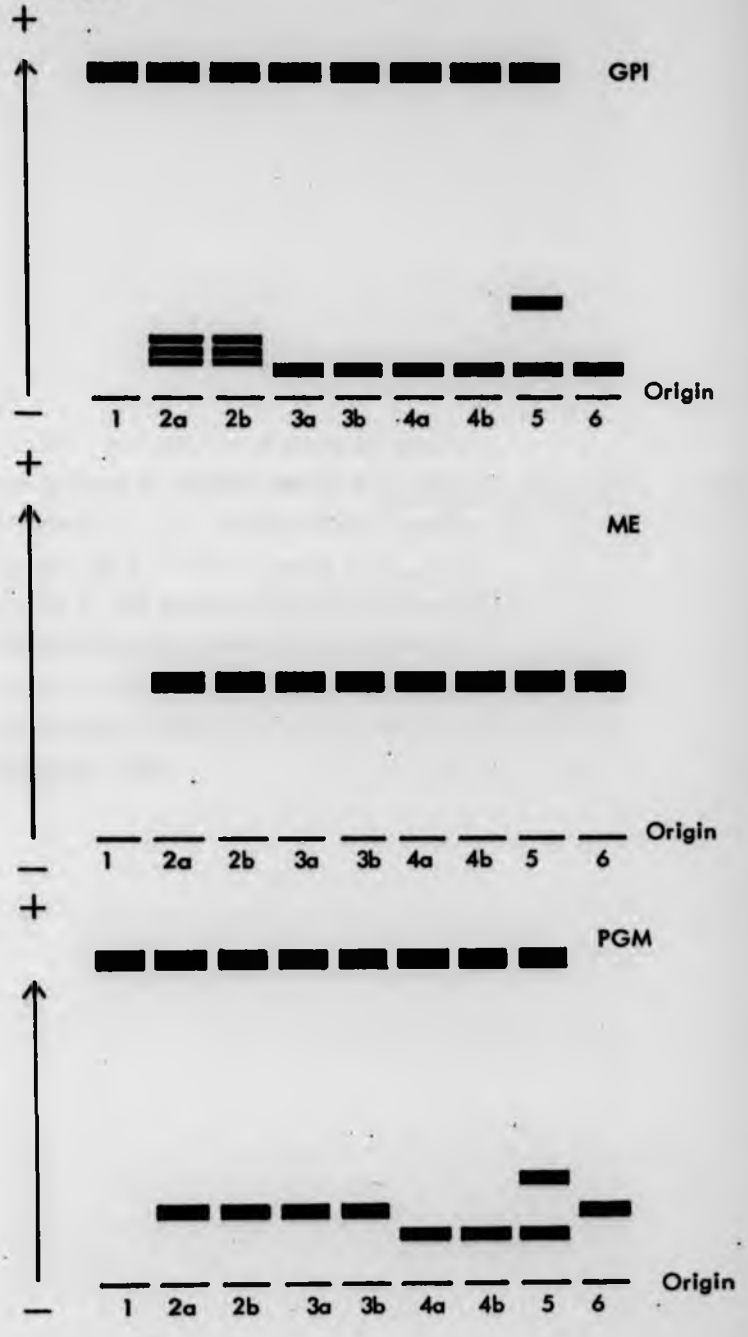
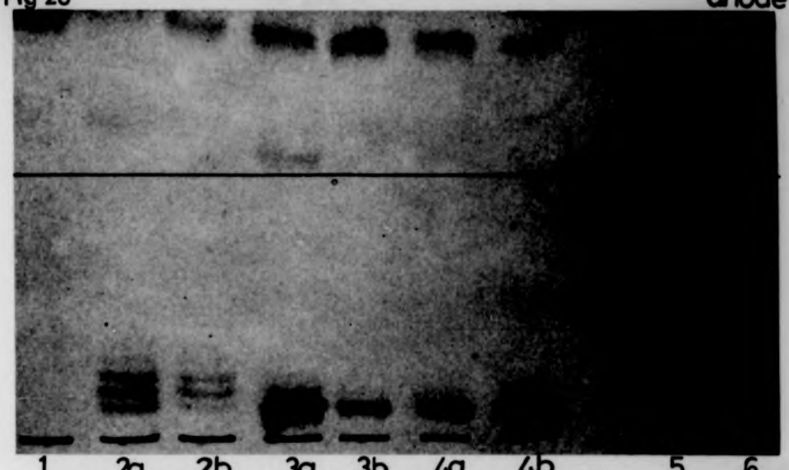

+
GPI
Origin
1 2a 2b 3a 3b 4a 4b 5 6
−
+
ME
Origin
1 2a 2b 3a 3b 4a 4b 5 6
−
+
PGM
Origin
1 2a 2b 3a 3b 4a 4b 5 6
−

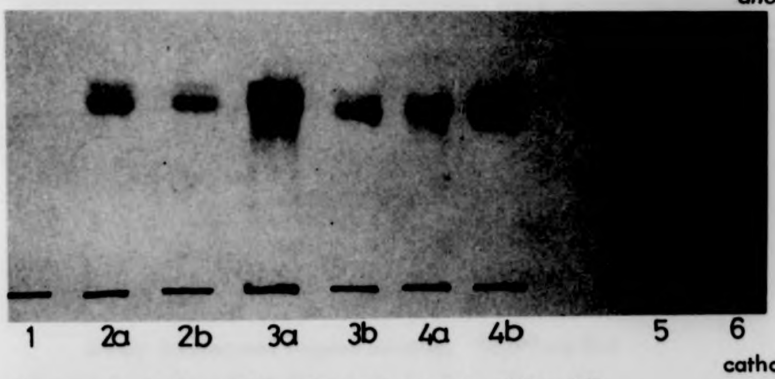
Fig 25

Diagramatic representation of starch gel electrophoretic patterns of soluble extracts of E. histolytica stained for GPI, ME and PGM. Position: 1, Es. coli - B; 2, Atkinson (group II); 3, Biswas (group II); 4, JEH (group I); 5, Thirer (Group III); 6, NIH:200 (axenic, group II); a, lysate prepared from unfrozen sample; b, from successively frozen and thawed samples. Positions 2–6 refer to E. histolytica stocks.

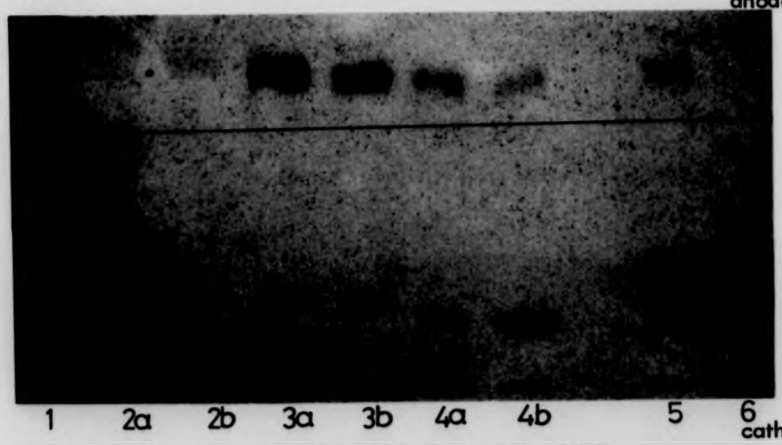
Fig 26



Photograph of electrophoretic patterns stained for GPI



Photograph of electrophoretic patterns stained for ME

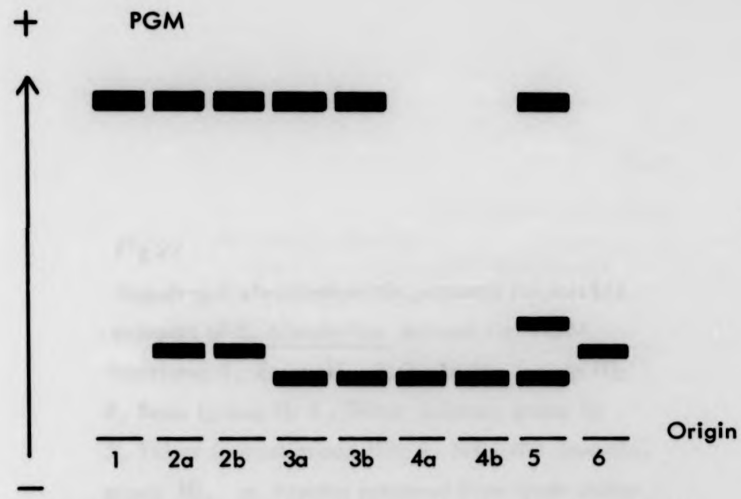


Photograph of electrophoretic patterns stained for PGM

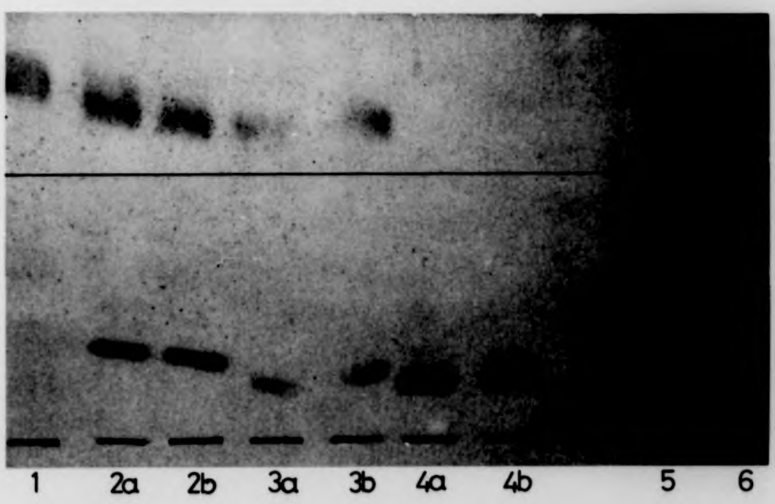
Fig 26

Photographs showing starch-gel electrophoretic patterns of soluble extracts of E. histolytica stained for GPI, ME and PGM. Position: 1, Es. coli - B; 2, Atkinson (group II); 3, Biswas (group II); 4, JEH (group I); 5, Thirer (group III); 6, NIH:200 (axenic, group II). a, lysate prepared from unfrozen samples; b, from successively frozen and thawed samples. Positions 2-6 refer to E. histolytica stocks.

Fig 27



A. diagrammatic representation of gel stained for PGM

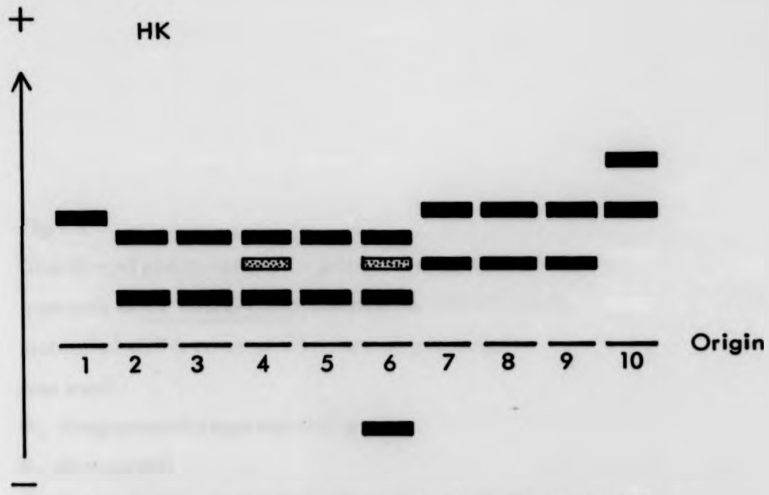


B. photograph of gel stained for PGM

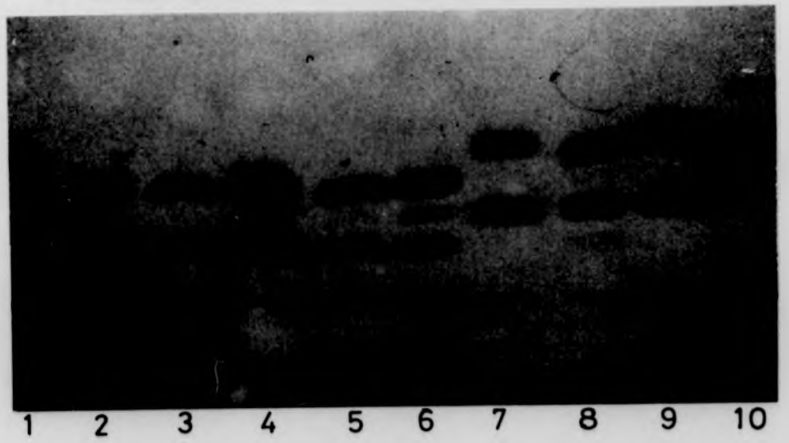
Fig 27

Starch-gel electrophoretic patterns for soluble extracts of E. histolytica stained for PGM. Positions: 1, Es. coli - B; 2, Irvine (group II); 3, Bean (group I); 4, Thirer (clone, group I); 5, Thirer (parent, group III); 6, NIH:200 (axenic, group II). a, lysates prepared from fresh unfrozen samples; b, lysates prepared from successively frozen and thawed samples.

Fig 28



A. diagrammatic representation of gel stained for HK



B. photograph of gel stained for HK

Fig 28

Starch-gel electrophoretic patterns of soluble extracts of E. histolytica stained for HK(=GK). A potential difference of 20 v/cm of gel length was used.

A, diagrammatic representation

B, photograph

Position: 1, Es. coli - B; 2, Bean (group I); 3, JEH (group I); 4, Thirer (parent, group III); 5, Thirer (clone, group I); 6, AM25 (group III); 7, NIH:200 (group II); 8, Irvine (group II); 9, Atkinson (group II); 10. E. coli. Positions 2-9 refer to E. histolytica stocks.

tional enzyme - HK. The groups of stocks are given in Table XLVI.

Table XLVI

The distribution of *E. histolytica* stocks into various isoenzyme variant groups

Group I	Group II	Group III
Thirer (clone)	Atkinson	Thirer (parent)
JEH	Biswas	AM25*
Bean	Irvine	
Thesiger	NIH:200	
Fenn		
Fox		
SN		
Ali		
Saigon		

* AM25 was a known group III; the lysate was kindly supplied by Mr P.G. Sargeaunt (L.S.H. & T.M.).

Using thin-layer starch-gel electrophoresis, there was no difference between the enzyme patterns of my *E. histolytica* isolates and the clones derived from them except in the case of Thirer where a clone showed a different band pattern from the parent. The parent could be placed in group III and the clone in group I, see Figs. 27B (PGM) and 28B (HK). This suggests that the parent Thirer was probably a heterogenous population which consisted of amoebae from groups I and III.

The enzyme patterns of amoebae were not affected by cryopreservation since after cryopreservation and subsequent growth in culture the electrophoretic patterns remained the same for all the enzymes used (Figs. 26 and 27).

Experiment 3. The effect of cryopreservation on the antigenicity of *E. histolytica*

Bryan *et al* (1976) reported that certain properties of cell membranes such as con A receptor sites were destroyed when they cryopreserved lymphocytes. Farrant (personal communication) also indicated that antigens may be stripped off cell membranes as a result of cryopreservation. However, these authors did not indicate whether these properties were restored after multiplication of the cells *in vitro*.

This experiment was performed to detect possible permanent changes in the surface antigens of various cryopreserved amoebal stocks. Antigens prepared from fresh, never frozen samples and from amoebae derived from successively frozen samples were compared after titration in an indirect fluorescent antibody test using anti-*E. histolytica* antiserum and fluorescein conjugated sheep antihuman immunoglobulin as described earlier.

The results showed that serum antibody titres remained practically unchanged in the groups studied. This suggests that the antigens concerned had not been irrevocably lost as a result of cryopreservation. See Table XLVII.

Table XLVII

Showing IFAT endpoints for *E. histolytica* before and after cryopreservation

Stocks	Titres at which positive	
	Before freezing	After freezing
Biswas	1/256 - 1/512	1/256
JEH	1/256	1/256
Irvine	1/512	1/256
Atkinson	1/256	1/256
NIH:200	1/256-1/152	1/256
Bean	1/256	1/256
Thirer (parent)	1/512	1/256

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Atkinson	1/256	1/256
NIH:200	1/256-1/152	1/256
Bean	1/256	1/256
Thirer (parent)	1/512	1/256

Generation-time of *E. histolytica*: Effect of age of culture, source of stock and cryopreservation

Experiment 4a: Age of culture

The generation-times of *E. histolytica* stocks were measured as described earlier. Preliminary experiments were carried out to test the effect of age of culture on generation-time.

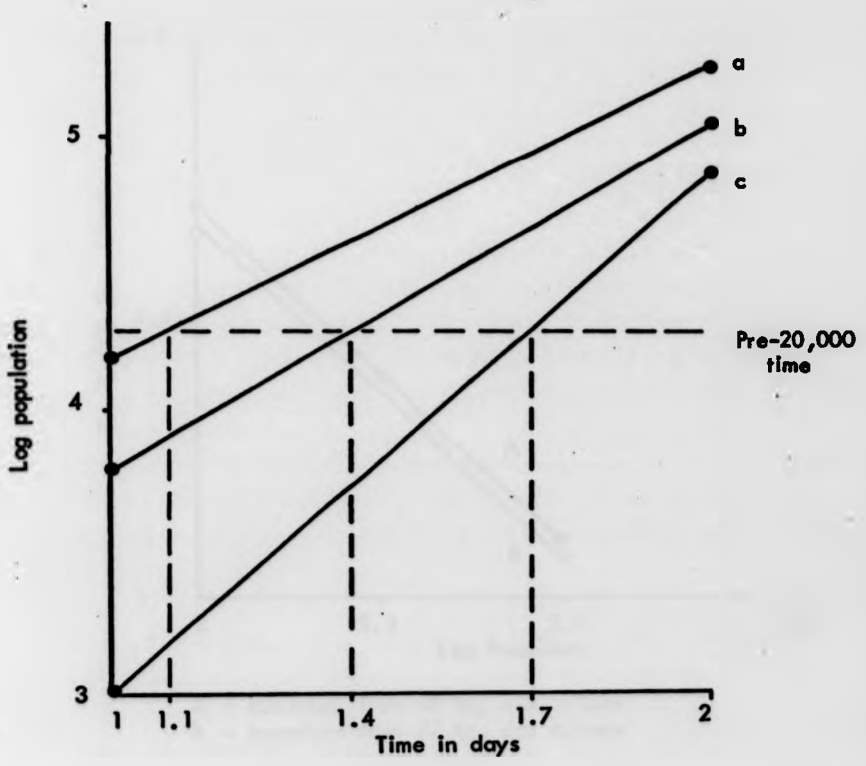
48 and 72-hour-old cultures of Thesiger were harvested and suspended in RSM. These were used in two separate experiments. For each experiment, 3 groups of 16 culture bottles were set up. Each group received an inoculum of 0.1 ml. Group 1 had 100 amoebae/inoculum, group 2 had 200 and group 3, 400. They were all incubated at 37° C. At 24-hourly intervals, four cultures from each group were removed from the incubator and the number of trypan-blue-excluding amoebae was estimated.

The average number of trypan-blue-excluding amoebae below and above 20,000 were plotted against time for each group of inocula. The mean pre-20,000 time was interpolated on the graph. Fig. 29 represents the graphical method used for the estimation of pre-20,000 time.

The results obtained from both experiments showed a negative correlation between inoculum size and the pre-20,000 time; as inoculum size increased, the pre-20,000 time decreased (Fig. 30). For example, the mean pre-20,000 times for the cultures which received inocula of 100, 200 and 400 amoebae from 48-hour-old cultures were 1.7, 1.4 and 1.1 days respectively. However, this linear relationship broke down when inocula were as low as 50 or as high as 800 (results from preliminary investigations). Inocula of 100, 200 and 400 amoebae were subsequently used in growth-rate measurements.

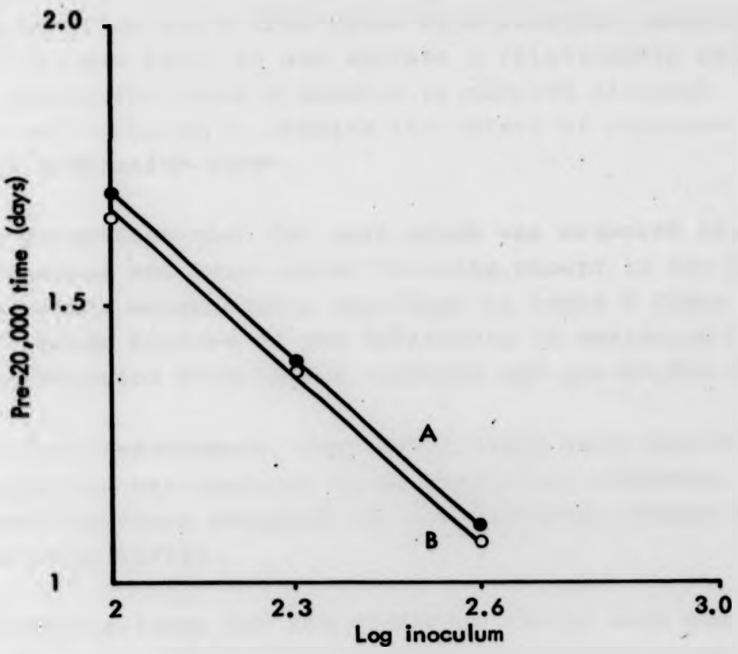
The slopes of the 2 regression lines were estimated as -0.45 using regression analysis, and the generation-time for

Fig 29
Graphical method for the estimation of pre-20,000 time (Thesiger)



a = Inoculum of 400 amoebae
b = Inoculum of 200 amoebae
c = Inoculum of 100 amoebae

Fig 30
The relationship between pre-20,000 time and log inoculum of cultures (stock Thesiger)



A - inoculum from 48 hr. old culture
B - inoculum from 72 hr. old culture

Thesiger was calculated as 0.135 day in both cases. Correlation coefficients were -1.0 (for 48 hour-old culture) and -0.9998 (for 72-hour-old cultures), $p < 0.001$ in both cases.

Experiment 4b. Source of stock and cryopreservation

The generation-times of 4 different amoebal stocks were measured. 2 of the stocks used were derived from asymptomatic carriers and 2 from cases with clinical amoebiasis. The exercise was first to see whether a relationship existed between generation-times of amoebae *in vitro* and clinical diseases and secondly, to examine the effect of cryopreservation on generation-times.

The generation-time for each stock was measured twice before freezing and twice after freezing, except in the case of Biswas where measurements were made at least 5 times before freezing because of the difficulty in obtaining a strong correlation between log inoculum and pre-20,000 time.

For each measurement, regression lines were calculated and correlation was analysed using regression analysis. The generation-times obtained for the different stocks are given in Table XLVIII.

Generation-times for the different stocks were compared before and after freezing using the standard error of the difference of means to give approximate standard normal deviate values.

When generation-times for each stock, measured on more than one occasion were compared, no significant difference was observed. Therefore it was valid to use mean values for comparison of generation-times.

When the mean generation-times for each stock were compared before/and after freezing, significant differences were

Table XLVIII

Measurements of generation-time of stocks of *E. histolytica* before and after cryopreservation

Origin of Stock	Amoebal Stock	Generation-time in days			
		Before freezing		After freezing	
		g*	mean g & SE	g	mean g and SE
Asymptomatic cases	JEH	0.135 0.135	0.135 ± 0.064	0.26 0.33	0.296 ± 0.01
	Thirer	0.195 0.195	0.195 ± 0.005	0.08 0.10	0.089 ± 0.013
Symptomatic amoebiasis	Biswas	0.45 0.43 0.39	0.428 ± 0.008	0.499 0.45	0.476 ± 0.026
	Irvine	0.287 0.299	0.293 ± 0.005	0.10 0.13	0.114 ± 0.008

*g = generation time.

observed except in the case of Biswas ($p < 0.001$).

It appears that freezing affects generation-times in different ways. The generation-times of stocks Thirer and Irvine decreased after freezing; that of JEH increased, and in the case of Biswas, it remained unchanged.

The very significant ($p < 0.001$) differences between generation-times of stocks Irvine and Biswas which were derived from clinical cases suggest that no correlation exists between generation-time and pathogenicity of *E. histolytica*.

Experiment 5. The effect of cryopreservation on the ability of *E. histolytica* to agglutinate with concanavalin A

Con A-induced agglutination was reported by Trissl *et al* (1977) to be related to pathogenicity of *E. histolytica* stocks. The agglutination reaction of stocks of amoebae derived from the different iso-enzyme variant groups, and origination from symptomatic and non-symptomatic case of amoebiasis was studied first to confirm that such a relationship existed and to test whether the ability to agglutinate with Con A was retained after cryopreservation.

Stocks JEH and Thesiger (group I), Irvine, Atkinson and NIH:200 (group II) and Thirer (group III) were studied. All were cultivated in association with *Es. coli - B* in RSM except NIH:200 which was cultivated axenically in Diamond's medium or in association with *Crithidia* in TTY medium.

The agglutination reactions were studied using con A at the final concentrations of 100, 50, 25 µg/ml and the procedure described earlier was followed.

All attempts to induce agglutination in the stocks cultivated in Robinson's medium failed. However, agglutination was easily induced in axenic and *Crithidia* cultures

of NIH:200. The degree of agglutination, as shown by clump size (see Figs. 31A,B,C,D), was proportional to con A concentration. The results as judged by the numbers of cells forming a clump are given in Table XLIX.

Table XLIX

Agglutination induced by con A of living *E. histolytica* stock NIH:200

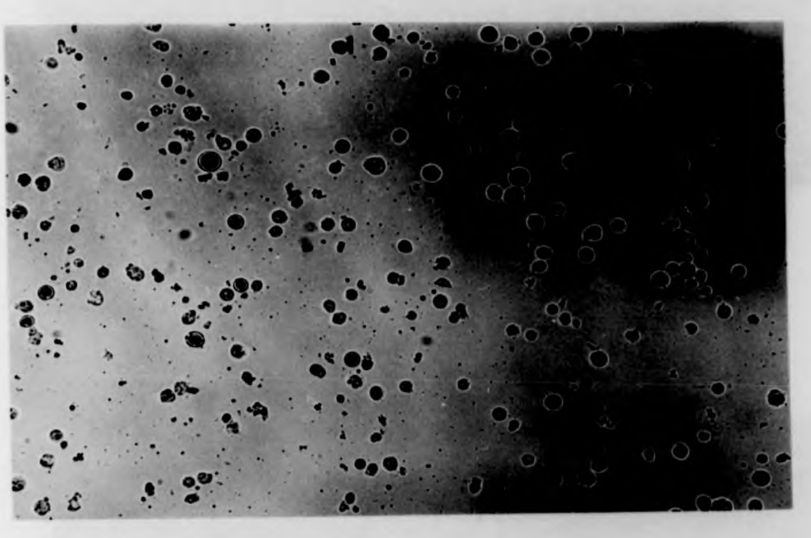
con A concentration	Number of amoebae forming a clump
100 µg/ml	40-hundreds - very large variable clumps
50.0 µg/ml	10-40 - small clumps interspersed with isolated amoebae
25.0 µg/ml	3-10 - many isolated amoebae interspersed with small clumps
Control (PBS)	0 - No clumps observed

It was thought that the presence of *Es. coli - B* in Robinson's medium might probably be responsible for inhibiting amoebal response to con A. An experiment was performed to test this.

A small, thick suspension of *Es. coli - B* in PBS was prepared and was added to a culture of axenic NIH:200 and incubated for 2 hours at 37° C. The amoeba/bacteria culture was then washed by centrifugation, resuspended in PBS and prepared as usual for a con A-agglutination experiment. No change was observed in the pattern of agglutination. There was agglutination of amoebae and bacteria together in the same clump.

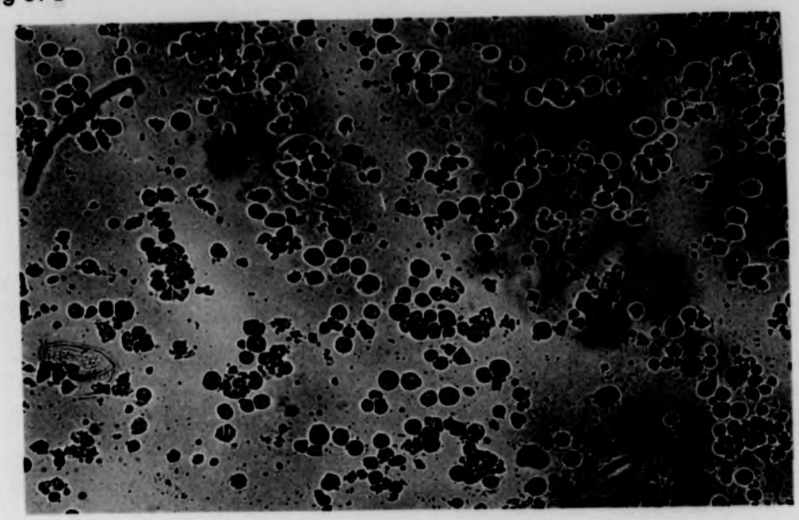
When bacteria failed to inhibit agglutination induced by con A, it was thought that perhaps the rice-starch granules were responsible for the inhibition by binding the available con A molecules. So, another experiment was per-

Fig 31 A



A. Control, amoeba in PBS, no agglutination x 100

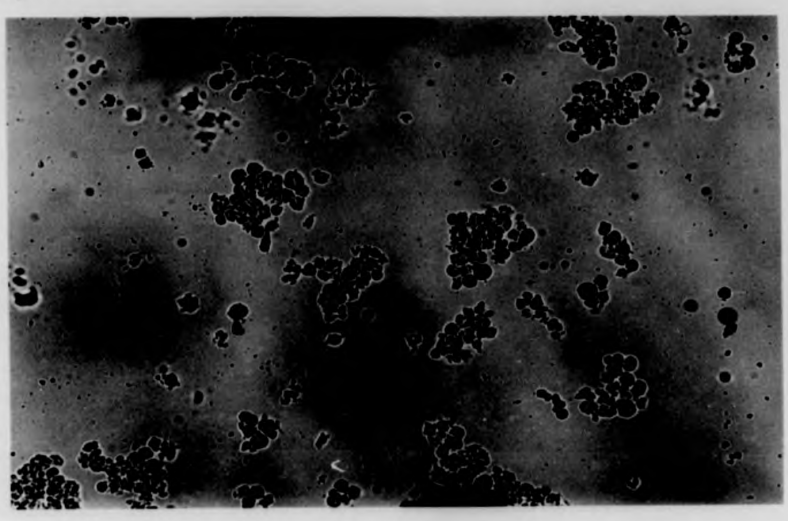
Fig 31 B



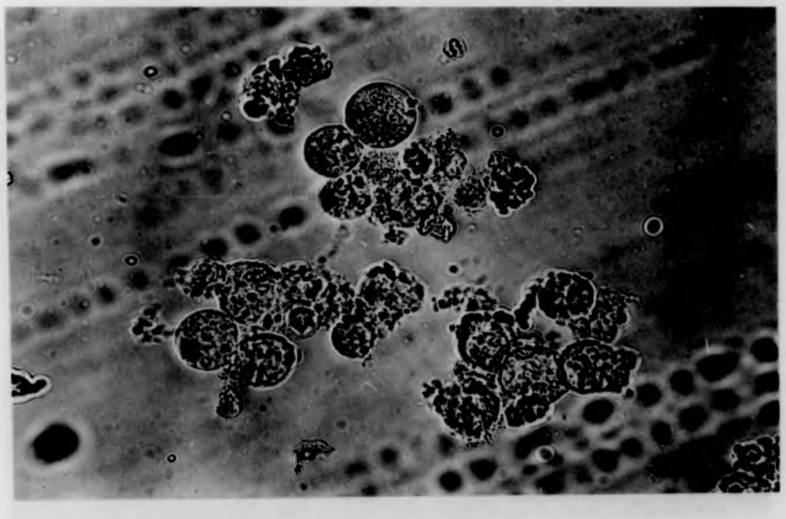
. Agglutination with 25 µg/ml con A x 100

Agglutination with 25 µg/ml con A. x 400

Fig 31C

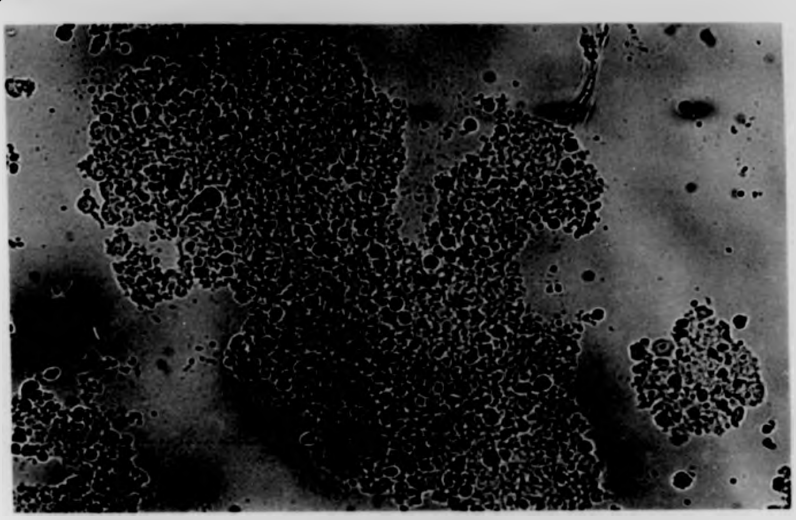


Agglutination with 50 µg/ml con A. x 100

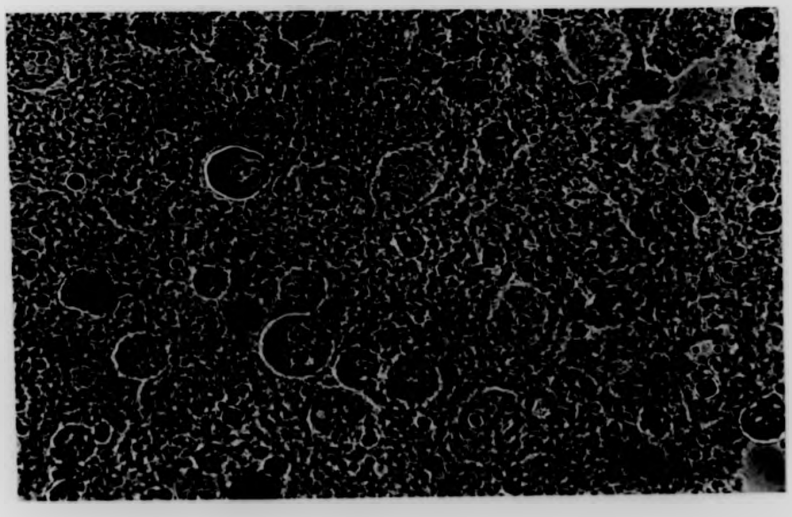


Agglutination with 50 µg/ml con A x 400

Fig 31 D



Agglutination with 100 µg/ml con A. x 100



Agglutination with 100 µg/ml con A x 400

Fig 31

Showing concanavalin A-induced agglutination of E. histolytica (NIH:200) axenically cultivated in Diamond's TPS-1 medium. Photomicrographs taken under phase contrast.

magnification x 400

A. control. PBS alone, no agglutination

B. agglutination with 25 µg/ml con A. Many isolated amoebae, few clumps, 3-10 amoebae/clump.

C. agglutination with 50 µg/ml con A. Few isolated amoebae, many clumps, 10-40 amoebae/clump.

D. agglutination with 100 µg/ml con A. No isolated amoebae, very large clumps, 40-hundreds of amoebae/clump.

formed using NIH:200 which had been incubated with rice starch. Again, no inhibition was observed; amoebae engorged with rice starch granules agglutinated with the free rice-starch granules. It is noteworthy that bacteria in cultures in Robinson's medium actually agglutinated; so did the starch granules but not the amoebae.

If starch and bacteria failed to inhibit agglutination of NIH:200 (axenic) something in Robinson's medium must therefore have been responsible. Axenic NIH:200 was adapted to Robinson's medium and was used to test this hypothesis. No agglutination was observed: NIH:200 had apparently lost its ability to agglutinate in the presence of con A during its cultivation in Robinson's medium.

Because of the difficulty encountered in recovering axenic NIH:200 from frozen storage, it was not possible to study in this stock the effect of cryopreservation on con A-induced agglutination. However, agglutination was not found after cryopreservation in any of the stocks that did not agglutinate in the presence of con A before freezing.

Experiment 6. The effect of cryopreservation on the toxicity of *E. histolytica* to human leucocytes

The toxic effect of live *E. histolytica* trophozoites on human leucocytes was studied using axenic stocks NIH:200 and stock JEH grown in Robinson's medium. The experiment was carried out to see whether a different reaction would be observed in stocks from symptomatic and non-symptomatic patients and also to test whether this toxic effect was retained after successive freezings and thawings.

After incubating the leucocytes for 30 minutes, the polymorphonuclear leucocytes spread out (Fig. 32A) and began to move. When amoebal suspensions were added, it was observed that the leucocytes moved towards the amoebae. It was also observed that the number of leucocytes responding to the

amoebae depended not only on the concentration of living amoebae in the suspension added but also on the distance between the leucocytes and the amoebae.

When there were too many amoebae in relation to leucocyte number, the leucocytes did not move so far, but when fewer amoebae were present in the suspension amoeba-directed movement was easily observed. It was observed that not all leucocytes reacted.

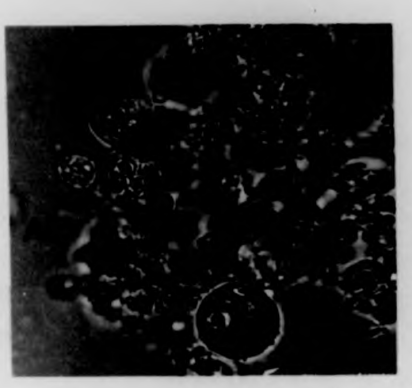
As observed by Jarumilinta and Kradolfer (1964) leucocytes rounded off 10-15 minutes after coming into contact with the amoebae. After about 30-45 minutes, the leucocyte cytoplasm completely disintegrated as shown in Figs. 32B, C and D.

Under the same experimental conditions *Es. coli - B* was not observed to exhibit a toxic effect on human leucocytes even after 45-60 minutes of contact.

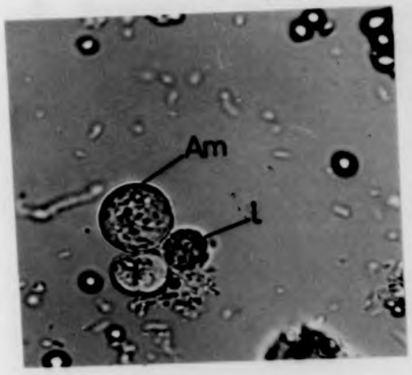
The response of leucocytes to amoebae and eventual death of leucocytes was not different in the two stocks studied, suggesting that the response was not dependent on stock and its origin but was a characteristic of the species. No change was observed in the behaviour of repeatedly frozen and thawed samples of stock JEH. It was, however, not possible to test change in the response of leucocytes to axenic NIH:200 after freezing because of the difficulty in recovering it.

Fig 32

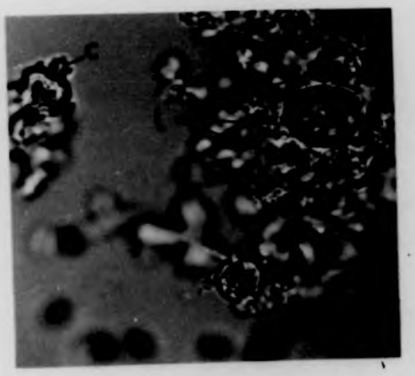
A



B



C



D

Fig 32

Showing changes in human polymorphonuclear leucocytes brought about by contact with E. histolytica (stock JEH) grown in Robinson's medium.

Photomicrographs taken under phase-contrast:magnification x 400

A. Normal leucocyte with extended pseudopodium

B. leucocyte (a) still active attached to trophozoite, leucocyte (b) has now rounded off 15 minutes after contact.

C. An amoebal trophozoite with rounded leucocyte attached to it.

D. Degeneration of leucocytes 45 minutes after contact with trophozoite (c) active leucocyte.

Am = amoeba, l = leucocyte

D DISCUSSION AND CONCLUSIONS

Cryopreservation of parasitic materials should be done as soon as possible after isolation to avoid changes in the biological characteristics through several serial passages. But, in most cases, before *E. histolytica* can be cryopreserved it is necessary to cultivate it *in vitro* until suitable numbers are obtained. The cultivation of amoebae from faecal material is not always easy since two important problems have to be solved:1) unsuitable concomitant bacterial flora have to be eliminated; 2) populations are likely to be mixed, therefore cloning must be done.

The conventional way of eliminating faecal flora is by incorporating in the culture medium antibacterial compounds such as acriflavine and trypaflavine (Dobell and Laidlaw, 1926a;Dobell and Neal, 1952) or antibiotics such as penicillin (Jacobs, 1947; Wittner, 1968), penicillin/streptomycin (Shaffer and Frye, 1948) or by treatment of cysts with mercuric chloride (Meleney *et al*, 1940; Rees *et al*, 1941), hydrochloric acid (York and Adams, 1926b; Cleveland and Sanders, 1930; Adler and Foner, 1941; Singh *et al*, 1963; Robinson, 1968b), emetine and chlorine (York and Adams, 1926b). Other methods which do not require the use of chemicals have also been used, for example micro-isolation of cysts using a micromanipulator (Rees *et al*, 1941; Chinn *et al*, 1942).

The treatment of amoebal cultures with antibiotics or other chemicals is not very satisfactory in view of the fact that some chemicals such as mercuric chloride and potassium permanganate are lethal to amoebic cysts (York and Adams, 1926b; Adler and Foner, 1941) and antibiotics do not always eliminate resistant vegetative forms of bacteria or viable bacterial spores (Neal, 1952). When this happens, treatment is usually repeated in successive cultures until the concomitant organisms have been eliminated (Neal, 1952).

The microisolation of cysts by micromanipulator, on the other hand, is a tedious process which requires skill and

experience (Rees *et al*, 1941). Although a simple method of cyst or trophozoite isolation has been developed (Farri, 1978) it is still time consuming. The sucrose-gradient method of isolating cysts from faecal concentrates is preferable because it is quicker and sucrose does not interfere with the biological characteristics of amoebae. It can also be used in separating mixed populations. For example, it was possible to separate the small cysts of *E. nana* from large cysts of *E. histolytica*.

The production of vigorous cultures of amoebae is impossible in the presence of fungal overgrowth such as *Blastocystis*. Elimination of associates by chemical treatment usually requires several serial passages which may result in biological changes of stocks; virulence, for example, may be lost (Thompson *et al*, 1954; Elsdon-Dew, 1958; Neal, 1958; Vincent and Neal, 1960; Wittner and Rosenbaum, 1970; Phillips, 1973; Zaslavaskaya, 1975; etc.). Elimination of concomitant organisms as soon as possible after isolation cuts down on labour, reduces the number of serial passages and hence chances of changes in biological characteristics.

The problem of *Blastocystis hominis* in *E. histolytica* cultures was solved by treatment with chemicals such as HCl in 0.1-0.2 M concentrations (Cleveland and Sanders, 1930; Adler and Foner, 1941; Robinson, 1968b) or acriflavine (Singh *et al*, 1963). Unfortunately, these chemicals and others such as 4.4% (w/v) sodium bicarbonate and 5,000 units Nystatin/ml have failed in my hands. I was, however, able to eliminate *Blastocystis* from my *E. histolytica* cultures by washing them in tap water (provided that sufficient bacteria were incorporated in the primary cultures). Meleney *et al* (1940) reported eliminating concomitant organisms from their cultures by first washing the cyst concentrates in sterile tap water before treatment with mercuric chloride. It is possible that the tap water they used had already lysed the *Blastocystis* before chemical treatment. However, Dobell and Laidlaw (1926b) were unable to eliminate *Blastocystis* from their cultures

after treatment with distilled water and neither was I. It is difficult to explain this observation.

Cultures established *in vitro* from human amoebic infections of the gut may consist of one, two or more species as shown with the original cultures of stock Saigon. Even cultures of a single species are likely to be genetically heterogenous. Krupp (1966) showed that within the true *E. histolytica* group some minor antigenic differences were detectable using immunoelectrophoresis. Genetic heterogeneity was also reported within a population of long established *E. histolytica* strains using trophozoite size as genetic marker (Goldman and Davis, 1965). In the isoenzyme characterization studies of *E. histolytica* using thin-layer starch-gel electrophoresis, it was discovered that stock Thirer was a mixed population, and possibly stock AM25 also. The heterogeneity of stock Thirer was detected when parent and clone showed different GPI and PGM bands in electrophoretic runs separately performed by me and by Mr J E Williams. A run for HK (GK) with the two samples placed side by side confirmed that parent Thirer was a mixed population because it had bands common to group I as well as group III (see Fig. 28). Although the cathodally migrating band of AM25 was never seen with any of the amoebal stocks already tested, it is likely to belong to some other species present in the isolate. There was no chance of proving this because of lysate shortage.

In view of all these points it is necessary to use clones for precise biochemical, physiological and genetic studies. It is also necessary to preserve original isolates as representatives of the variability of populations in nature.

The need to cultivate *E. histolytica* in a suitable medium after retrieval from low temperature storage has been emphasized (Neal *et al*, 1974; Raether and Uphoff, 1976). The axenic medium TPS-1 is clearly an unsatisfactory medium for

this purpose since it requires a large number of viable cysts (15-250/ml of medium) to initiate axenic cultures (Singh *et al*, 1973; Dutta, 1976) and 5,000 trophozoites/ml to maintain them (Diamond, 1968b). Besides, initial growth is extremely slow. Dutta (1976) reported that an inoculation of 250 amoebae/ml would produce, in his modified TPS-1 medium, maximum yield in about 2 weeks. The best medium therefore has to be sought in the more conventional culture medium in which concomitant organisms are present.

In an attempt to find the best culture medium which will support growth of *E. histolytica* from small inocula, the growth curves of three stocks were studied in Robinson's medium (Robinson, 1968a), Locke-egg-serum (LES) medium (Boeck and Drbohlav, 1925) and modified Locke-egg-serum (M-LES) medium (Harinasuta and Harinasuta, 1955).

The growth curves of all the different isolates in the different media followed the classical growth curve obtained with bacteria, with a prolonged lag phase which lasted up to 24 hours, a log phase and a stationary phase. There was also a decline phase as already reported (Balamuth and Howard, 1946; Jacobs, 1950; Harinasuta and Harinasuta, 1955 and Abioye, 1971).

The best initial growth was obtained for all the stocks in Robinson's medium (see Fig 13), although the maximum growth reached within 48 hours of inoculation into Robinson's medium was not significantly higher than it was in LES medium. In M-LES medium, the growth of Irvine was similar to that in LES medium, but JEH and Thirer showed remarkably poor growth: maximum population was reached 72 hours after inoculation.

The occurrence and length of the lag phase depended on the type of medium, the inoculum size and the stock being investigated. For example, a lag phase observed with a low inoculum (1.7×10^4/ml) of Fenn was very much shortened when the inoculum size was increased to about 4×10^4 amoebae/ml

(Fig 9). No lag phase was observed in the growth of Thesiger when the initial inoculum was increased from 0.25 x 10^4 to 1 x 10^4/ml (cf Figs 7 and 8). However, it is not clear why an inoculum of 0.25 x 10^4/ml should show a longer lag phase than the one of 0.2 x 10^4/ml (Fig 10) of the same stock Thesiger when experimental conditions were practically the same except, maybe, that the estimated number of viable amoebae as judged by trypan-blue-exclusion was unreliable.

The same initial inocula of stocks Irvine, JEH and Thirer exhibited a lag phase to a varying extent in LES and M-LES media but no lag phase was observed in Robinson's medium (cf Figs 13 A, B and C). Also, when the growth curves of various stocks were observed in Robinson's medium, starting with the same initial inocula of 1,000 amoebae/ml, a prolonged lag phase which lasted for 24 hours was observed for Irvine, Thirer and Biswas but was much shorter for JEH and Thesiger (see Fig 10).

However, Balamuth and Howard (1946) thought that the yield of amoebae in each culture medium would be a function of the medium itself rather than the size of initial population, if the limiting factors were in the growth-promoting capacities of the nutrients.

The lag phase which was always observed in amoebae grown in LES or M-LES media regardless of inoculum size was shortened when the culture media were inoculated with bacteria and preconditioned at 37° C before inoculation. Preconditioning at 37° C of Robinson's medium had no significant effect on either the growth pattern or the maximum yield, suggesting that the fresh medium already contained all the nutrients and conditions required for growth. However, a number of factors may be responsible for the very slow initial growth of *E. histolytica* in LES and M-LES media. McConnachie (1955) and Botero (1961) reported that, when amoebae were transferred from one type of culture medium to another, they usually did not grow well in the second

medium. The failure of my amoebae to grow well initially in these media was unlikely to be due to this factor because the stocks were cultivated in the different media for 3 weeks with 2 weekly passages before they were used. Besides, harvests from the 3 different culture media were pooled for the comparative growth studies. It is likely that other factors may have been responsible.

Harinasuta and Harinasuta (1955) reported that the lag phase of their strains of amoebae decreased when the number of bacteria increased or when the cultures were preconditioned with bacteria before amoebae were inoculated. The lag phase increased when the number of bacteria was too small or too large. The number of *Es. coli - B* used in LES and M-LES media in this study were not counted but it was comparable to that used in Robinson's medium in the same experiments. Perhaps an increase in bacterial flora would have improved growth of amoebae in these media but then results would not have been comparable with those obtained with RSM.

Another factor could be that *Es. coli - B* alone is incapable of providing the initial growth conditions (apart from low redox potential) for *E. histolytica* in LES and M-LES media. The best results obtained by Harinasuta and Harinasuta on the growth of the *E. histolytica* stocks which they used were obtained from those grown with a mixture of bacterial flora. Strain PA which was grown with *'B. coli' (Es. coli)* alone showed inferior growth throughout their studies.

The patterns of pH changes in the different fresh and preconditioned media were also studied here. The pattern followed that observed by previous workers with very minor differences (Shaffer and Frye, 1948; Jacobs, 1950; Harinasuta and Harinasuta, 1955; Rees *et al*, 1960). In fresh LES medium, there was an initial drop in pH from 7.6 to 4.7 followed by a gradual increase to 5.6. In fresh Robinson's medium there was an initial drop in pH from 6.9 to 6.2 which then remained

steady for the rest of the experiment (Fig 14a). The pH changes observed in the preconditioned media were more or less the same as in fresh media except that the pH values of LES and M-LES media at the time of inoculation were 5.1 and 6.2 respectively. That of Robinson's medium remained the same.

The finding that there was no correlation between initial drop in pH and the commencement of amoebal growth agrees with the observations of previous workers (Chang, 1946; Balamuth and Howard, 1946; Jacobs, 1950), although there was no correlation between initial drop in pH and the rapid multiplication of bacteria which was shown by the very low redox potential. It was concluded that the onset of amoebic growth is independent of pH values within the range studied.

The relationship between O-R potential and amoebic growth was studied in fresh and preconditioned media. The initial potentials in fresh Robinson's, LES and M-LES media were +100, +230 and +320 mv respectively. These dropped to their lowest levels below -300 mv within 12 hours, but rose again within 24 hours to a low positive level (+70 mv) in Robinson's medium and to lower than -150 mv in LES and M-LES media where they remained with slight fluctuations for the rest of the experiment (See Fig 14a). The pattern of O-R potential observed in the preconditioned media did not differ from those observed in the fresh media except in slightly lowered initial values. The initial fall of potential in preconditioned RSM (to -100 mv) was not as low as observed in the fresh medium but it did rise to a low positive level within 24 hours as observed earlier.

Many workers who studied the effect of O-R potential on the growth of *E. histolytica* with mixed bacterial flora have shown that the best growth of amoebae occurred when O-R potential was strongly negative (Chang, 1946; Jacobs,

1950; Harinasuta and Harinasuta, 1955) and that, at low positive and slightly negative levels, the trophozoites were killed (Chang, 1946). Working with axenic cultures, Singh *et al* (1973) reported that a negative O-R potential was necessary to initiate axenic cultures of *E. histolytica* from small inocula. The same authors (1974) showed that a strong negative O-R potential not only cut down the lag phase but also helped in obtaining maximum population of *E. histolytica* in axenic cultures. However, Wittner (1968) suggested that amoebae probably do not depend entirely on anaerobic metabolism but that they also utilize atmospheric oxygen in axenic cultures since they tend to migrate to the top of the medium as the O_2 at bottom probably becomes depleted. Montalvo *et al* (1971), using Shaffer and Frye medium with attenuated bacterial flora, showed that *E. histolytica* is capable of consuming oxygen in cultures in the absence of any exogenous substrate because of its high reserve of glycogen.

The low O-R potentials obtained in this study with amoebal cultures in LES and M-LES media with *Es. coli - B* were comparable to those obtained by previous workers with mixed bacterial flora. Jacobs (1950) found that when *E. histolytica* was grown in Locke-egg-ringer (LER) medium with *Clostridium perfringens*, the O-R potential during amoebic growth remained very low, below -240 mv, but when the bacteria were changed to organism 't', the potential during growth was at a low positive level and remained so throughout. A comparable result was obtained here with amoebae grown in RSM with *Es. coli - B*. At the time of maximum proliferation, the potential rose to +50 mv and fluctuated between low positive and slightly negative levels thereafter. The results of Jacobs with organism 't' in LER medium and mine with *Es. coli - B* in RSM seem to suggest that there was no correlation between rapid amoebal growth and reducing potentials of the media. It was also suggested that a range of O-R potentials probably exists at which *E. histolytica* can proliferate provided there is an adequate supply of substrate (Jacobs, 1950).

In view of these various and rather conflicting results, it is suggested that some factors other than the O-R potentials are operative in providing the conditions for maximum growth of amoebae in cultures, especially in RSM. Factors such as the substrate present in the culture medium, the capacity or quantity of reducing substances have been suggested to cause fluctuations or variations in O-R potential measurements (Jacobs, 1950). Boyd and Reed (1931) found that an addition of glucose to the broth in which bacteria *(Es. coli)* were grown caused a more precipitous fall in O-R potential to a negative limiting value than occurred in broth without glucose. The glucose in LES and M-LES media may have provided the additional supply of substrate for the bacteria and might have kept the potentials down at very low levels. The kind of nutrient present in a medium has also been found to influence the respiratory needs of the organisms present in it (Jacobs, 1950). This may explain why RSM failed to maintain a negative redox potential; *Es. coli - B* being a facultative anaerobe could revert to anaerobic respiration after initial growth as an aerobe in Robinson's medium.

Chang (1946) reported that the life-span of *E. histolytica* in culture *in vitro* depended on how long the O-R potential in the medium is maintained low enough to permit growth of amoebae, provided there is enough food supply for the activity of both bacteria and amoebae. The results with amoebae grown in RSM with *Es. coli - B* showed that a low O-R potential was not necessary for long life, because the O-R potentials fluctuated between slightly positive and slightly negative values and amoebae survived for up to 2 weeks in culture without passage.

Although it cannot be claimed that Robinson's medium offers the best growth conditions for *E. histolytica*, it can be concluded that, apart from the low initial O-R potential, it contains certain components, probably nutrient substrates yet unknown, which promote early proliferation of the amoebae and will support growth from an inoculum containing one amoeba.

An important aspect of this work was to develop an assay method by which different conditions could be compared. The ID_{63} assay method, originally developed for trypanosomes (Lumsden *et al*, 1963), enables quantitative determination of *E. histolytica* viability and provides a means by which optimal conditions can be established after retrieval from low temperature storage. It allows conclusions to be drawn about the effects of various treatments on the amoebae.

Quantitative measurements based on the ability of amoebae to exclude or take up dyes have been generally used as a measure of *E. histolytica* viability (Diamond, 1964; Neal *et al*, 1974; Raether and Uphoff, 1976).

With regard to exclusion of vital stains, it is necessary to be careful in the interpretation of viability results (Neal *et al*, 1974; Raether and Uphoff, 1976; Raether *et al*, 1977). Stulberg *et al* (1962) found no correlation between viability assessment of some tissue-culture cells based on trypan-blue-exclusion and their ability to attach to the plating surface and multiply (which they called the plating efficiency). Neither did Raether and Uphoff (1976) find any correlation between the percentage of unstained, cryopreserved and thawed axenically cultivated amoebae and their potential to establish themselves in culture.

In my experience, the number of trypan-blue-excluding amoebae did not correlate with the number of viable organisms given by ID_{63} estimates, neither did resistance to staining with trypan-blue correlate with growth after thawing. There were two very marked instances of this lack of correlation, 1) when *E. histolytica* was frozen in 7.5 and 10% (v/v) glycerol and 2) when amoebae were frozen in 5, 10 and 20% (w/v) PVP. In these instances, after thawing, up to 25% of the glycerol-treated and 100% of the PVP-treated trophozoites resisted staining and yet no growth was observed when cultured.

Additives such as PVP may perhaps completely coat the surface of cells and prevent the uptake of dyes. Perhaps there exist other non-permeating polymeric cryoprotectants which behave in like manner. In such cases careful interpretation of results must be made and other methods of assay must be sought because assessment of viability based on dye-exclusion, for such cryoprotectants, will not only be unreliable but impossible. An assay method with an end point such as the ID_{63} assay is recommended.

Although it was argued that the mathematical basis of Lumsden's tables 'is not statistically justified' (Overdulve and Antonisse, 1970a), my results from 2 dilution series after infectivity titrations using Lumsden's method were comparable to those using the Exact Probability test. For example, results from two dilution series of a frozen amoebal suspension ($^{log}/ID_{63}$ = 1.6 ± 0.3) and an unfrozen control sample ($^{log}/ID_{63}$ = 4.4 ± 0.5) were compared using the Exact Probability test and the ID_{63} method.

Log dilutions	$\overline{1}$	$\overline{2}$	$\overline{3}$	$\overline{4}$	$\overline{5}$
A Unfrozen control = r_1 number of cultures positive	6	6	6	1	0
B Frozen sample = r_2 number of cultures positive	2	0	0	0	0
Number of cultures inoculated	6	6	6	6	6

where $r_1 + r_2$ are numbers of positive cultures.

The viability of the two samples can be compared using the formula

$$X_1^2 = \frac{(r_1 - r_2)^2 \cdot 12}{R \cdot (12 - R)}$$

where $R = r_1 + r_2$; X_1^2 = Chi squared of dilution 1.

When the five Chi squared values have been separately calculated for each dilution, they are added. The significance of the differences between A and B can be tested using the number of degrees of freedom (= number of dilutions) using the standard normal deviate (SND) table (Armitage, 1971). In this case $p < 0.001$.

When the ID_{63} estimates were compared in the approximate SND test using the formula:

$$SND = \frac{x_1 - x_2}{SE(d)}$$

where x = ID_{63} estimate, and
$SE(d)$ = Standard error of the difference
$SE(d) = \sqrt{SE\ (x_1)^2 + SE\ (x_2)^2}$

the significance of the difference between the two samples A and B was comparable to that of the Exact Probability test because p was also <0.001.

It can be concluded that, in spite of the comments of Overdulve and Antonisse, ID_{63} results are amenable to the usual statistical analysis.

It was also argued that it is inherent in the ID_{63} method that at least half of the number of mice used do not contribute substantially to the result (Overdulve and Antonisse, 1970a). Admittedly, this is correct but a careful preliminary determination of the approximate ID_{63} value will give an idea of the dilutions in the vicinity of the ID_{63} to be inoculated. These authors also argued that some factors other than the infectivity of the organism e.g. technical failures, inhomogeneity of animal population and their response to infection may obscure results and therefore make exact determinations of infectivity impossible, and it is only when these factors are reduced to a minimum that the ID_{63} can be used with success.

These arguments may be true where animals are used as the recipients of inocula but, fortunately, such problems as antibody formation and heterogeneity of recipients are usually not encountered when cultures are used. In any case, when homogenous suspensions of organisms are used, as was the case in this study, and inoculations are made into culture media under identical experimental conditions, the ID_{63} assay can be successfully used for measuring by titration the viability of *E. histolytica* populations.

Using the ID_{63} method, it was shown that young cultures (48-hours-old) of *E. histolytica* in Robinson's medium consist of significantly higher proportions of viable amoebae than the 96-hour-old cultures although the number of trypan-blue-excluding amoebae is greater in the latter. This is probably because at 48 hours the number of daughter amoebae being produced is slightly higher than the number dying, and at 96 hours the opposite is happening - more amoebae are dying than are being produced. It is therefore not surprising that it was more difficult to isolate clones into RSM from 72-hour-old cultures than from the 48-hour-old ones. Although the viability of 24-hour-old cultures was not measured, it is believed that the percentage survival at this time would exceed that at any other time. The ease with which clones were produced from 24-hour-old cultures suggests that this is the case.

The ID_{63} method was also used to demonstrate that amoebae could withstand temperatures as low as 0^{o} C for at least 2 hours without loss of viability.

The cryopreservation of *E. histolytica* at very low sub-zero temperatures is not new. What is generally lacking in the literature is the systematic study of the various factors which tend to influence the viable recovery of frozen organisms after thawing. The aim of this work was to study the various factors involved in cryopreservation of *E. histolytica* with a view to producing a high percentage survival of the amoebae after thawing.

Using the ID_{63} assay method, it was shown that a cooling rate of 1° C/min, following treatment with 7.5% (v/v) DMSO equilibrated at 37° C for 15 minutes, offered the best cryoprotection to *E. histolytica*. Maximum percentage viability observed after retrieval from storage at -196° C was 12.5%.

In this study, the effect of 6 cryoprotectants of different types, namely DMSO, glycerol, PVP, sorbitol, methanol and ethanol, were evaluated in unfrozen amoebal samples. It was found that sorbitol, methanol and ethanol at the concentrations used were not toxic to the amoebae. Although effective in preventing freezing damage, glycerol, DMSO and PVP were found to be toxic in themselves.

The toxic effects of cryoprotectants are related to their concentration, the temperature and duration of exposure, so that when the concentration increases toxicity increases (Karrow, 1969). The toxic effect of glycerol on unfrozen samples of *T. vaginalis* was demonstrated by Lumsden *et al* (1966). These authors found that there was an immediate distortion of the organisms when placed in a suspension containing 10% (v/v) glycerol. Collins and Jeffery (1963) also observed an apparent pre-freeze toxicity of 10% (v/v) glycerol to *P. gallinaceum* since there was a drop in their ID_{63} estimate. The toxic effects of DMSO have also been reported. Warhurst (1966) using an assay based on 'pre-2% period' reported that the treatment of *P. berghei* with 7.2% (v/v) DMSO without freezing reduced viability to 40-45%, suggesting a harmful effect of DMSO (he assumed that the viability of the untreated, unfrozen sample was 100%) and that, after freezing with DMSO to -78° C, the percentage viability decreased to about 3-5%. Herbert *et al* (1968) reported also that the infectivity of a suspension of trypanosomes treated with cryoprotectants (glycerol and DMSO) prior to rapid freezing and storage at -196° C was less than when the suspension was frozen without cryoprotectants. Overdulve and Antonisse (1970b) found that incubation of *B. rodhaini* with 5 and 10% (v/v) DMSO at 0° C for 2 hours without

freezing caused a reduction in infectivity to mice. The results obtained from this study confirm the results of previous workers.

The mechanisms of cryoprotectant damage were not investigated here, but it has been suggested (Chang and Simon, 1968) that DMSO affects enzyme systems of cells without affecting the permeability characteristics. It seems that not much is known about toxicity of DMSO or glycerol to cells.

Flux across cell membrane is temperature-dependent and varies not only with pH but also with the concentration of the cryoprotectant (Meryman and Hornblower, 1972). So that when cryoprotectants are administered at a lower temperature, there is a decrease in their toxicity at any given concentration. This temperature effect was demonstrated for glycerol on rat heart muscles (Karrow and Webb, 1965), on bull spermatozoa (Sherman, 1963) and on *E. histolytica* (Smith, 1961). In this study, glycerol was found to be non-toxic to *E. histolytica* at 0° C. But, as temperature increased, there was a progressive loss of viability; at 37° C the toxic effect was noticed most. These observations presumably relate to the increase in permeability of cell membranes to glycerol as temperature rises. As for glycerol, the toxic effect of DMSO is positively correlated with increase in temperature and concentration. The temperature effect on DMSO toxicity was demonstrated on rat heart muscle (Karrow and Webb, 1965) and on *B. rodhaini* (Dalgliesh, 1972). Dalgliesh found that when *B. rodhaini* was incubated with 3 or 4 M (approximately 21 or 28% (v/v)) DMSO at 38° C, there was a complete loss of infectivity to mice, whereas at 4° C loss of infectivity was slight. At comparable molar concentrations of glycerol (22 or 29%(v/v)) infectivity was not eliminated, but a more marked reduction was noticed at 38° C than at 4° C. It seems likely that the low infectivity obtained after the exposure of *B. rodhaini* to glycerol at 38° C was due to the toxic effect of

glycerol at this temperature. Persidsky *et al* (1965) demonstrated the temperature effect for PVP on rat bone-marrow cells. In this study the temperature effect was more pronounced in glycerol than in DMSO or PVP. It was shown that PVP was the least toxic of the three cryoprotectants below 37° C probably because it did not penetrate the cells, but as soon as it did at 37° C, and depending on its concentration (about 15%(w/v) or above), its toxic effect was exerted.

It appears that cryoprotectants have conflicting effects on parasites, toxicity on the one hand and prevention of freezing damage on the other. Cryoprotectants which penetrate cells protect them during freezing by preventing an excessive increase in the concentration of damaging salts as water in the suspension turns to ice. Therefore, for any cryoprotectant to exert its full protection it must be present within and around the cells (Lovelock and Bishop, 1959). In terms of cultivability of amoebae in Robinson's medium after freezing and thawing, this theory is supported by results obtained in this study for DMSO, PVP and glycerol. Except for PVP, these cryoprotectants are considered to offer protection to other cells during freezing only when they have permeated the plasmalemma. This is probably also the case with *E. histolytica*. Although not harmful in themselves to amoebae in the concentrations used, sorbitol, methanol and ethanol were not effective as cryoprotectants and it is not clear whether this was due to non-penetration of cells or to inadequate concentration. Perhaps a more detailed study would elucidate the underlying factors. It is equally important to stress that, during the whole exercise, amoebae did not survive freezing once without cryoprotectant no matter the rate of freezing used.

There is no evidence in the literature that PVP has been successfully used to cryopreserve parasitic protozoa, although Persidsky and Richards (1962) and Persidsky *et al* (1965) have reported its usefulness in preserving rat bone-marrow cells at a concentration of 15% (w/v) and at a free-

zing rate of 1° C/min. Their results indicate that PVP exerts external cryoprotection to bone marrow cells. The results obtained here using different temperatures of equilibration suggest that PVP needs to be taken in by *E. histolytica* before it confers cryoprotection. Lumsden *et al*, (1966) reported that when *T. vaginalis* was cooled in the presence of 10% (w/v) PVP by dropping the ampoule into methanol containing CO_2, none of the organisms survived after thawing. It is suggested that PVP can be cryoprotective for parasitic protozoa only when it has permeated the cell membrane and in the case of *E. histolytica* when cooling rate was 1° C/min. Although 15% (w/v) PVP was protective for *E. histolytica*, a lower or higher concentration was found to be ineffective. Perhaps at a lower concentration, a longer equilibration time would be required, at a higher concentration, a shorter equilibration time would be required, these were not fully investigated. PVP cannot permeate cell walls by simple diffusion because of its high molecular weight. Moreover, the high toxicity of PVP at 20% (w/v) may itself prevent active uptake by pinocytosis.

Glycerol, at 5% (v/v) concentration was also protective to *E. histolytica* but, like PVP, the percentage viability was so low that infectivity was lost at the lowest serial dilution. Fulton and Smith (1953) cooled *E. histolytica* stepwise to -79° C in the presence of 5, 10 and 15% (v/v) glycerol. After equilibration at 37° C for 1-2 hours, they were able to recover viable organisms from suspensions treated with 5 or 10% glycerol and frozen. They found 5% (v/v) more satisfactory than 10% (v/v) glycerol. I was unable to recover from low temperature storage *E. histolytica* which had been equilibrated with various concentrations of glycerol at 37° C. This is possibly explained when one considers that a simple exposure of *E. histolytica* to 7.5% (v/v) glycerol at 37° C for more than 15 minutes without freezing lowers the viability to less than 1% (as assessed by the ID_{63} method). And, unless there are many millions of amoebae in the frozen suspensions, it is unlikely that any would survive

after thawing. The success of Fulton and Smith may be due to this factor. In the first place they used a larger volume (0.5 - 1ml) of amoebal suspension which probably contained millions more amoebae than I have used (unfortunately, they did not state the number of amoebae they froze); secondly, their assay of viability was simply based on cultivability of the ampoule content in culture medium after thawing. It is probable that the few amoebae which survived gave rise to their cultures. Kasprzak and Rydzewski (1972) recovered viable *E. histolytica* from suspensions frozen at 1^{o} C/min to -196^{o} C after they had been exposed to 5 and 10% (v/v) glycerol at 4^{o} C for 30 minutes. They did not indicate the number of amoebae they froze, nor did they mention anything about how viability was assessed. Their result is therefore difficult to interpret.

DMSO was found to be cryoprotective for *E. histolytica* at concentrations of up to 15% (v/v) and viable recovery was always achieved regardless of equilibration temperature or time. It is apparent from my results that the degree of protection varies with concentration and equilibration conditions, but an unawareness of this has led previous workers, who based their viability assessment on dye-exclusion and/or cultivability in culture medium after thawing, to believe that their various conditions were optimal. For example, Diamond *et al* (1963) believed that the optimum condition for cryopreservation of *E. histolytica* was an equilibration with 5% (v/v) DMSO at 35^{o} C for 30 minutes, while Gordon *et al* (1969) held that 8% (v/v) DMSO was required with an equilibration at room temperature for up to 1 hour. Neal *et al* (1974) recommended the use of 7.5% (v/v) DMSO with an equilibration at 23^{o} C. They did not vary equilibration temperature but suggested that equilibration time should be examined. The results obtained in this study indicate that the optimum condition for the cryopreservation of *E. histolytica* is a concentration of 7.5% (v/v) DMSO and an equilibration at 37^{o} C for not longer than 15 minutes provided freezing is done at 1^{o} C/min.

I have restricted myself to a maximum equilibration period of 30 minutes in this work, because an increase in equilibration time before freezing has been shown to increase the probability of toxic reactions at a given cryoprotectant concentration (Karrow *et al*,1965). My results, however, indicate the need to investigate further the equilibration period, especially with regard to low concentrations of DMSO, glycerol and perhaps PVP.

Neal *et al* (1974) did not find a 'dramatic' difference in the recovery of *E. histolytica* which had been exposed to 7.5% (v/v) DMSO at 23° C for various lengths of time (0.5-5.7 hours); neither were Fulton and Smith (1953) able to detect differences in the recovery rate of amoebae equilibrated at 37° C for different periods (0.5-2 hours) in the presence of 5% (v/v) glycerol. An explanation for this would again be the lack of a sensitive method of assessing viability. Where an insensitive assay is used results are unlikely to vary dramatically unless there is a complete absence of viable amoebae in the frozen suspension after thawing. For example, Fulton and Smith failed to recover live amoebae from suspensions which had been held at 37° C for longer than 30 minutes in the presence of 10% (v/v) glycerol.

Callow and Farrant (1973) found no change in the infectivity of *L. tropica* to mice when exposed to the same 1.5 M (11% v/v) glycerol at 28° C for varying lengths of time. The difference in their results for *L. tropica* and in the results of Fulton and Smith (1953) for *E. histolytica*, as well as those reported here with regard to the sensitivity of organisms to glycerol under varying equilibration conditions, confirm that living cells of different types and different species will have different optimum conditions for cryopreservation (Pegg, 1976).

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Since the suggestion of Robertson and Jacob (1968) that the combination of DMSO with other cryoprotectants might give a cryoprotective synergism considering the ability of

DMSO to assist the penetration of compounds which are usually biologically impermeant, many workers have tried to cryopreserve *E. histolytica* in a combination of cryoprotectants. Diamond (1964) reported the improved recovery (up to 40%) of *E. invadens* when frozen with a combination of 4.5% (w/v) glucose and 15% (v/v) DMSO over 1% obtained from using 5% (v/v) DMSO alone. Djerassi and Roy (1963) found that rat platelets were better preserved in 5% (w/v) dextrose with 5% (v/v) DMSO. The use of these compounds alone at the given concentrations did not confer any protection. However, Karrow *et al* (1965) considered that cryoprotective synergism did not result when they failed to recover rat heart muscles from freezing in the presence of 12.5% (v/v) DMSO in dextran whereas each individual protectant conferred protection on its own. My results with different combinations of cryoprotectants (DMSO + PVP, DMSO + glycerol, DMSO + glucose) did not suggest the existence of cryoprotective synergism but rather the reverse. DMSO and glucose at the concentrations used by Diamond was relatively unsuccessful for *E. histolytica*. Viability was lost at the lowest serial dilution, whereas, when 7.5% (v/v) DMSO was used with 2.25% (w/v) glucose, viability was measurable by the ID_{63} method although estimates were still very much lower than when DMSO was used alone.

From the present study, DMSO proved consistently to be a more efficient cryoprotectant than glycerol or PVP for the preservation of *E. histolytica*. However, the proportion of amoebae which survived low temperature storage remained low, between 0.3 and 12.5%. Various workers at different times reported variability in the percentage viability of frozen amoebae. Results have even varied with the same workers at different times. Diamond (1964) recovered 30-40% of *E. invadens* after freezing them to -196° C in the presence of 15% (v/v) DMSO and 5% (w/v) glucose, while Neal *et al* (1974) reported between 1 and 3.7% survival (measured by Evan's-blue-exclusion) for *E. histolytica* frozen with 7.5% (v/v) DMSO, and Raether and Uphoff (1976) using the same technique as Neal *et al* recovered 10-44%.

The varying results obtained by different workers can be partly explained by the unreliability of the assay methods, the differences in the culture media and the differences between species and stocks of amoebae. However, the reasons why I obtained variable results at different times cannot be easily arrived at, especially when efforts were made to keep experimental conditions standard. The only obvious cause could have been differences in the batches of serum used in cultivation although it is not clear what effect this might have on survival. A certain amount of variation is, however, to be expected in biological work.

While efforts were made to cryopreserve cultures of the same chronological age (48-hours-old), one cannot be sure of the exact stage of cultures for, at 48 hours, populations may be in their middle or late log phase depending on the stocks of amoebae, the medium in which they are cultivated and the number of viable (cultivable) organisms present in the initial inoculum. It is likely that at some points during growth, amoebae are less vulnerable to freezing than at other times.

E. histolytica carries out phagocytosis and ingests rice-starch granules. The organisms, like other phagocytic cells, possess a large reserve of glycogen (Montalvo *et al*, 1971) and a large number of lysosomes (Cappell and Anderson, 1971). It has been reported that organisms which have many lysosomes such as *T. foetus* (Feinberg and Morgan, 1953) are impossible to cryopreserve (McEntegart, 1954). Mammalian granulocytes, like *Entamoeba*, are known to have a large reserve of glycogen and many lysosomes and they are known to survive freezing very poorly (Cavins *et al*, 1968; Knight *et al*, 1975). Neal *et al* (1974) suggested that the presence of many starch granules in the cytoplasm of *Entamoeba*, especially *E. terrapinae*, may have contributed to their poor record of recoveries. It seems very probable that when glycogen is present in *E. histolytica* in excessive amounts they become more susceptible to freezing damage. It is also likely that phagocytic cells

like *E. histolytica* are damaged during freezing because the lipoprotein membranes of their lysosomes are very sensitive to high concentrations of electrolytes (Lee and Allen, 1972) and are denatured by freezing (Lovelock, 1957). Damage to lipoprotein membranes of lysosomes as a result of freezing and thawing (Raether *et al*, 1977) leads to the release of hydrolytic enzymes into the cytoplasm of the cells resulting in cell damage due to autolysis (Cappell and Anderson, 1971).

Not much time was spent in the present study on the cryopreservation of amoebae derived from axenic or *Crithidia*-grown cultures. As a result of this I did not become sufficiently experienced in handling axenic and crithidial cultures and this in turn might have contributed to my lack of success in this aspect.

The success of previous workers could be attributed to the fact that a huge number of amoebae were preserved; for example, Gordon *et al*, (1969) preserved up to 20×10^6 (NIH: 200) amoebae/ml while Raether and Uphoff (1976) preserved between 1.1×10^6 and 1.2×10^7 amoebae/ml. The percentage viabilities quoted by these authors were based on dye-exclusion followed by cultivation *in vitro*.

The rate of freezing giving optimal survival has been shown to be dependent upon the cell type and the nature and concentration of the cryoprotectant present (Farrant and Morris, 1973; Pegg, 1976). An arbitrary cooling rate of 1° C/min had been employed with viable recovery of many parasitic protozoa including *Babesia* (Mieth, 1966), *Eimeria* (Norton and Joyner, 1968), *Trichamonas* (McEntegart, 1954; Resseler *et al*, 1965), *Trypanosoma* (Lumsden *et al*, 1963; Cunningham *et al*, 1963), *Plasmodium* (Jeffery and Rendtorff, 1955; Warhurst, 1966; Schneider *et al*, 1968), *Naegleria* and *Acanthamoeba* (Willaert, 1976), *Entamoeba* species (Diamond, 1964; Neal *et al*, 1974; etc.), *Tritrichomonas* (Levine and Marquardt, 1955).

Although this rate of cooling was satisfactory for *E. histolytica* the viability after thawing was low, and it was therefore decided to investigate experimentally the effect of different freezing rates. Controlled cooling rates of 0.2°, 0.5°, 1.0°, 2.0°, 5.0° and 10.0° C/min were used for *E. histolytica* suspensions in various concentrations of DMSO, PVP and glycerol using optimum equilibration times. Suspensions with 5% (v/v) glycerol survived freezing rates of 0.5° and 1° C/min, those with 15% (w/v) PVP survived freezing rate of 1° C/min alone, while those suspended in DMSO survived a range of freezing rates between 0.2° and 2.0° C/min. At 2° C/min, the percentage survival was so low that it was not measurable by the ID_{63} assay method. None of the suspensions survived freezing rates of 5° C/min and above. Using DMSO, PVP and glycerol a rapid rate of cooling to subzero temperatures was injurious to the amoebae. Diamond (1964) was unable to recover *E. invadens* cooled to -50° C at 8° C/min in the presence of 15% (v/v) DMSO and 5% (w/v) glucose. However, he was able to recover *E. histolytica* suspensions in 7.2% (w/v) glucose directly plunged into and stored in liquid nitrogen after one and seven days.

The two-step freezing technique originally developed by Luyet and Keane (1955) for lymphocytes has been used with different types of cells (Polge and Soltys, 1957; Rapatz and Luyet, 1963; Farrant *et al*,1974; Walter *et al*, 1975; Wilson *et al*, 1977). Protection is achieved because freezing of extracellular water to ice causes an osmotic gradient which leads to the dehydration and shrinkage of cells held at high subzero temperatures. This prevents intracellular ice formation when the cell is eventually stored at very low temperature and minimizes damage due to intracellular ice on thawing (Walter *et al*,1975). Under the experimental conditions in this study, the two-step freezing regime was successful only to a limited extent; it did not give improved protection to *E. histolytica* against freezing and thawing. It is possible that the holding time at the sub-zero temperature was not ideal; it could have been too long or too short. A more detailed study in this direction may result in an

improvement in viable recovery of amoebae. On present information a cooling rate of 1° C/min gives the best viable recovery of *E. histolytica*.

Thawing rate has been practically neglected as one of the conditions for successful recovery of parasitic protozoa from low temperature storage. An arbitrary fast thawing regime at temperatures between 37° C and 40° C has been used by several investigators. Diamond (1964) investigated the effect of thawing rate on samples of *E. histolytica* cooled to -170° C in the presence of 15% (v/v) DMSO and 5% (w/v) glucose. He thawed suspensions in an ice bath and at 37° C and 45° C and found a significant 85-95% decrease in the percentage 'survival' of amoebae thawed in the ice bath compared with samples thawed at 37° C. Although Diamond's estimates of viability were based on exclusion of dye, his results were comparable to mine. I found, using thawing temperatures of 0°, 25° and 37° C, that there was a significant difference in survival between thawing at 0° C and at 37° C ($p < 0.001$). Although there was a difference in the ID_{63} estimates for suspensions thawed at 25° C and 37° C, 37° C being superior, it was not statistically significant. It is therefore suggested that a thawing temperature of 37° C should be used on retrieval of *E. histolytica* from low temperature storage.

The viability of thawed *E. histolytica* suspensions decreased considerably when they were left for longer than five minutes at the thawing temperature of 37° C, and viability was almost lost when they were left for 30 minutes. It is apparent therefore that a period of 'structural reconstitution' (Lumsden *et al*, 1968) is detrimental to thawed amoebal suspensions. Fulton and Smith (1953) showed that a gradual withdrawal of glycerol from *E. histolytica* suspension was detrimental to the amoebae. I found that when thawed suspensions of *E. histolytica* preserved with PVP or glycerol were washed with PBS before subculturing there was a complete loss of viability which was probably due to dilution shock (Farrant and Morris, 1973). Washing in PBS, therefore, is not recommended. In the case of DMSO, washing is not necessary since the presence of DMSO in

culture was not observed to affect the growth of *E. histolytica* in this study, and infectivity titration for the ID_{63} assay made in Robinson's medium ensures adequate dilution of the cryoprotectant. Dilution shock is unlikely to affect DMSO-treated amoebae because of the relative ease at which it enters or leaves the cell.

There was no evidence from the results that *E. histolytica* which survived freezing treatment showed any increased resistance to subsequent freezings. In addition successively frozen and thawed samples without intermediate subculture failed to grow in culture medium, suggesting a complete loss of viability.

There are many reports on the progressive loss of viability in cells stored at temperatures above -100° C for prolonged periods (Manwell and Edgett, 1943; Levaditi, 1952; McEntegart, 1954; Eyles *et al*, 1956; Polge and Soltys, 1957; Moline *et al*, 1962; Jeffery, 1962; Diamond, 1964, etc.).

It is theoretically expected on physical principles that storage at below -130° C will result in essentially indefinite preservation (McEntegart, 1954) because formation and growth of ice crystals do not occur below this temperature (Diamond *et al*, 1961). Also, as absolute zero is approached, atomic and molecular movement is minimized and degradation of proteins, etc., is reduced. While -196° C storage has been routinely and successfully used for storing a variety of parasitic protozoa including *E. histolytica* without apparent loss of viability (Diamond, 1964; Neal *et al*, 1974, etc.), the results with *E. coli* and possibly *E. ranarum* have shown a reduction in viability with time (Neal *et al*, 1974). It is rather interesting, though disturbing, to find, using the ID_{63} method, that *E. histolytica* shows progressive loss of viability when stored at -196° C.

It is not clear what is responsible for this loss. Loss of viability over lengthy storage time may not have been

detected in the past, probably because the method by which viability was assessed was insensitive, i.e. by exclusion of dyes and subculture without titration. On the other hand, the number of amoebae included per ampoule has often been enormous (for example Gordon *et al* (1969) used 20 million amoebae/ml) so that, if loss was gradual, it was still possible to recover viable amoebae after several years. In this study, the maximum number of viable (cultivable) amoebae preserved per ampoule rarely exceeded 25×10^4. Assuming that this suspension loses 80% viability every 3 months, and that there is a linear relationship between loss of viability and time (although there is no evidence that deterioration in viability is linearly related to time), it would still be possible to recover viable organisms after 21 months in liquid nitrogen for there will still remain 3 viable organisms (and one trophozoite is capable of initiating a culture provided the conditions in the medium are suitable). An experiment is therefore desirable to examine the relationship between loss of viability and storage at -196° C. If, however, it is found that deterioration in viability is linearly related to time, then it would be necessary to predetermine expiry date when stabilates are laid down, as suggested by Neal *et al* (1974).

Various other factors may have been responsible for the loss in viability with time. It was suggested that oxidation probably occurs in frozen samples during storage at low subzero temperatures which might result in the production of lipid peroxides in the presence of serum (Ormerod, personal communication). These peroxides may be harmful to amoebae. Kilgour (personal communication) showed that oxidation of glucose phosphate isomerase takes place at -196° C and this results in the progressive loss of this enzyme's activity. Karrow and Webb (1964) reported that enzymatic degradation continues at temperatures far below 0° C since, for example, blood cells 'decay' significantly in a month even at -80° C. It is believed that, if such enzyme activity is allowed to continue, cells may be killed by autolysis or self-intoxication. It is therefore suggested that this aspect of cryo-

preservation be properly investigated and the problem solved, and,unless this is done, the idea of indefinite storage may become a fiction. However, in spite of all these, the freezing method described here makes the storage of *E. histolytica* cultures possible for a useful period of time.

In addition to the determination of viability in terms of growth in Robinson's medium, the preservation of cellular characteristics of *E. histolytica*, such as its ability to encyst in culture, its cytopathic effect on living cells, its agglutination induced by con A, its growth rate, characteristics of the isoenzymes and antigenic properties,were also investigated. The importance of these studies was demonstrated by the work of Bryan *et al* (1976) which suggested that cryopreservation might cause loss of certain surface properties such as con A receptor sites in lymphocytes. In this connection, certain techniques were used whereby the preservation of cell characteristics was evaluated and they are separately discussed below.

Dobell and Neal (1952) reported that the presence of certain *Es. coli* strains in amoebic cultures inhibited the production of cysts in their HSre culture media. My results with *Es. coli - B* grown in association with *E. histolytica* in Robinson's medium showed that amoebae could encyst and excyst in Robinson's medium although only a small number of cysts were formed. In view of the relatively low numbers of trypan-blue-excluding cysts found in cultures which had been treated with hydrochloric acid, it was always necessary to subculture to prove the presence of viable cysts. Some amoebae were found not to encyst in Robinson's medium and those which did continued to do so after successive freezings and thawings with intermediate cultivation, indicating that the ability of stocks of amoebae to encyst in Robinson's medium is not altered by cryopreservation.

Gordon *et al* (1969) were unable to detect any permanent antigenic changes in axenically cultivated *E. histolytica* using

the indirect haemagglutination test after cryopreservation. The results obtained here from testing antigenic properties of cryopreserved *E. histolytica* by the indirect fluorescent antibody technique confirm that no detectable antigenic changes occurred as a result of cryopreservation.

E. histolytica stocks have been shown to exert a cytopathic effect on living tissue culture cells (Eaton *et al*, 1970) and mammalian polymorphonuclear leucocytes (Jarumilinta and Kradolfer, 1964) with which they come into close contact. The results obtained here using human leucocytes confirm those of Jarumilinta and Kradolfer. The cytopathic effect of *E. histolytica* was observed to be exerted only when the amoebae and the leucocytes were in very close proximity. It was also observed that, when leucocytes became attached to the amoebae, hardly any movement was observed in the latter. Rounding off of leucocytes took up to 15 minutes after contact with amoebae and complete degeneration was not observed until after 30 minutes of contact.

Bos and Van den Griend (1977) showed that the speed at which degeneration of leucocyte was effected depended on the pathogenicity of the amoebal stock; pathogenic stocks were reported to exert a cytopathic effect quicker than the non-pathogenic ones. However, I was unable to correlate clinical condition of the patient from whom stocks of amoebae were isolated with leucocytotoxicity, for it took roughly the same length of time for each of the stocks tested to exert its cytopathic effects. It appears probable that leucocytotoxicity is a character of the species rather than that of the stocks, and this character should not be used as a criterion for assessing pathogenicity in amoebiasis. Leucocytotoxicity remained unaffected by cryopreservation.

The ability of amoebae to agglutinate in the presence of con A is considered to depend on the presence of particular glycoproteins on the cell membrane and was considered by Trissl *et al* (1977) to relate to their pathogenicity. These workers showed that the 'pathogenic' amoebae had a low sur-

face charge and agglutinated more strongly with con A than did the 'non-pathogenic' ones with high negative surface charge. The results obtained here appear to contradict those of Trissl and her colleagues for it was not possible to induce the agglutination reaction in amoebae from patients with proved clinical amoebiasis. However, it was possible to induce the agglutination reaction in stock NIH:200 which had grown in TPS-1 axenic medium or in association with *Crithidia*.

It was at first thought that the competition of bacteria and starch granules for the binding sites on the con A molecules prevented amoebae from agglutinating, but agglutination was obtained with axenically cultivated NIH:200 which had been incubated with bacteria and starch for 2 hours prior to treatment with con A. Moreover, NIH:200 from axenic culture grown for several subcultures in Robinson's medium also failed to agglutinate.

Although an explanation for this observation cannot be easily found without proper investigation, it is probable that growth in Robinson's medium and perhaps other media affects the surface properties of amoebal membranes in a way that does not affect their viability. An explanation for this could be that the erythromycin which is incorporated in RSM actually inhibits the ribosomal synthesis of specific cell-surface proteins (Gale *et al* 1972). Unfortunately this idea has not been put to test experimentally. The observations made in this work cast doubt on the use of con A agglutination as a means of testing 'pathogenicity' in *E. histolytica*. Since it was not possible to induce the agglutination reaction in descendants of frozen and thawed amoebae, it can be concluded that cryopreservation did not change this surface property of *E. histolytica*.

Using cellulose-acetate electrophoresis, Reeves *et al* (1967), Reeves and Bischoff (1968) and Montalvo and Reeves (1968) were able to distinguish between GK, ME, PGM, GPI and

NADP diaphorase patterns of typical and atypical *E. histolytica* strains and also between *E. histolytica* and reptilian amoebae and bacteria, but they were unable to distinguish between typical *E. histolytica* strains .

Sargeaunt and Williams (1978), using thin-layer starch-gel electrophoresis, did not only distinguish between species of *Entamoeba* but also between typical *E. histolytica* stocks by their GPI, PGM and ME patterns. They found that the typical *E. histolytica* fell into 3 iso-enzyme variant groups which they designated I, II and III. More recently, they were able to associate *E. histolytica* belonging to group II with clinical amoebiasis in the patients from whom the amoebae were originally isolated (Sargeaunt and Williams, in press). My observations using the enzymes used by Sargeaunt and Williams and in addition HK (GK) confirm and extend the findings of Sargeaunt and Williams.

When the HK patterns of *E. histolytica* belonging to the 3 different groups were studied each showed 2 prominent bands, but group III had an additional faint band between the two prominent bands (See Figure 28B). The pair of *E. coli* bands moved faster than those of *E. histolytica*, but the pair of bands associated with group II moved slightly ahead of the pair of prominent bands of groups I and III which had similar electrophoretic mobilities. Once again, it was demonstrated that amoebae belonging to group II and derived from cases of clinical amoebiasis could be differentiated from the other typical *E. histolytica* stocks.

Es. coli - B had a single HK (GK) band which appeared to correspond with the faster-moving band of group II and the slower-moving band of *E. coli*. The lack of variation in the enzyme patterns obtained for GK in 'typical' *E. histolytica* by previous workers was due to the fact that they used strains of amoebae which were isolated from cases of clinical amoebiasis (except for one strain from a monkey). However, the GK double band found in these strains of *E. histolytica* was also found in this study. It is reasonable to

suggest that isoenzyme characterization is probably the most effective and sensitive method for differentiating stocks of intestinal amoebae. It was not possible to detect changes in the GPI, ME, PGM or HK isoenzyme patterns of *E. histolytica* as a result of cryopreservation.

Lumsden (1972) suggested that cryopreservation probably selects in a way that is unrelated to the biological characteristics of the population. This does seem to be the case because successively frozen and thawed samples (with intermediate subcultures) did not show any significant increase in their survival after freezing, neither did freezing select fast- or slow-growing amoebae for, if it did, the results obtained from growth-rate comparisons between fresh and frozen cultures would have been consistent.

One might expect that stocks isolated from clinical cases would grow faster than those from non-clinical cases, but there was no correlation between amoebic generation-time *in vitro* and the presence of clinical symptoms of amoebiasis. Stocks Irvine and Biswas were both isolated from cases of clinical amoebiasis and their generation-times *in vitro* were shown to differ very significantly ($p < 0.001$); Irvine was very fast growing whereas Biswas was very slow. The differences in their generation-times could, however, be related to the fact that Biswas is a long-established stock and Irvine was only recently isolated.

However, conclusions cannot be drawn from these results because it is not clear why or how freezing affects growth-rate in different ways. I can only suggest with Kasprzak and Rydzewski (1972) that growth-rate is probably a characteristic of the stocks which changes 'at will' or spontaneously regardless of the clinical conditions of the patient from whom they are isolated. These changes could also be brought about by some extrinsic factors such as variation in culture medium, for the same batch of serum was not used for all the experiments. The length of time during which the stock had been maintained *in vitro* after freezing and thawing may also have an affect on changes in growth-rate.

E. SUMMARY

The following general principles can be recommended for the preservation of *E. histolytica* at low sub-zero temperature.

1. *E. histolytica* should be cryopreserved as quickly as possible after primary isolation in culture to avoid possible changes in biological characteristics which could be caused by prolonged serial passages.

2. The original culture material should be cryopreserved in addition to clones since it represents the variability in the natural population.

3. Isolates should be suspended in a suitable culture medium for cryopreservation. The suspension should contain at least 10×10^6 viable amoebae/ml assuming an 80% loss in viability per 3 months.

4. 7.5% (v/v) DMSO should be used. Neat DMSO should never be added direct to suspensions because the reaction is exothermic! It should be prepared in double concentration using culture medium, then diluted with amoebal suspension 50:50.

5. It is advisable that the volume of stabilate should not exceed 1 ml/ampoule.

6. Suspensions should be equilibrated at 37° C for 15 minutes with occasional shaking.

7. Time should be allowed for the suspension to reach 0° C to reduce thermal shock on further cooling. A controlled-rate freezing regime of 1° C/min from 0° C to -60° C is recommended; the frozen suspensions should be transferred to liquid nitrogen storage.

8. Thawing should be rapid and should be performed with continuous agitation in a water bath at 37° C.

9. Thawed samples should not be washed and should be inoculated into preconditioned cultures as quickly as possible to avoid further loss from the toxic effect of DMSO.

10. There is now evidence for the need to predetermine expiry date so that further subcultures and stabilates can be made.

Appendix

Statistical analysis of results on the viability of thawed *E. histolytica* suspensions.*

A logarithmic transformation was used in the data analysis. Results from experiments with a temperature of 0° C were not included, as they were clearly low and not repeated. The remaining data, logarithmically transformed, were analysed by non-orthogonal analysis of variance using the computer program GLIM.

Interactions between the three factors, equilibration temperature (E), per cent DMSO (D) and equilibration time (T), were found not to be statistically significant. This was established for the three-factor interaction by comparing the fit of the model with main effects and all interactions (the completely saturated model) with the fit of the model with main effects and two-factor interactions only. The two-factor interactions were tested by the change in fit of this second model as each of these interactions were removed separately. The two-factor interactions were tested again by comparing the fit of the model with main effects only with the model of main effects and each of these interactions separately.

The main effects were each tested statistically by comparing the fit of the model with the three main effects with the fit of the model with the other two main effects only. All three were found to be statistically significant (for E, $p < 0.001$, for D, $p < 0.01$, and for T, $p < 0.05$). In the case of DMSO (D), the largest contrast was between 5% and 7.5% and, in the case of time (T), the largest contrast was between 2 minutes and 15 minutes.

The best estimates of the responses at the various combinations of treatment are therefore given by fitting the model:

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log (response) = DMSO effect + Temperature effect + time effect and these estimates are compared with the group geometric means in Table XXVII.

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A simple technique for preparing clone cultures of *Entamoeba histolytica*

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Cultures established *in vitro* from human amoebic infections of the gut may consist of one, two or more species of amoebae. Even cultures of a single species are likely to be genetically heterogeneous. For biochemical, physiological and genetic studies, it is desirable to work with clones.

Methods of obtaining clones of *Entamoeba histolytica* by micro-isolation using a complex micromanipulator have been described by previous workers (REES, 1942; REES *et al.*, 1950; BAERNSTEIN *et al.*, 1957; REES *et al.*, 1960).

Isolated trophozoites have been cultivated in Shaffer and Frye medium (SHAFFER & FRYE, 1948) seeded with *Trypanosoma cruzi* (PHILLIPS, 1950; PHILLIPS & REES, 1950) or in cultures with minced chick embryo without bacteria (BAERNSTEIN *et al.*, 1957) or with bacteria in HSre medium and starch (DAS, 1972).

To my knowledge, the largest volume of medium in which *E. histolytica* has been isolated is that which a cavity slide would contain (DAS, 1972); otherwise micro-volumes contained in 4 × 50 mm or smaller microtubes have been used (PHILLIPS, 1950; BAERNSTEIN *et al.*, 1957). No one has successfully inoculated single trophozoites into normal volumes of culture medium. REES and his colleagues (1960) inoculated single trophozoites into 3·5 ml of their culture medium 50 times without success. I have successfully isolated clones from 10 strains of *E. histolytica* into 3 ml of Robinson's culture medium (ROBINSON, 1968).

Origin of amoebae used

Strains Thesiger, SN, JEH, Thirer, Bean and Fox were all isolated from semi-formed to formed stools of apparently asymptomatic cyst-passers.

Table I—Success rate in cloning *E. histolytica* strains by micro-isolation

Strain of amoeba	No. of attempts at cloning	No. positive	
		No. inoculated	
Biswas	2	0/10	2/10
Thesiger	1	2/10	
SN	1	7/10	
Irvine	2	4/10	8/10
Bean	1	8/10	
Fox	2	2/10	4/10
Thirer	2	0/10	2/10
JEH	2	2/10	6/10
Atkinson	1	7/10	
Ali	1	1/10	

Strain Irvine was isolated from a patient proved to have amoebiasis (liver abscess serology positive). Strain Atkinson was isolated as haemotophagus trophozoites from rectal scrapings of a patient with bloody diarrhoea who was serologically positive for amoebiasis. Biswas strain was isolated from the normal stool of an apparently asymptomatic carrier whose serological tests later revealed invasive amoebiasis. CFT was positive at a titre of 1/96 (SARGEAUNT, personal communication).

The IFAT of all the strains used in this report were tested against human anti-*E. histolytica* serum at a titre of 1/100. All were antigenically similar. Biswas strain is reported to be pathogenic in rats (NEAL & JOHNSON, 1968).

Method

Using the liquid phase of Robinson's medium, ten-fold serial dilutions of thoroughly mixed 24 to 48-hour-old amoebic suspensions were made until a dilution was reached in which not more than one trophozoite could be picked in a drop of suspending medium. A sterile 10 µl capillary tube was placed in a suspension from which clones were to be prepared; the capillary tube was allowed to take up a small volume of the suspension and, with the help of a holey blower, about 1·0 µl of suspension was dropped on to a sterile coverslip (approximately 7 × 7 mm). This was quickly examined under a low power microscope to confirm the presence of a healthy-looking, motile trophozoite with few starch granules within its cytoplasm. A coverslip carrying a single trophozoite was quickly transferred into a 5 ml bijou bottle containing the warmed culture medium with added starch. The cultures were incubated at 37°C and were checked for growth of amoebae on days five, seven and ten. Cultures which remained negative after day ten were discarded and recorded as negative.

The success rate of this method of cloning depends not only on the age of the culture but also, to a very large extent, on the percentage of amoebae infective to cultures in the original suspension (see Table I). Using the infectivity titration technique described for trypanosomes by LUMSDEN *et al.* (1963), it was possible to show that not all actively motile or trypan-blue-excluding amoebae were infective to cultures (author's unpublished work).

DAS (1972) recorded 70% success with *E. histolytica* using one strain (Jawa). A success rate of up to 80% has been achieved with strain Bean at the first attempt and with strain Irvine at the second; both these results were obtained from 24-hour-old cultures. Success has been limited with some

strains when 48-hour-old cultures were used but improved with 24-hour-old cultures.

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1 **Measurement of the 'viability' of cryopreserved *Entamoeba histolytica***

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Adaptation of the ID_{63} infectivity-assay method, originally developed for trypanosomes (LUMSDEN, W. H. R. *et al.*, 1963; *Expl. Parasit.*, **14**, 269-279), enables quantitative measurement of the survival of *Entamoeba histolytica* after cryopreservation.

48-hour cultures of *E. histolytica*, Strain JEH, maintained in Robinson's medium (ROBINSON, G. L., 1968; *Trans. R. Soc. trop. Med. Hyg.*, **62**, 285-294) were used for these experiments and infectivity to cultures before and after freezing in liquid Nitrogen (1°C per minute) was tested.

The reproducibility of infectivity titrations was compared with estimates of viability using trypan-blue exclusion in five replicate amoebal suspensions. The results show that the dye-exclusion estimates of numbers of amoebae in the suspensions were consistent, varying over 0·09 on the log scale, about 1·23-fold arithmetically. The estimates of amoeba numbers by dye-exclusion were significantly higher than the ID_{63} estimates ($P = 0·03$).

Considering the five replicates, the average difference between dye-exclusion and infectivity results was 0·612 on the log scale, indicating that only one out of four dye-excluding amoebae was infective to cultures.

The method was then used to estimate the number of amoebae surviving cryopreservation after treatment with varying concentrations of DMSO, equilibration temperatures and time. The results showed that, without titration, comparison of the efficiency of different methods of cryopreservation is not possible, and that equilibration at 37°C in 7·5% or 10% DMSO for 15 minutes gave the best survival (10% of unfrozen control).

2 **Clonal derivation of *Entamoeba histolytica***

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Isolates of *Entamoeba histolytica* were cultivated monoxenically in Robinson's "secondary" medium containing 16·6% v/v serum using two methods. One method involved sucrose-gradient centrifugation, the other, antibiotic treatment. Single trophozoites of eight strains of *E. histolytica* were successfully cultivated and serially passaged. Clones were derived by the limiting dilution method or by micro-isolation of individual trophozoites, or both. A technique for growth-rate measurement based on the time elapsing before the amoebal population reached an arbitrary low number was described. Generation time for strain Thesiger was estimated to be 7·2 hours.

1 Transactions of the Royal Society of Tropical Medicine and Hygiene, 72, 437, 1978.

2 Transactions of the Royal Society of Tropical Medicine and Hygiene, 71, 386, 1977.