

MONOXENIC CULTIVATION OF ENTAMOEBA HISTOLYTICA
AND RELATED SPECIES WITH TRYPANOSOMA CRUZI
AND OTHER ORGANISMS.

A thesis submitted for the Degree of Doctor of Philosophy
in the Faculty of Science, University of London.

by

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ABSTRACT

A technique for monoxenic cultivation of Entamoeba histolytica, based on the methods of Pan (1960), and Diamond (1968a) has been developed. This was studied extensively, to establish the optimum cultural conditions. Qualitative assessment of growth with various factors was further clarified by quantitative evaluation.

The diversity of cultural associates which would support amoebic growth was demonstrated. This included a detailed study of amoebic growth resulting from association with Trypanosoma cruzi, including different stages of its life cycle and its physiological state. The results emphasised the importance of the healthy metabolism of the associate in the amoeba-trypanosome system. The role of the associate was discussed.

The culture system was tested for other related amoebae, using T. cruzi and other organisms as possible associates. A study of the basic cultural requirements of entamoebae and species preferences was made by comparing growth of E. invadens with E. histolytica under various circumstances.

The effect of this type of cultivation on the virulence of E. histolytica was shown and an attempt at elucidating the role of bacteria in virulence was made.

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CHAPTER 1 LITERATURE SURVEY

a) Systematics

Entamoeba histolytica

family : Endamoebidae

order : Amoebida

subclass : Rhizopoda

class : Sarcodina

subkingdom : Protozoa

The above, commonly accepted, classification is that of Kudo (1966). The sarcodines are known characteristically for their ability to form pseudopods, which are used for locomotion and feeding. Cytoplasm may be differentiated into endo- and ectoplasm. The former contains the nucleus, food vacuoles and various granules. Typically, members of this class are holozoic. Asexual reproduction occurs by means of binary fission. A few cases of sexual reproduction have been reported. With one exception to the rule, encystment occurs. The five orders of the Rhizopoda subclass vary in their possession of rhizopods, lobopods and tests, but contrast with the subclass Actinopoda which possess axopodia.

The family Amoebida have lobopodia formed by streaming or fountain movement of the cytoplasm. There is no thick pellicle and the hyaline cytoplasm is clearly differentiated from the granular cytoplasm. Many are found in the alimentary canal of various animals.

The nucleus of the Endamoebidae is vesicular with a small

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karyosome, centrally or eccentrically placed. Chromatinic granules are attached to the nuclear membrane. Entamoeba histolytica is well known for the pathogenesis it often causes to man when it infects primarily the colon and the liver. The active trophozoite is 7-35 μ in diameter. The cytoplasm is well differentiated and the nucleus, when stained, has small peripheral granules and a small karyosome, and an indistinct network with a few chromatin granules.

The life cycle continues from trophozoite by binary fission resulting in a smaller than usual form known as the precystic form. This secretes a resistant wall and becomes encysted. The mature cyst has a diameter of 5-20 μ , is tetranuclear and contains chromatoid bodies and a large glycogen vacuole which is gradually absorbed and disappears. Excystment occurs under suitable conditions and the tetranuclear cyst escapes through a pore in the wall. It then undergoes a series of divisions to produce eight uninuclear amoebulae. These grow into trophozoites.

Other related organisms, E. hartmanni, E. moshkovskii, E. invadens, E. coli, E. ranarum, Dientamoeba fragilis, will be used in this work. Histomonas meleagridis also will be used. The various characteristics of these different species will be described in detail in chapter 5 pp205-209.

In 1956 Connell isolated from the stool of a patient, from Laredo, U.S.A. a strain which was morphologically indistinguishable from typical E. histolytica (Dreyer 1961). This strain was found to be capable of growth at room temperature, as well as

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37°C. Since that time other similar strains have been isolated and a considerable comparison made of their biological properties with those of normal E. histolytica amoebae. Richards, Goldman & Cannon (1966) found that none of eight normal E. histolytica strains would grow at low temperatures in hypotonic medium, whereas all of five E. histolytica-like strains would grow at 10°C and in medium diluted 1:64 with distilled water. The work of Neal & Johnson (1966 & 1968) confirmed these studies with regard to temperature. They also failed to produce experimental infection in weanling rats with E. histolytica-like amoebae. Lunde & Diamond (1969) used axenic amoebae to produce antigen from both types of E. histolytica. The E. histolytica-like amoebae were demonstrated to have fewer antigenic components (Immuno-electrophoresis test) and the antigens from these amoebae were less active (in the haemagglutination test) than normal E. histolytica. Goldman (1969) has presented a review of the biological comparisons of E. histolytica-like strains. He suggests that these strains differ enough from normal E. histolytica to justify constitution of a separate species. However he also feels that insufficient isolates have been found to justify this step, at present, but in the meantime perhaps they could be considered as belonging to part of the E. moshkovskii complex; E. moshkovskii itself being found in apparently free-living habitats as a result of its wide tolerances. De Carneri (1969) on the other hand disagrees with Goldman feeling that E. moshkovskii cannot be

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considered except as a free-living amoeba. This is supported by the location of isolates away from any human infection with E. histolytica. Interesting additional evidence has recently been presented by Gelderman, Keister, Bartgis & Diamond (1971). On the characteristic of deoxyribonucleic acid composition, they found sufficient differences between typical E. histolytica, Laredo strain and E. moshkovskii to constitute three separate species.

b) Cultivation

The terminology used is that recommended by Dougherty (1953) for describing cultivation of an organism with and without associated organisms of other species.

Terms are as follows:-

<u>Term</u>	<u>No. of associated organisms</u>
Axenic	None
Monoxenic	One
Polyxenic	Several

Verbs also have been proposed thereby giving words such as axenise and monoxenise.

Entamoeba histolytica was first cultured by Boeck & Drbohlav (1925) using Locke-egg serum and Locke-egg albumin media. Many different media have been designed and documented since then using a wide variety of natural products. Eggs, (Balamuth & Sandza 1954), sera (Dutta & Mohan Rao 1966), liver extract and infusion (Harinasuta & Harinasuta 1955, Cleveland & Collier 1930) and blood (McQuay 1957) have been used frequently. An extract of intestinal contents (Svensson 1963) is one of the more unusual media components which have been described. Today Locke-egg serum medium as well as Dobell & Laidlaw's medium (1926) are commonly used. This latter medium consists of a solid horse serum slope overlaid by Ringer and egg albumin supplemented with rice starch. Three review articles very adequately cover the cultivation of E. histolytica with associated bacteria (Neal 1967, Balamuth & Thompson 1955 and Nakamura 1953).

Attenuation of a strain may be achieved by long term cultivation in these media (Neal & Vincent 1956) .

Chinn, Jacobs, Reardon & Rees (1942) felt that bacteria play a significant part in excystation, multiplication and encystation of E. histolytica and suggested that studies of bacterial action may lead to knowledge necessary to culture the amoeba without bacteria.

In order to study the nutrition and physiology of E. histolytica in vitro it was soon realised that the picture was severely confused by the presence of an unknown mixed bacterial flora. However, several experiments have shown that this formed an essential part of the medium (Dobell & Laidlaw 1926). Therefore attempts were made to grow E. histolytica with only one species of bacterial associate. This was done by treatment of cysts with chemicals (Balamuth & Wieboldt 1951, Rees, Baernstein, Reardon & Phillips 1953, Dobell & Neal 1952, Singh, Das & Saxena 1963, Rao, V.G. 1951). The disadvantages of this technique are that bacterial spores often present in samples are not eliminated.

Microisolation of cysts has also been used to eliminate concomitant bacteria (Rees, Baernstein, Reardon & Phillips 1953, Rees 1961). An obvious method of obtaining sterile E. histolytica though one that is unavailable to most workers, is by isolating amoebae from liver abscesses (Freedman et. al. 1958).

By the use of the techniques mentioned above, E. histolytica may be grown with one or several chosen bacteria. Some attempts at the elucidation of the amoeba-bacterial relationship of E. histolytica have been made by a detailed study of the physiology

of the concomitant bacteria (Dobell & Neal 1952, Bradner 1953). Studies of growth of E. histolytica with only one bacterial species have been made by Rees et. al (1941) Rees et. al. (1953) Ghosh (1970), Dobell (1947), Dobell & Neal (1952).

The flora of established E. histolytica vary widely. NRS strain flora has 3 aerobic and no anaerobic organisms (Balamuth & Wiëboldt 1951) whereas strain K28c flora has 8 aerobic and 7 anaerobic organisms (Dobell & Neal 1952). The individual components of these flora vary in their amoebic support and Balamuth & Wiëboldt found best support was afforded by the complete flora. Rees et. al. (1953) assessed a wide variety of available bacteria for amoebic support and found that E. coli and Clostridium perfringens and organism "t" were the best. Cultures associated with organism "t" needed supplemented cholesterol and B vitamins.

Growth of E. histolytica with inhibited bacteria as associates was first achieved by Jacobs (1947). He used penicillin to inhibit C. perfringens in monobacterial cultures of E. histolytica prepared from cysts obtained sterile by micro-isolation.

This technique has been successfully modified and utilised for considerable studies on growth requirements of E. histolytica by two groups of workers; Shaffer in Chicago and Reeves in New Orleans (Shaffer & Frye 1948, Reeves et. al 1957a) Shaffer has used his culture techniques to assess nutritional requirements (Shaffer & Key 1960, Zweig & Shaffer 1961) and also observed the

in vitro reaction to presence of erythrocytes (Shaffer & Iralu 1961). Reeves has used his technique of culture supplemented with washed inhibited streptobacilli for biochemical studies of E. histolytica growth factors. He demonstrated the need for pyridoxine, pantothenate and iron (Reeves et. al. 1959, Latour & Reeves 1965). Shaffer & Iralu (1963) established the limit of thermal lability of a growth factor to be 53°C for MS-F and 55°C for CLG media. Ahmed (1969) has used the Shaffer-Frye system to study a growth factor produced by Bacteroides symbiosis. Albach et. al. (1966) have made extensive studies of the incorporation of thymidine-methyl- H^3 from labelled bacteria. At least some of the cytoplasmic H^3 -thymidine is synthesised from ingested bacteria or bacterial degradation products. Karlsson used the same basic culture technique to characterise the physical properties of the growth factor derived from the inhibited bacteria. (Karlsson, 1952).

Various attempts have been made to replace the bacteria with tissues, tissue culture or tissue extracts. Reeves, Meleney & Frye 1957 developed a culture system similar to that of Shaffer and Frye but used chick embryo tissue juice instead of inhibited streptobacilli. Apparently no intact cells or bacteria were present in the culture medium. Shaffer, Sienkewicz & Washington (1953) obtained propagation of E. histolytica with chick embryo tissues in the absence of bacteria. Liver tissue was the best. Shaffer (1953) was also able to grow E. histolytica in medium prepared from chick embryos; its success was due to subcellular particles.

Krupp (1969) used the Shaffer-Frye technique as a basis for cultures with rabbit and hamster liver and spleen cell fractions, but was unable to obtain long term maintenance. H. meleagridis has been successfully grown in modified T.C. medium enriched with fresh hamster liver and metal ions (Lesser 1961). Meeroovitch (1961) grew E. invadens in organotypic cultures of chick embryo intestine in perfusion chambers.

Another approach to the problem of E. histolytica cultivation was made by Everitt, Sadun & Carrera (1953). They inoculated amoebae into developing chick embryos. The best result obtained was survival of amoebae for 72 hours when inoculated by the amniotic route.

Other organisms used as associates in the culture of E. histolytica have been protozoa. Probably the earliest reports of such an attempt is that of Rees (1949). He was unable to grow E. histolytica with Trichomonas foetus beyond one division. Baba, H. (1957) also failed to grow E. histolytica with a protozoan, Trichomonas gallinae. Phillips (1950 and 1962) has had considerable success in producing E. histolytica growth when associated with Trypanosoma cruzi. He could not reproduce it with Leishmania. However Pan (1960) found that T. cruzi, T. lewisi, Leishmania donovani, L. enriettii, L. brasiliensis and Endotrypanum schaudinni were all capable of supporting E. histolytica growth. Benham & Havens (1958) used continuous culture methods for E. histolytica-T. cruzi cultures. Excellent monoxenic growth of E. histolytica has been achieved by Diamond

(1968a) using Crithidia sp. (isolated from Arilus cristatus), as associate, which has the advantage of being nonpathogenic to humans. Westphal & Michel (1971) using a large variety of protozoal organisms that would grow in Diamond's TTY-SB medium, found that only T. foetus would support the growth of E. histolytica; this only for 27 days.

Stoll first reported axenic cultivation of an entamoeba (1957). Using E. invadens cultures already growing in Miller's medium with hamster liver fragments (Miller 1953), he transferred and maintained the organism in a medium containing liver infusion broth and acidified raw extract. Since then, Diamond has successfully cultured the following Entamoeba species axenically:- E. terrapinnae, E. invadens (1960) E. histolytica (1961, 1965), E. histolytica-like amoebae (1968) and E. moshkovskii (Diamond & Bartgis 1970). Diamond adapted entamoebae to axenic culture by first culturing in the absence of bacteria with T. cruzi or Crithidia sp. as associates. Studies have been made of growth in modified and simplified axenic media (Diamond 1968b) McConnachie 1968, Stoll 1959, Wittner 1968).

Reference has been made already to the studies of growth factors etc. responsible for growth of E. histolytica in the Shaffer-Frye system pp 12-13.

Some study has been made of the effect of numbers of associates on amoebic growth. Reeves (1964) found a minimum ratio of bacterial cells to E. histolytica trophozoites in MS-F cultures for growth. This ratio was smaller if living bacteria were used instead of penicillin inhibited. Meerovitch (1961) made mention of this point indirectly by stating that the growth of E. invadens

in organotypic culture of chick embryo intestine depends upon the growth of the tissue cells. Phillips' comment that E. histolytica growth was correlated with the initial amoebic inoculum, might well be due to variation of an amoeba: associate ratio, (1962). Pan used a range of flagellate and amoebic inocula and it is not possible to correlate amoebic growth with initial concentration of flagellates in his experiments. Diamond (1968a) suggested that the standard of E. histolytica growth he achieved in monoxenic culture was poorer than that of Phillips & Pan since he used a lower initial concentration of T. cruzi. Diamond used $1.5-2.0 \times 10^6$ flagellates/ml. while Pan used $4.0-7.4 \times 10^6$ flagellates/ml. Phillips used concentrations around 1.0×10^7 flagellates/ml.

Further studies on the nature of the nutrients afforded for E. histolytica by T. cruzi association have been made by examination of the effect of heat on the flagellates and on their subsequent amoebic support. In Phillips' first papers on amoeba-trypanosome cultures (Phillips 1950, Phillips & Rees 1950) it was reported that treatment of the associates at 46°C for 1 hour 15 minutes still allowed amoebic growth. No amoebic growth resulted if the T. cruzi was heated to 52°C for 10 minutes. A more detailed study demonstrated that T. cruzi heated for 10 minutes at 48°C would not continue to multiply although respiration still continued and the organisms would support E. histolytica growth, (Nakamura & Anderson 1951). Phillips demonstrated that E. histolytica growth could continue with

T. cruzi that had been treated so that they showed no oxygen uptake, motility or reproduction but still reduced methylene blue. Physical contact between E. histolytica and T. cruzi and some continued metabolism was necessary to support amoebic growth (Phillips 1953).

Since E. histolytica is a parasite of humans the optimum temperature for in vitro culture is 35°-37°C. Almost all of the culture systems, mentioned so far, have used temperatures in this range. Pan (1960) used 33°-35°C. Besides finding the optimum temperature for cultivation, studies have also been carried out to establish critical and survival temperatures. This has often been followed up by attempts to adapt various entamoebae to grow at exoptimal temperatures. It was hoped that some indication as to the taxonomic position of the E. histolytica-like strains might result. Siddiqui (1963) found that thermal death points for typical E. histolytica, E. histolytica-like amoebae, E. moshkovskii and E. invadens were 43°, 41°, 41° and 39°C respectively. He adapted a strain of E. histolytica to grow at 30°C and also adapted E. invadens to grow at 35°C. The trophozoites of adapted strains varied in size from the original strain and the growth curves appeared to be temperature dependant. He concluded that the Laredo strain of E. histolytica-like amoebae is a mutant of E. histolytica having some properties similar to E. moshkovskii and E. invadens.

Cabrera & Porter (1958) found that strains of E. histolytica varied in their survival and critical temperatures. Interchanging

the associated bacterial flora had no significant effect but a monoxenic strain survived better with a larger bacterial flora. This work was followed by adaptation to 29° and 42.5°C by Cabrera (1958). An interesting result occurred. Two strains which had been avirulent to hamster liver became virulent when grown at the lower temperature. This faculty was not lost when the strain was again cultured at 37°C. Bacteria from the adapted strain added to the original strain slightly enhanced its virulence. E. invadens also has been temperature adapted in vitro (Lachance 1963, Gordeeva 1966).

The pH preferred by E. histolytica is thought to be 7.0-7.2. Boeck & Drbohlav's medium is initially pH 7.2-7.8 but 24 hours of bacterial growth reduced it to pH 6.0, (Nakamura 1953, Neal 1967). McConnachie (1962) studied the tolerated and optimum pH range for E. invadens in axenic culture. The amoebae would grow between pH 5.5-8.0 but the optimum range was pH 6.0-7.5.

E. histolytica exhibits considerable tolerance to a wide range of tonicity and changes therein. A medium containing 0.94% salt content is optimal. Chang (1942) considered that M/30 phosphate and 0.4% sodium chloride content was necessary. Reduced growth occurs at M/40 phosphate (Nakamura 1953).

In conventional media with bacterial associates amoebic growth is anaerobic (Neal 1967). As low a concentration as 0.1% O₂ reduced growth and 2.0% O₂ killed the amoebae. The lag phase observed in amoeba-bacterial cultures can be very considerably reduced by preconditioning the medium with bacterial growth

which reduces the oxidation reduction potential to very low levels. (Neal 1967). The requirement by E. histolytica for anaerobic conditions in monoxenic and axenic cultures is a complex subject. Phillips, (1962), Diamond (1968a & b) and Jackson & Stoll (1964) all used reducing agents in their media. Nakamura (1952) used Phillips' system for successful growth of E. histolytica without adding sodium thioglycollate or using a petroleum/wax seal. Pan also grew amoeba-trypanosome cultures without the use of reducing agents or seal. Wittner (1968) measured the oxygen tension at 3 levels in axenic culture tubes. He observed a progressive depletion of oxygen during culture maintenance and suggested that amoebic migration up the tubes was caused by this lack of oxygen at the lower levels.

The mode of feeding of E. histolytica in vitro has long been thought to be obligate particulate, as in vivo. However axenic cultivation necessitates the consideration of this feeding as facultative particulate. In bacteria associated cultures supplemented with starch, starch grains are generally observed in the food vacuoles of the amoebae. This entamoeba and E. invadens are also known to ingest erythrocytes (Lowe & Maegraith 1970, Zaman 1970. In cultures based on the Shaffer-Frye system, ingestion of the bacterial protoplasts has been observed, (Albach & Shaffer 1967). It has already been mentioned that Phillips demonstrated the requirements for physical contact between amoebae and trypanosomes in his cultures. Westphal & Michel (1970) have published a complete account of E. histolytica phagocytosing associated trypanosomes in Diamond's monoxenic culture system.

c) Virulence

Examination of the literature on strain variation as expressed by pathogenicity reveals that this is a complicated problem in itself. It has also been additionally complicated by other factors which have been elucidated and understood more clearly as studies have progressed. Hoare (1952) described the incidence of "minuta" forms of trophozoites which were never associated with clinical symptoms but nevertheless felt that they formed an integral part of the life cycle as they only were capable of producing cysts. It has since been found that cysts can be divided into two categories, 7-9 μ and 12-15 μ in diameter. The strains of this small race with cyst diameter 7-9 μ are now classified as E. hartmanni (Elsdon-Dew 1968).

Apart from the two occasions of apparently non-invasive strains which have been clarified above, considerable variation in virulence occurs between strains of E. histolytica. Neal (1951) reported that strains isolated from patients with acute intestinal symptoms were virulent to weanling rats. He found that strains from contact carrier patients might or might not be invasive to rats. Further evidence (Neal & Vincent 1955) showed that strains from contact carriers were generally non-invasive. Mizgireva (1966) found that all isolates were invasive to some extent; the virulence order was demonstrated firstly, from symptomatic patients, secondly, from convalescent carriers, thirdly, from contact carriers and fourthly, from healthy carriers.

Several theories for strain variation have been suggested.

Mizgireva (1966) found some exceptions to the general rule mentioned above and thereby suggested that virulence is a variable characteristic. More recently the same author and Gleiberman (Misgireva & Gleiberman 1969) proposed that strain variations might be considered as adaptive changes. Okamoto (1953c) observed that generally speaking more virulent strains produced larger cysts. Experimental support for the theory of adaptive variation is provided by the work of Neal (1957). The virulence of non-invasive strains could not be increased either by encouragement of the completion of the organism's life cycle, by encystment, or by giving the experimental hosts protein deficient diets, or by adding bacterial flora from virulent strain cultures or by animal passage. As a long attenuated strain could be made invasive by five liver passages, it would seem that the non-invasiveness of a strain from asymptomatic patients is irreversible. A recent review covering all these aspects of virulence variation is that by Neal (1971).

It has already been mentioned (p 10) that one of the effects of continued cultivation is virulence loss. Mixed bacteria associated E. histolytica cultures have been reported to lose their virulence during long periods of maintenance. (Thompson et. al. 1954). This was noted not only in the rat intestinal infection but also in hamster liver infection over a longer period. This virulence loss was not obviously affected by the culture medium, associated bacteria or breed of rats. Other workers have also recorded similar virulence loss (Biagi & Marvan 1968, Taylor

et. al. 1959). Passage through dogs (Thompson et. al. 1954) and through rats (Healy & Gleason 1966) will revive virulence. Neal & Vincent (1956) developed a method of virulence revival by successive passage through hamster livers. Since the liver in vivo is sterile no other bacteria are added to the associated flora during this process. The same workers traced the virulence of a strain over a period of 12 years, in which liver passage had been necessary on three occasions. After each occasion the virulence declined more rapidly than after the previous occasion.

Taylor et. al. (1959) using guinea pigs as experimental hosts compared the virulence of the same strain maintained in various cultures, including monobacterial, amoeba-trypanosome and association with chick embryo extract. Only one isolate, that growing with Bacterium coli, approached the type of virulence of the isolate with the mixed bacterial flora.

Phillips & Bartgis (1954) made a study of virulence loss by E. histolytica during maintenance in association with T. cruzi. Prolonged cultivation resulted in complete loss of virulence. This could be restored by returning the isolate to the bacterial culture or by reisolation from the original stock bacterial culture. Virulence was then retained for 3-8 weeks. Similar reports of virulence loss have been made for axenic cultures (Wittner, Rosenbaum & Einstein 1970).

The problem of virulence has been studied extensively. The role of bacteria in virulence has been of particular interest. Luttermoster & Phillips (1952) used the rabbit for experimental

intestinal infection. 85%, 35% and 1 in 24 rabbits were infected by polyxenic, monobacterial and trypanosome amoebic cultures respectively. Virulence was restored by returning the amoebae to culture with the mixed bacterial flora. Balsam & Shaffer (1955) had similar results using Shaffer-Frye cultures and hamster liver inoculation of amoebae.

As well as the great value of monoxenic and axenic cultures, in virulence investigations, further assistance has been afforded by the use of germ-free animals. Germ-free guinea pigs mono-contaminated with each of 7 species of bacteria, were inoculated with E. histolytica from amoeba-trypanosome cultures (Phillips & Gorstein 1966). Lesions occurred in each group but some were worse than others. The amoebic infection was superimposed on bacterial inflammation so interpretation of results was difficult. Conventional guinea pigs had all developed fatal amoebic enteritis. (Phillips 1964).

Another approach employed, was the treatment of either the amoeba culture or the experimental animal with penicillin and streptomycin. This prevented the induction of experimental infection (Sarkisyan 1967). The same author also found, like Neal (1956) that addition or substitution of bacterial flora from virulent strains did not enhance virulence of avirulent strains.

Recent work by Wittner & Rosenbaum (1969, 1970) has added further information on the labile characteristic of virulence. They demonstrated the absence of infection of hamster livers caused by axenic amoebae and virulence restored by bacterial

reassociation in the same test. Reassociation with each of three bacterial species was only necessary for 6-12 hours prior to inoculation. Use of treated bacteria or extracts resulted in failure to produce abscesses. These experiments were also carried out using rat intestinal infection as test. It was observed that after axenisation, E. histolytica had low levels of intracytoplasmic hydrolytic enzymes compared with conventionally cultured E. histolytica. The return of virulence was found to correlate with increased hydrolytic enzymic activity and this followed the same time pattern as the virulence characteristics.

CHAPTER 2 MATERIALS AND METHODS

a) Materials and methods used for cultivation or preparation of associate organisms

The strains of various species of associates used in the experiments have been listed in table 1.

Table 1 Strains of Associates and strain histories

<u>Specific Name</u>	<u>Strain</u>	<u>Strain history</u>
<u>Trypanosoma cruzi</u>	<u>Y</u>	Silva, L.H.P. da. & Nussenzweig, V. 1953, Folia clin. Biol. <u>20</u> 191-208
" "	<u>Sonia</u>	Garnham, P.C.C. 1956. Trans. Roy. Soc. trop. Med. Hyg. <u>50</u> , 613
" "	<u>Kessel</u>	Obtained as culture from Prof W. Balamuth, Univ. of California. Obtained by him from Dr. Hamilton Anderson, Medical School of Univ. of California at San Francisco
" "	<u>Peru</u>	Marsden, P.D., Voller, A., Seah, S.K.K., Hawkey, C. & Green, D. 1970. Rev. Soc. Bras. Med. trop <u>IV/3</u> , 177-182
" "	<u>M1</u>	Isolated by Dr. Stella Gonzales Cappa from 5 year old girl in Mendoza, Argentina Feb. 1970
<u>T. lewisi</u>	<u>St. Albans</u>	Isolated by Dr. J.R. Baker from wild <u>Rattus norvegicus</u> at Winches Farm August 1960
<u>Crithidia sp.</u>	<u>ReF.1:PRR</u>	Diamond, L.S. 1968. J. Parasitol. <u>54</u> (4), 715-719
<u>Leishmania enriettii</u>	<u>YE</u>	Obtained as culture leptomonads from Prof. R.G. Yeager (Tulane University) Dec. 1960. Originally Brazilian.
<u>L. tarentolae</u>	<u>tar</u>	Cultures obtained from Dr. Trager (Rockefeller Institute, New York) in 1971.

Table 1 continued....

<u>Specific Name</u>	<u>Strain</u>	<u>Strain History</u>
<u>Euglena gracilis</u>	}	These organisms were bought from the Culture Centre of Algae and Protozoa, Cambridge
<u>Ochromonas danica</u>		
<u>Ankistrodesmus convolutus</u>		
<u>Nannochloris coccoides</u>		
Unknown yeast		Contaminant discovered in amoeba-trypanosome culture
<u>Escherichia coli</u>	<u>A'</u>	Dobell, C. & Neal, R.A. 1952
" "	<u>B</u>	Robinson, G.L. 1968
<u>Hartmanella castellanii</u>	<u>R</u>	Obtained from Dr. S.R. Das as culture. Culture obtained by him from Dr. D. Warhurst, Liverpool. Isolated from mammalian tissue culture infected with "Ryan Virus"
<u>Eimeria tenella</u>	<u>Weybridge</u>	Isolated by L.P. Joyner (Weybridge) about 1950.
Tissue culture	<u>HEp-2</u>	Human carcinoma of larynx. ATCC Reference CCL No. 23. Flow Laboratories Ltd.
" "	<u>Girardi</u>	Human heart. ATCC Reference CCL No. 27. Flow Laboratories Ltd.

Cultivation Procedures

Trypanosoma cruzi - epimastigotes

Leishmania enriettii - promastigotes

L. tarentolae - promastigotes

All these organisms were grown in Boné's medium supplemented with 5% rabbit serum. Subculture was preceded by microscopic examination to ensure the absence of contaminants. Subculture was carried out once weekly using a 10% inoculum. The incubation temperature was 25°C.

Crithidia sp - epimastigotes

These organisms were grown in Boné's medium without serum. Subculture procedure was carried out as described for T. cruzi epimastigotes using a 3% inoculum. The incubation temperature was 25°C.

T. lewisi - epimastigotes

The St. Albans isolate was received from the London School of Hygiene & Tropical Medicine as infected rat blood stored in liquid nitrogen. It was thawed rapidly and inoculated directly into rats intraperitoneally. After one week infected blood was removed from a rat by cardiac puncture and subinoculated into further rats. Weekly passage continued for a few weeks while culture isolation took place.

Culture isolation was carried out by addition of one ml. infected blood to 10 ml. Boné's medium with rabbit serum. This was subcultured weekly using a 10% inoculum and an incubation temperature of 25°C. After one week most trypanomastigotes had become amastigotes. By three weeks mostly epimastigotes were present. The culture was maintained for 3 months.

T. cruzi - amastigotes

Strain Y epimastigotes from Boné's culture were used to initiate amastigote culture. Pan's F7 medium was used. Initial and subsequent subcultures were made with a 10% inoculum. Incubation was at 33°C. By the seventh subculture in F7, amastigotes predominated heavily. However such amastigotes were not used in amoeba culture experiments until the tenth subculture when 98% of the population was amastigotes.

T. cruzi - metacyclic trypanomastigotes

Strain M1 epimastigotes from Boné's culture were used to initiate metacyclic trypanomastigote culture. Modified HLL medium was inoculated with epimastigotes to give an initial concentration of 1.5×10^7 organisms/ml. The culture was incubated at 21°C for two days. Thereafter it was maintained at 28°C. Daily examination, using phase contrast and permanent preparations (Field & Sandesham's stain) was carried out. Between days 14-16 post subculture 72-79% metacyclic trypanomastigotes were present. Several similar cultures were prepared at intervals so that they might be used to provide the highest concentration of metacyclic trypanomastigotes when used in amoeba culture experiments.

Euglena gracilisAnkistrodesmus convolutus

These organisms were grown in Euglena medium. Subculture procedure was carried out as described for T. cruzi epimastigotes using a 1 in 10 inoculum. Cultures were incubated at room temperature (ca 25°C) in a position subject to indirect sunlight.

Ochromonas danicaNannochloris coccoides

These organisms were grown in Ochromonas medium. Subculture procedure was carried out as described for T. cruzi epimastigotes using a 1 in 20 inoculum for Ochromonas and a 1 in 10 inoculum for Nannochloris. Cultures were incubated as described for Euglena.

Hartmanella castellanii

These amoebae were grown in 100 ml. quantities of nutrient broth in 250 ml. round bottles at 25°C. Subculture was made after microscopic examination using a 1 in 10 inoculum. Subculture was made every 10 to 14 days.

Unknown yeast

This was discovered as a contaminant of amoeba-trypanosome cultures growing in TP-S-1 medium (see chapter 2b). It was maintained by 1 in 20 subinoculation into TP-S-1 medium (10 ml. quantities in 6" x $\frac{1}{2}$ " screw cap tubes). Incubation was at 37°C.

Escherichia coli

For long term maintenance, which required subculture only once every 3-6 months, cultures were grown on nutrient agar slants. A platinum wire loop was used to remove bacteria from a single colony in the old culture and dispense them in an even distribution on the sterile slant. Incubation was at 25°C.

For short term maintenance and production of large numbers of bacteria nutrient broth (20 ml. quantities) was used. A platinum loop was again used to transfer colonies of bacteria from an established agar culture into the broth. Incubation for one week

at 25°C was employed. Further broth cultures could be initiated either as described above or by using a 1 in 20 to 1 in 10 inoculum of bacterial broth culture.

Tissue culture - HEp2 and Girardi

Monolayer cultures in 100 ml. medical flats were obtained. Microscopic examination was carried out to assess tissue culture growth using an inverted microscope. Drops of medium were examined microscopically to ensure freedom of contamination. The old culture medium was poured off and the monolayer rapidly rinsed in 0.05% versene containing 0.15% trypsin. This was poured off leaving approximately 0.5 ml. in the bottle. The monolayer was allowed to slough off and the cells separate. This process was checked microscopically. As soon as the cell layer was sloughed off 9.5 ml. of fresh Eagles BHK medium, warmed to 37°C, was added. The cells were thoroughly mixed using a pipette. Subculture bottles were prepared by seeding 9 ml. of medium with one ml. of cell suspension inoculum as prepared above. Incubation of culture vessels was with the flat side of the bottle horizontal at 37°C. Subculture took place twice weekly.

Preparation of Red Blood Cells (RBC)

A rabbit was bled from the heart using aseptic conditions throughout. The blood was transferred from the syringe into a sterile centrifuge bottle containing several small steel balls. To defibrinate it, the blood was shaken vigorously for several minutes. Whole blood was stored at 4°C until required.

Preparation of T. cruzi trypanomastigotes

T. cruzi strain Y was maintained in Evans mice by weekly subinoculation. A drop of tail blood from an infected mouse was examined microscopically before killing and bleeding from the heart.

Heparin was used as anticoagulant. Between 0.1 and 0.15 ml. of infected blood was inoculated intraperitoneally into clean mice. To prepare T. cruzi trypanomastigotes a number (approximately 10) of infected mice were used all carrying a heavy infection. Using aseptic techniques throughout infected blood was obtained as above and put into long narrow centrifuge tubes. The blood was allowed to stand at 4°C for 30 minutes. It was then centrifuged at 25 x g for 1 hour. The clear plasma supernatant was removed using a pasteur pipette and was centrifuged at 500 x g for 5 minutes. The plasma was removed and the trypanomastigotes washed twice in PBS pH 7.2. Trypanomastigotes prepared thus were resuspended in PBS for counting and used the same day.

Preparation of E. tenella sporozoites

Oocysts of E. tenella Weybridge strain were obtained. They had been prepared from faeces and after normal washing procedures were stored in 2% potassium dichromate solution in shallow layers in Roux flasks stoppered with cotton wool plugs. They were allowed to sporulate at 28°C for two days.

Oocysts were cleaned using "Chlorox" treatment. The potassium dichromate was removed by centrifugation. (800 x g for 10 minutes) The oocysts were suspended in 10-25% sodium hypochlorite solution in an ice bath. When the foaming had died down the oocysts were examined microscopically for cleanliness. The "Chlorox" mixture was diluted to less than 5% with sterile distilled water. The oocysts were washed free of "Chlorox" by repeated centrifugation and addition of fresh water until no smell of "Chlorox" remained. Oocysts for

immediate use were resuspended in PBS A and the remaining ones were suspended in 2% potassium dichromate as before and stored at 4°C.

The oocysts employed for sporozoite preparation were used at a minimum concentration of $1 \times 10^7/\text{ml}$. The oocyst suspension was transferred to the grinding vessel of a teflon tissue homogeniser in an ice bath. The pestle was driven at 12,000 r.p.m. Between 2-5 minutes was required to crack most of the oocysts, liberating sporocysts. Microscopical examination indicated the required grinding time. The sporocyst suspension was diluted 1 in 10 vol./vol. in warm PBS A containing 0.5% Difco bile salts and 0.25% trypsin (1 : 250 powder) in screw-capped tubes. The mixture was incubated in a water bath at 39°C for 1 to $1\frac{1}{2}$ hours. Microscopical examination at intervals after one hour showed when the majority of sporocysts had hatched releasing sporozoites. The tubes were removed from the water bath gently and the sporozoite suspension was removed using a pipette. A denser layer containing oocyst debris remained at the bottom of the tubes. The trypsin/bile solution was washed from the sporozoites by centrifuging for 5 minutes at 1,000 x g. The sporozoites were resuspended in warm PBS. A sterile glass bead column had been prepared in the 37° warm room. It was 3 inches long by 1 inch in diameter and made up of beads 100 μ in diameter. It was prepared by flushing with sterile distilled water until the pH of the glass bead column could be shown to be pH 7.0 when PBS containing phenol red was poured through. The sporozoite suspension was poured into the PBS soaked column followed by PBS as required. Fractions were collected until no cloudiness was seen. Most sporozoites were obtained in fractions 1 and 2.

If necessary they were centrifuged to concentrate them. Passage through the column of glass beads removed any debris, sporocysts and most bacterial contamination from the sporozoites.

Production of *E. tenella* 2nd generation merozoites

Ten day old fertile eggs were inoculated via the chorioallantoic route with 10,000 *E. tenella* sporozoites each. The eggs were incubated at 105°F for five days. On the fifth day, surviving eggs were treated as follows. The shell and the vitelline membrane were broken and the allantoic fluid was removed aseptically using a capillary pipette. The allantoic fluid was left to stand on the bench for one hour, during which time most of the red blood cells settled, leaving the merozoites suspended in the upper portion of the fluid.

Appendices for chapter 2a

Boné's Medium

Boné, G.J. & Parent, G. 1963. J. Gen. Microbiol., 31, 261-266

	<u>wt. in gms</u>
Na Cl	4.0
glucose	2.0
KCl	0.4
NaPO ₄ 12H ₂ O	5.0
Bacto Difco tryptose	15.0
Oxoid liver infusion	1.0
haemin	0.02
glass distilled H ₂ O	1000 ml.

The haemin was ground in a pestle and mortar in a few drops of N/1 NaOH. The liver infusion was dissolved in about 30ml. of the water and the insoluble residue removed by centrifugation at 2,000 r.p.m. for 10 minutes. The haemin and liver infusion supernatant were then added with the other ingredients to the water. When all the ingredients had dissolved the pH was adjusted to pH 7.8. Medium was dispensed in 10ml. quantities in 50 ml. medical flats and in 95ml. quantities in 250 ml. round bottles. Sterilisation was by autoclaving for 30 minutes at 112°C.

Medium was supplemented with 5% rabbit serum, after sterilisation, where necessary. (95ml. quantities).

Modified HIL medium

Modified from HIL medium - Castellani, O., Ribeiro, L.V. & Fernandes, J.F. 1967. J. Protozool., 14(3), 447-451

	<u>wt. in gms.</u>
KCl	0.4
Na ₂ HPO ₄	8.0
glucose	2.0
haemin	0.025
Oxoid brain/heart infusion	10.0
glass distilled H ₂ O	875ml.

The formula for this medium was modified due to the unavailability of some constituents in original HIL medium. The haemin was ground in a pestle and mortar in a few drops of N/1 NaOH. The haemin was then added with the other ingredients to the water. When all the ingredients had dissolved the pH was adjusted to pH 6.7 medium was dispensed in 8 ml. quantities in 50ml. medical flats. Sterilisation was carried out by autoclaving for 40 minutes at 112°C.

Medium was supplemented with 10% lactalbumen hydrolysate (5% solution commercially available) and 10% calf serum.

Ochromonas Medium

As recommended by the Catalogue of Laboratory Strains of Free-Living and Parasitic Protozoa. J. Protozool., 5(1), 1-38 (1958).

	<u>wt. in gms</u>
tryptone	1.0
liver extract	1.0
glucose	1.0
distilled H ₂ O	1000ml.

The ingredients were mixed and dissolved. The pH was adjusted to pH 5.5 - 6.0. Medium was dispensed in 10ml. quantities in McCartney bottles. Sterilisation was by autoclaving at 121°C for 15 minutes.

Euglena Medium

As recommended by the Catalogue of Laboratory Strains of Free-Living and Parasitic Protozoa. J. Protozool., 5(1), 1-38 (1958)

	<u>wt. in gms.</u>
Na acetate	1.0
beef extract	1.0
tryptone	2.0
yeast extract	2.0
CaCl ₂	0.01
distilled H ₂ O	1000ml.

The ingredients were mixed and dissolved. The pH was adjusted to pH 6.0. Medium was dispensed in 10ml. quantities in McCartney bottles. Sterilisation was by autoclaving at 121°C for 15 minutes.

Pan's F7 Medium

Pan, C.T. 1968. Am. J. Trop. Med. Hyg., 17(6), 823-832

	<u>Volume in mls</u>
Modified 199 medium	85
Chicken serum (Flow Laboratories)	5.0
Chick-embryo extract (Flow Laboratories)	5.0
Haemolysed rabbit blood (1 part:4 parts distilled H ₂ O)	5.0

Rabbit blood was obtained aseptically by cardiac puncture. It was defibrinated and haemolysed by addition of sterile distilled water as above. Modified 199 medium was bought in as x10 concentrate and diluted with distilled water as required. 2.5ml. of 4.4% sodium bicarbonate solution was also added to provide buffer at an appropriate pH. After all the constituents named above had been combined, medium was dispensed in 10ml. quantities in 50 mL. medical flats. Sterilisation was not necessary as all the original constituents were sterile and aseptic techniques were used throughout. The pH of the resultant medium was measured and found to be pH 7.7.

Nutrient Broth - Modified Wright's Medium

This medium was available commercially. It was used in 10ml. quantities in McCartney bottles (25ml.) and in 200ml. quantities in 250ml. round bottles.

Nutrient Agar Slopes

This medium was available commercially. Slopes in McCartney bottles and agar in 100ml. quantities were used. The large quantities were used for the preparation of sterile plates and blood agar plates (see below).

Blood Agar Plates

Sterile defibrinated rabbit blood was prepared as described previously p.30 . The nutrient agar was heated to liquify it and when it had cooled to just below 50°C it was supplemented with 10% defibrinated whole blood. Plates were poured immediately, in 4 inch petri dishes.

Eagles BHK Medium

quantities in ml.

Eagles BHK x 10 concentrate	8.0
Glass distilled H ₂ O	72.0
Tryptose phosphate broth	10.0
Sodium bicarbonate (4.4%)	1.5
Calf serum	10.0

b) Materials and methods used for cultivation of entamoebae and related organisms

The strains of various species of amoebae that were used have been listed in table 1.

Table 1 Strains of amoebae and strain histories

<u>Specific name</u>	<u>Strain</u>	<u>Strain History</u>
<u>Entamoeba histolytica</u>	<u>Biswas</u>	Neal, R.A., Robinson, G.L., Lewis, W.L. & Kessel, J.F. 1968. Trans. Roy. Soc. trop. Med. Hyg., <u>62</u> (1), 69-75
" "	<u>Rahman</u>	
" "	<u>Menzes</u>	
" "	<u>Jawa</u>	
" "	<u>Rustom</u>	
" "	<u>Sharma</u>	Isolated Feb. 1963 by G. L. Robinson
" "	<u>4490/63</u>	
" "	<u>200:NIH</u>	
" "	<u>Ambrose</u>	
" "	<u>Burden</u>	
" "	<u>Laredo</u>	Isolated by Dr. F.H. Connel 1956. Subline of original maintained by Dr. Goldman and subculture sent to W.L.T.M. in June 1965 Goldman, M. 1969. Bull. WHO, 40(3), 355-364
<u>E. hartmanni</u>	<u>Chattergee</u>	Isolated 17 May 1966 by G.L. Robinson
<u>E. coli</u>	<u>Tooti</u>	Isolated 19 Dec. 1968 by G.L. Robinson
"	<u>Said</u>	Isolated 18 March 1969 by G.L. Robinson
<u>E. invadens</u>	<u>BAH</u>	Isolated 26 March 1953 from dead <u>Boa constrictor</u> by Dr. R. A. Neal

Table 1 continued....

<u>Specific Name</u>	<u>Strain</u>	<u>Strain History</u>
<u>E. ranarum</u>	R —	Isolated 31 Oct. 1953 from <u>Rana temporaria</u> by Dr. McConnachie
<u>E. moshkovskii</u>	DC	Neal, R.A. (1953) Parasitology <u>43</u> , 253-268
<u>Dientamoeba fragilis</u>	Gokul	Isolated 4 Sept. 1968 by G.L. Robinson
<u>Histomonas meleagridis</u>	382III	Culture received from Dr. L. Joyner 1959
" "	Ft	Isolated prior 1965 from infected turkey at Frant by M. Clarke.

Routine cultivation of amoebae growing in association with bacteria in HSre+S, Ehs+S and HSM+S media, and Robinson's medium

The media and temperatures used for maintaining different strains of amoebae in vitro are listed below.

<u>Strain</u>	<u>Medium</u>	<u>Temperature</u>
<u>4490/63</u>	Jones' medium	37°C
<u>382 III</u>		
<u>Ft</u>		
<u>Laredo</u>	Ehs+S	25°C
Other <u>E. histolytica</u> strains except <u>200:NIH</u>	HSre+S	37°C
<u>Chattergee</u>		37°C
<u>BAH</u>		25°C
<u>R</u>		25°C
<u>DC</u>		25°C
<u>Gokul</u>		37°C
<u>E. coli</u>	Robinson's medium	37°C

Cultures maintained at 37°C were subcultured twice weekly and those maintained at 25°C, once weekly.

A small drop of sediment was removed with a capillary pipette from a grown amoebic culture. It was examined microscopically and the growth of amoebae assessed as follows:-

<u>+</u>	1 amoeba seen
1+	2-5 amoebae seen/low power field
2+	5-10 " " / " " "
3+	10+ " " / " " "

The specimen was also examined macroscopically for bacterial growth and fungal contamination.

An appropriate quantity of sediment was transferred to the bottom of a tube of fresh culture medium using a capillary pipette. Thus one third of the sediment was used from a 3+ culture and most of the sediment was used from a 1+ culture. Care was taken to avoid unnecessary aeration of the medium. The cultures were incubated at the appropriate temperatures. Duplicate cultures were maintained for each strain. A daily record of cultures was kept. If a strain was growing consistently poorly the fresh medium was conditioned by 24 hours growth at 37°C of A' bacterium originating from a loop inoculation. Where the bacterial growth was observed to be poor 1 ml. of A' culture in nutrient broth was added to the culture tube. Fungal contamination and excess bacterial growth was checked by the addition of up to 0.2 ml. of 1:500 solution of acriflavin. Old amoebal cultures were kept until grand-daughter cultures had been initiated so that they could be used if necessary if daughter cultures failed to grow.

Bulk cultivation of the above cultures for production of large numbers of amoebae

For this cultivation, Ehs+S and HSre+S media in 250 ml. round bottles and HSM+S medium in 40 ml. quantities in 50 ml. medical flat bottles were used. All culture media were conditioned with either 24 hours A' bacterial growth at 37°C or 3 days A' bacterial growth at 25°C. This bacterial growth was initiated by a loopful of bacterial material.

All the sediment from two 3+ routine cultures was required to initiate bulk cultures. Subcultivation techniques were similar to those described above.

Harvesting of amoebae from bulk cultures was carried out as follows. As much of the supernatant liquid phase as possible was poured off into a sterile bottle without losing the sediment. The sediment was then mixed thoroughly using a capillary pipette or gentle shaking. The sediment was then used as the amoebic concentrate and the supernatant could be used as diluent when necessary.

Initiation of amoebic cultures, associated with bacteria, from amoebae stored in liquid nitrogen.

An ampoule containing approximately 0.75 ml. of amoeba concentrate was removed from the liquid nitrogen container and thawed rapidly in the hands.

Two culture tubes of the appropriate medium had been conditioned with A' bacterial growth. When the amoebic concentrate was thawed it was removed from the ampoule with a capillary pipette and distributed equally between the two culture tubes. After two or three days the cultures were examined in the normal fashion and subcultured into fresh tubes using most of the sediment unless growth was 3+ when less was used for each daughter culture. Thereafter subculture was carried out routinely.

Isolation of *E. histolytica* strains from human stools using mixed bacteria associated cultures

E. histolytica specimens were obtained from two children in which cysts had been observed in the stools.

On arrival at the laboratory wet smears were prepared from the stools using 1% aqueous eosin stain and Lugol's iodine respectively. Thorough microscopical examination was made and the presence of *E. histolytica* cysts was confirmed. Cysts of *Endolimax nana* were

also observed in one sample.

Using HSre+S medium which had been conditioned with A' Escherichia coli growth two cultures were prepared from a small quantity of each specimen. One of the cultures contained 0.2 ml. of 1:500 acriflavin solution. The cultures were incubated at 37°C.

After three days incubation the culture sediments were examined microscopically. Both isolates were observed to contain E. histolytica trophozoites. A heavy inoculum was used for subculturing, one line with added acriflavin. Routine subculture continued for 5 weeks (10 subcultures) when it was decided that healthier amoebic growth had been achieved in the acriflavin containing cultures. The other line was discarded. The acriflavin line was itself subcultured to produce two daughter cultures, one with acriflavin (0.1 ml.) and one without. After a further two weeks the line without acriflavin was selected for continued propagation. In one strain, although a few E. nana trophozoites had been observed within the first two weeks, none were seen thereafter.

Liquid nitrogen storage of amoebae

The sediment from four cultures was pooled and counted. It was diluted to give a concentration of 7×10^5 trophozoites/ml. and 7.5% dimethylsulphoxide added. This amoebic inoculum was dispensed in 0.7 ml. ampoules which were sealed and the temperature reduced 1°C per minute until -20°C had been reached. Thereafter they were transferred to the liquid nitrogen storage container.

Routine cultivation of amoeba-trypanosome cultures in modified A-TPH medium (E. histolytica only)

15 ml. of A-TPH medium was added to 5 ml. of double egg yolk infusion. To this was added 4 ml. of 7 day old Boné's T. cruzi culture, which had been examined microscopically to ascertain bacterial sterility. The amoebic culture tubes were first examined macroscopically for gross contamination. They were then examined microscopically using the lower power magnification (x 225) of an inverted microscope. The amoebae could be seen directly through the glass walls of the culture vessel. Each tube was examined and the amoebic growth assessed as follows:-

<u>+</u>	1 amoeba seen/low power field
1+	2-5 amoebae seen/low power field
2+	5-10 " " / " " "
3+	10+ " " / " " "

The A-TPH, egg yolk infusion and T. cruzi medium was dispensed in 5 ml. quantities in screw cap 4" x $\frac{1}{2}$ " tubes. Using a capillary pipette an appropriate quantity of sediment from an old amoebic culture was transferred to the bottom of the tube of new medium. Care was taken to avoid excess aeration of the cultures. The quantity of sediment transferred related approximately to the observed amoebic growth. Thus only one third of the sediment was used from a 3+ culture, whereas all of the sediment from a + or 1+ culture was used. A minimum of 3 tubes for each strain was maintained at any one time. The amoebic culture tubes were incubated vertically at 37°C.

Cultures were examined microscopically daily and were subcultured routinely twice weekly. If the amoebic growth had been

too rapid three subcultures were made in one week and the inoculum was reduced. Once a month sterility tests were made.

Bulk cultivation in modified A-TPH medium

100 ml. medical flat bottles were used. A-TPH, double egg yolk infusion and Boné's T. cruzi culture were made up in the same relative proportions as used in tube cultures to make 82 ml. of medium. The bottles were inoculated with sediment from 4 small tubes of 3+ growth. Harvesting was by careful pouring off of the medium and chilling in ice water for 5 minutes followed by gentle shaking.

Routine cultivation of amoeba-trypanosome cultures in TP-S-1 and TTY-SB media (All amoebae listed as successful in table 1 chapter 5a)

As the methods involved in amoeba-trypanosome cultivation in both TP-S-1 and TTY-SB media are so similar, they will be dealt with simultaneously. Either medium may be substituted for the other providing that the same medium is used throughout.

The amoebic cultures were examined macroscopically and microscopically as described previously (pp 46). The T. cruzi grown in Boné's medium were also examined microscopically to check freedom of contamination and adequate growth of T. cruzi epimastigotes. For 37°C cultures subculture was carried out twice weekly, once by replacement of the old medium and once by inoculation of fresh tubes of medium with amoebae from the old cultures. The second method was carried out by preparing the completed medium in the clean culture tubes and using a capillary pipette to transfer some amoebic sediment as described for modified A-TPH medium (p 46). Subcultivation by replacement of the medium was carried out by pouring off the old medium and then preparing the fresh medium in the old tube containing amoebae. The amoeba-trypanosome medium was prepared from 1 part of 7 day old T. cruzi culture and 5 parts of

medium. Thus when 4" x $\frac{1}{2}$ " screw capped tubes were used 1 ml. of T. cruzi culture was added and when 6" x $\frac{1}{2}$ " screw capped tubes were used 2 ml. of T. cruzi culture and 10 ml. of medium was added.

Culture tubes were incubated in a vertical position. The temperature was 37°C for all amoebae except E. invadens, E. ranarum, E. moshkovskii and Laredo strain of E. histolytica where it was 25°C.

Subcultivation was rarely necessary three times a week for 37°C cultures. Subculture of room temperature cultures was made once weekly, the two methods described above being used alternately.

Triplicate cultures were maintained for each strain.

Bulk cultivation in TP-S-1 medium with T. cruzi

Medical flat bottles (100 ml.) were used, containing 75 ml. TP-S-1 medium. 15 ml. of a T. cruzi culture were placed in the bottle. The sediment from 4 small tubes of 3+ growth or 3 large tubes of 3+ was used as inoculum. Harvesting the amoebae was carried out as described for bulk cultivation in modified A-TPH medium pp 47 .

Initiation of monoxenic amoeba-trypanosome cultures from amoeba-bacteria cultures

The method, based on Pan (1960), described was used both for A-TPH and TP-S-1 media. 4" x $\frac{1}{2}$ " screw capped tubes were used throughout.

Bulk amoeba-bacteria cultures were set up and incubated as described previously (pp 43). When these cultures were 3-4 days old amoebae were harvested. An amoeba inoculum was prepared. This ranged from $2-5 \times 10^4$ /ml. for non-invasive E. histolytica to $5-20 \times 10^4$ /ml. for other amoebae. As is explained in chapter 3d,

inocula of non-invasive E. histolytica were prepared at $5-20 \times 10^4$ amoebae/ml. until on one occasion when this had been impossible, success had resulted from a far lower concentration.

Culture tubes of amoeba-trypanosome medium were prepared as described under routine cultivation methods pp 46 . Penicillin (buffered "Solupen") and streptomycin sulphate were added to give a concentration of 100,000 i.u./ml. and 10,000 μ g/ml. respectively. One ml. of amoeba inoculum was added to each tube. All cultures were examined daily both macroscopically for bacterial growth and microscopically for amoebic growth. After 3 days subculture was made regardless of whether amoebae had been observed. Subculture was not made only in cases where the bacterial growth was excessive, and obviously not inhibited by the antibiotics. Subculture was carried out by the medium replacement method described on pp 47 . The concentration of penicillin and streptomycin remained the same. The cultivation continued by subculturing after four days incubation and with the same methods and concentration of penicillin and streptomycin. The amoebic and bacterial growth was noted daily during the first three weeks of monoxenic cultivation. If the bacterial growth appeared to have been halted by the end of the third subculture the fourth subculture was made with 10,000 i.u. penicillin/ml. and 10,000 μ g streptomycin/ml. On the fifth subculture, cultures which were observed to contain healthy amoebae on the walls of the culture tube were subcultured by inoculation into fresh culture tubes and medium using capillary pipette passage. The old culture tube was not discarded but filled with replacement amoeba-trypanosome medium. Any cultures in which amoebae were not observed to be

crawling on the walls of the tube were subjected to a more thorough examination. A drop of sediment was removed and examined microscopically. If no amoebae were observed the culture was discarded. If amoebae were observed subculture was carried out by capillary pipette passage and by medium replacement in the old tube.

Subcultures 6 and 7 also routinely contained 10,000 i.u. penicillin and 10,000 $\mu\text{g/ml}$. streptomycin. Subculture was by capillary pipette passage unless growth was observed to be poor (1+ or less). If during this time bacterial contamination was observed to reappear the concentration of antibiotics was boosted to that of the early concentrations used in initiation.

By subculture 8 a reasonable assessment of success could be made. If amoebic growth was good (3+) and bacterial growth absent the antibiotic concentration was reduced to 100 i.u. and 100 $\mu\text{g/ml}$. respectively. Subculture continued by alternate capillary pipette passage and medium replacement as described under routine maintenance pp. 47 until subculture 10. Daily observations were made but only contraindications were noted and remedial action taken if possible - e.g. addition of extra antibiotics if there was suspicion of bacterial growth.

Subculture 11 was made without the use of antibiotics. A further 10 subcultures were generally made before tests for bacteriological sterility were made.

The progression outlined above was adhered to for almost all the successfully cultured strains, but there were a few exceptions. If the progression had to be delayed it was resumed at the last change in antibiotic content thereby prolonging the operation by three subcultures. Strains which were not successfully isolated in

monoxenic culture were discarded when amoebae had not been observed after thorough examination in three subcultures. Further attempts were made.

During initiation experiments that were monitored quantitatively one set (minimum number of 4 tubes) of tubes was never disturbed but reserved for counting. For the counting procedures see chapter 2c.

Exchange associate experiments

All amoeba-trypanosome cultures were initiated using T. cruzi epimastigotes as associates. Once the cultures had become established and had been passed for sterility some were used to assess the use of other associates. The stock amoeba-trypanosome isolates were maintained in culture throughout the period of study.

The various methods used to culture or prepare prospective associates have been described in chapter 2a. The associate to be tested was counted and sufficient quantity was added to TP-S-1 medium to give a resultant concentration of 2.0×10^6 associates/ml. If the original concentration of the associate culture was so low that the ratio of associate culture:TP-S-1 medium was greater than 1:5 the associate culture was concentrated by centrifugation. The speed and time chosen for this, varied according to the size and motility of the organisms. Thus L. tarentolae cultures were spun at 750 x g and H. castellanii cultures were spun at 200 x g for 5 minutes. Subculture from the original amoeba-trypanosome culture was made into the medium containing the new associate at the normal time of subculture.

Subculture continued using the new associate only with no return to T. cruzi epimastigote as associate in the series. After a minimum of 10 subcultures, the last 5 having shown consistent amoebic growth, the cultures of amoebae were considered to be

established with the new associate. Further experiments were generally conducted at this stage (see pp.67).

All cultures grown with associates other than T. cruzi were derived directly from amoeba-T. cruzi epimastigote cultures.

Maintenance of 200:NIH in TP-S-1 axenic culture

This strain was obtained from Dr. L. S. Diamond growing in an axenic condition.

When it was received two lines of subculture were started, one in normal TP-S-1 medium and one in TP-S-1 medium with 0.02% ionagar and 0.1% gastric mucin. Subculture was made by first chilling the amoebic culture tubes for 5 minutes in ice cold water. These contained 3+ growth as observed through the walls of the tube using the inverted microscope. The chilled cultures were inverted gently 10 times, to loosen the amoebae from the walls. They were then centrifuged at 100 x.g. for 5 minutes. The amoebic sediment was removed with a capillary pipette and inoculated into 4 tubes. Thus two cultures of TP-S-1 and 2 cultures of TP-S-1 with ionagar and gastric mucin resulted from one culture. The next subculture was made from one tube of each type of medium to produce daughter cultures of the appropriate type. Subculture continued in this manner for some months using one third of the sediment from one tube instead of one quarter. The second line of cultures in ionagar and gastric mucin supplemented medium was discarded since the simple TP-S-1 was easier to handle.

Subculture continued for a period of 3 years during which another simpler method of subculture was adopted. This was the use of capillary pipette passage of the amoebic sediment to 2 or 3 fresh

tubes of medium without prior chilling and centrifugation procedures.

Sterility tests

Nutrient broth (Modified Wright's medium) was bought in as 10 ml. quantities in McCartney bottles. The contents of two bottles were sometimes combined to give 20 ml. in a bottle.

Nutrient agar was available commercially. It was melted by placing the bottle in a beaker of water with some cotton wool at the bottom and the water brought to the boil. The liquid nutrient agar was used to pour nutrient agar plates and also blood agar plates which were prepared as described in chapter 2a p39.

Sterility tests were prepared from 3 or 4 day old monoxenic cultures. The amoebic sediment was not included in sterility test inocula.

Nutrient broth cultures were inoculated with 1 ml. monoxenic supernatant to 20 ml. of broth. Both 10 ml. and 20 ml. quantity cultures were set up in duplicate. Half the cultures were incubated at 37°C and half at 25°C.

Another sterility test was set up to test for anaerobic organisms specifically. Nutrient broth which was in the process of cooling was used. To approximately 40 ml. of agar, 2 ml. of monoxenic culture was added and nutrient agar plates poured. Half the plates were incubated at 37°C and half at 25°C.

Stab cultures were also made in McCartney bottles containing 15 ml. of nutrient agar and blood agar respectively. These cultures were prepared by stabbing the agar with a capillary pipette containing a drop of monoxenic culture supernatant. Again incubation was at

two temperatures.

All sterility tests were observed daily. After 1 week at 37°C and 2 weeks at 25°C they were discarded if no cloudiness or growth was observed. Slight cloudiness observed in the nutrient broth 10 ml. quantity cultures was followed up by nutrient agar and blood agar plate tests. The nutrient broth culture was used for streaking out with a platinum loop. Incubation was at the same temperature as the broth cultures from which they were derived.

Any observation of bacterial contamination in the sterility tests was related back to the monoxenic amoebic cultures and appropriate action was taken.

Sensitivity tests for bacterial contaminants

These were used for any monoxenic cultures which became contaminated and also for polyxenic cultures which could not be cleaned by straightforward addition of penicillin and streptomycin.

Tests were carried out to ascertain the number and variety of contaminants and their aerobic or anaerobic nature.

Aerobic organisms

Where possible nutrient agar cultures were used for this but if better bacterial growth had occurred in blood agar this latter medium was used. Agar plates were inoculated with 0.5 ml. of broth culture of the contaminants. After 2 hours incubation at 37°C a "Multodisc" antibiotic disk was carefully placed on the agar. Incubation was continued at 37°C and when the bacteria had grown (24 hours generally) a thorough examination both macroscopical

and microscopical was made. Several different types of "Multodisc" were used so that a wide range of antibiotics could be assessed.

Anaerobic organisms

Broth cultures were used to inoculate liquified nutrient or blood agar and plates were poured as described on pp. 39 . Using an alcohol flamed cork borer of appropriate size, small wells were cut in the agar and the agar removed. The wells were filled with 0.2 ml. of antibiotic solution. Several different antibiotic solutions were used of which the most common were erythromycin, "Septrin", neomycin and novobiotin. Incubation at 37°C was for a minimum of 24 hours or until bacterial growth was observed in control plates without wells. Bacterial sensitivity to various antibiotics was noted.

In vitro tests used to choose antibiotic and concentration thereof

The antibiotic sensitivity tests provided an indication of a suitable antibiotic that would inhibit growth of certain culture contaminants. As the antibiotics chosen from these tests might be amoebicidal also, in vitro tests were carried out to estimate their prospective use in monoxenisation experiments.

Antibiotic solutions were prepared at various concentrations and sterilised by filtration through a "Millipore" cellulose acetate filter. In vitro tubes (4" x $\frac{1}{2}$ " tubes with oxoid caps) were filled with 4.5 ml. of HSM+S medium and incubated overnight to check sterility. Amoebae were grown in bulk in HSM+S medium. When the amoebae culture was 3 to 4 days old 2 drops of nutrient broth culture of A' were added to each in vitro tube. They were incubated

at 37°C for 3 hours minimum. The amoebae were harvested and an inoculum of 1×10^5 amoebae/ml. was prepared. 0.5 ml. of drug solution was added to each in vitro tube and mixed with the medium by careful inversion. Then 0.1 ml. amoeba inoculum was added to each tube. Tubes were incubated for 3 days at 37°C and then examined.

Examination of an in vitro test was done by removing a drop of sediment from each tube with a capillary pipette. The capillary pipette was placed in boiling water between each sampling to prevent any carryover of amoebae between tubes. The drop of sediment was examined microscopically for growth of amoebae and bacteria. The pH of the medium was also taken.

The amoebae used for this test were in amoeba-bacteria cultures and therefore the total absence of bacteria and amoebae at the end of the test could be confusing. Death of amoebae could be directly due to the antibiotic or indirectly due to the antibiotic by starvation. However, as the antibiotics selected by the sterility and sensitivity tests were active on organisms unaffected by the wide range antibiotics penicillin and streptomycin all bacterial growth was rarely inhibited.

The antibiotic solution and concentration for further use was chosen only if 3+ amoebic growth had been observed in in vitro tests.

Treatment of contaminated monoxenic amoebic cultures

Bacterial contamination

Where possible contaminated cultures were discarded and parallel cultures were used for subculture. This was not possible during unsuccessful monoxenisation, where failure was attributed to

persistent bacterial growth.

The nature of the bacterial contamination was established by the use of sterility and sensitivity tests. Antibiotics were added to the cultures in concentrations that would not affect the amoebae adversely. Subsequent sterility tests indicated success or failure of the treatment. If failure resulted another antibiotic was used if possible or the culture was discarded.

Fungal contamination

If at any time fungus or mould was observed in monoxenic amoebic cultures they were discarded immediately. A fresh isolation was subsequently prepared if necessary.

Infection of monoxenic amoebic cultures with mycoplasma

On one occasion poor growth of amoebae was observed for a period of three weeks although there was no obvious abnormality in the culture conditions. Sterility tests had not shown the presence of any bacteria and no fungus had been observed.

Mr. T. Varley of the Bacteriology Department, Wellcome Research Laboratories, kindly tested the cultures for the presence of mycoplasma. These tests were negative. After a few weeks the amoebic growth improved and it was concluded that the problem could have been caused by poor rinsing of tubes in the washing-up procedure.

Appendices for Chapter 2bMedia used for polyxenic, bacteria associated, amoebic culturesHSre+S

Horse serum slopes

Ringer egg albumen overlay

Sterile rice starch

5gm. rice starch was placed in an Universal bottle, the bottle capped and taped. It was autoclaved at 121°C for 15 minutes. When cool 20ml. sterile distilled water was added to suspend the starch. One ml. starch suspension was added to 100ml. overlay medium. Both serum slopes and overlay were bought in. Serum slopes were made in McCartney bottles for routine use and also in 250ml. round bottles for bulk growth of amoebae. About 12ml. overlay containing starch was poured onto the serum slopes in McCartney bottles. The serum base in the 250ml. round bottles which was 1cm. deep was overlaid with approximately 100ml. Ringer egg albumen.

Ehs+S

Coagulated egg slopes

Ringer horse serum

Sterile rice starch

Egg slopes were bought in, in McCartney bottles and also in 250ml. round bottles as described for serum slopes. Ringer horse serum was also bought in. Sterile rice starch was prepared as described above and added to the overlay medium. The overlay medium was added as described for HSre+S.

HSM+S

Jones (1946)

Jones' E. histolytica medium

Sterile rice starch

Jones' medium was bought in in 100ml. quantities in 100ml. medical flat bottles. It was supplemented with sterile rice starch as described for HSre+S. The completed medium was dispensed into sterile 6" x $\frac{1}{2}$ " screw capped tubes, approximately 10ml. quantities.

Robinson's Medium

Robinson, G.L.(1968)

The constituent solutions and medium together with B strain of Escherichia coli were kindly supplied by Mr. Sargeunt, L.S.H.T.M.

Slopes of agar were overlaid with 2.25ml. of phthalate solution, 0.03ml. of erythromycin solution and 0.1ml. of bactopectone solution. 1ml. of E. coli B culture in nutrient broth and horse serum (equal volumes) was added. A drop of starch suspension was also added. Subculture was performed by capillary pipette passage. see pp. 42.

Media used for monoxenic amoebic cultures

Modified A-TPH medium

Modification of Pan's A-TPH medium (1960) J. Infect. Dis. 106, 284-9

	<u>wt. in gms.</u>
Trypticase	30.0
NaCl	7.0
Na ₂ CO ₃	0.3
Glucose	5.0
Distilled H ₂ O	1000ml.

The ingredients were weighed and dissolved in the water. The medium was dispensed in 13.5ml. aliquots in McCartney bottles. Sterilisation was by autoclaving at 10lb. pressure for 15 minutes. When cool, 0.75ml. of sterile horse serum (heat inactivated) and 0.75ml. haemolysed, defibrinated rabbit blood were added. The rabbit blood had been obtained as described previously and haemolysed by addition of 4 parts distilled water to one part of rabbit blood. Both the rabbit blood before addition to the medium and the complete medium were stored at -22°C.

Double Egg Yolk Infusion

Balamuth & Sandza (1954) Proc. Soc. Exp. Biol. & Med. 57, 161-163

The medium as described below produced 250ml. of infusion. Twice the number of eggs were used compared with that reported. Four infertile hens eggs were hard boiled for 15 minutes. On cooling, the whites were discarded and the yolks crumbled into a beaker containing 125ml. of 0.8% NaCl solution. This mixture was boiled

for 15 minutes with frequent stirring. The volume was made up to the original quantity by adding distilled water. The mixture was filtered through muslin and then filtered through a Zeist filter using suction. The clear liquid was autoclaved at 15lb. pressure for 20 minutes. On cooling a slight precipitate formed which was removed by simple filtration. 125ml. of M/15 phosphate buffer (pH 7.5) was added thereby making the total salt concentration M/30 phosphate solution in 0.4% NaCl solution. The medium was dispensed in 5ml. quantities in McCartney bottles. It was autoclaved as before for sterilisation and stored at 4°C.

TTY-SB

Diamond, L.S. (1968). J. Parasit., 54(4), 715-719

	<u>wt. in gms.</u>
Tryptose (Difco)	1.0
Trypticase	1.0
Yeast extract	1.0
Glucose	0.5
L-cysteine monohydrochloride	0.1
Ascorbic acid	0.02
Sodium chloride	0.5
Potassium phosphate, monobasic	0.08
Potassium phosphate, dibasic, anhydrous	0.08
Distilled water	95.0ml.

The ingredients were weighed and the tryptose dissolved in the water by heating to 50°C if necessary. The remaining ingredients were dissolved in the order given above. The pH of the medium was adjusted to pH 7.2. Medium was dispensed in 95ml. quantities in 100ml. medical flat bottles and sterilised by autoclaving at 112°C for 30 minutes. When cool the medium was supplemented with 5.0ml. of horse serum and 0.25ml. of whole rabbit blood.

TP-S-1

Diamond, L.S. (1968) J. Parasit., 54(5), 1047-1056

	<u>wt. in gms.</u>
Trypticase	1.00
Panmede, liver digest	2.00
Glucose	0.50
L-cysteine monohydrochloride	0.10
Ascorbic acid	0.02
Sodium chloride	0.50
Potassium phosphate, monobasic	0.06
Potassium phosphate, dibasic anhydrous	0.10
Distilled water	87.5ml.

The ingredients were weighed and dissolved in the water in the order listed above. The pH was adjusted to pH 7.0 using IN sodium hydroxide. The medium was dispensed in 87.5ml. quantities in 100ml. medical flat bottles and sterilised by autoclaving at 112°C for 30 minutes. When the medium was cool it was supplemented with 2.5ml. of vitamin mixture (NCTC 107) and 10ml. horse serum.

Vitamin Mixture NCTC 107

McQuilkin, W.T., Evans, Virginia J., & Earle, W.R. (1957). J. Nat. Can. Inst., 19, 885-907

	<u>wt. in mgms.</u>
Nicotinic acid (niacin)	12.5
p-aminobenzoic acid	25.0
Nicotinamide (niacinamide)	12.5

	<u>wt. in mgms</u>
Pyridoxine HCl	12.5
Pyridoxal HCl	12.5
Aneurine HCl	5.0
Calcium pantothenate	5.0
i-Inositol	25.0
Choline chloride	250.0
Riboflavine	10.0
D-biotin	10.0
Folic acid	10.0
Calciferol	10.0
Vitamin A alcohol	10.0
Menadione	10.0
Tween 80	500.0
Disodium α -tocopherol phosphate	10.0

The vitamin mixture was prepared from five stock solutions which were stored at -22°C until use. The stock solutions were prepared as follows:-

1. Water soluble B vitamins

- a. 12.5mg. nicotinic acid and 25mg. p-aminobenzoic acid were dissolved in boiling distilled water and brought to a total volume of 25ml.
- b. 12.5mg. nicotinamide, 12.5mg. pyridoxine HCl, 12.5mg. pyridoxal HCl, 5mg. thiamin (aneurin) HCl, 5mg. calcium pantothenate, 25mg. i-inositol and 250mg. choline chloride were dissolved in distilled water and brought to 25ml.

c. 10mg. riboflavin was added to 10ml. of 0.075 N HCl, then 5ml. of 0.2 N NaOH was added and moderate heat applied. The total volume was brought to 20ml. with distilled water. 10ml. of the riboflavin solution was combined with 25ml. of solution 1a and 25ml. of solution 1b. The final solution was brought to a final volume of 100ml. with distilled water.

2. Biotin Solution

10mg. D-biotin was dissolved in 50ml. of distilled water acidified with 1ml. N HCl. This was brought to a volume of 100ml. with distilled water.

3. Folic acid solution

10mg. folic acid was dissolved in 100ml. of distilled water, heated to 80°C.

4. Lipid soluble vitamins A,D & K

An alcoholic tincture of each of these three vitamins was used for the first stage of this solution. 10mg. vitamin D (calciferol) was dissolved in 2ml. ethyl alcohol (200° proof) in a 100ml. volumetric flask. 10mg. vitamin A alcohol was then dissolved in this tincture. 10mg. vitamin K (menadione) was dissolved in 1ml. of alcohol in a separate flask, from which 0.1ml. was pipetted into the volumetric flask above. 10ml. of a 5% aqueous (0.5g/10ml.) solution of Tween 80 was added to the pooled alcoholic tinctures. The solution was brought up to 100ml. with distilled water.

5. Vitamin E. solution

10mg. disodium α -tocopherol phosphate was made up to a volume of 100ml. with distilled water

To prepare the vitamin mixture, NCTC 107, the five stock solutions were combined in the following proportions:-

2 parts water soluble B vitamins (solution 1)

1 part biotin solution (" 2)

1 part folic acid solution (" 3)

10 parts lipid soluble vitamins A, (" 4)

D & K

1 part vitamin E solution (" 5)

The complete vitamin mixture was sterilised by passage through a "Millipore" membrane filter (pore size 0.22 μ). It was stored at -22°C.

c) Materials and methods chosen for assessment of growth and analysis of associate support

Establishment of monoxenic cultures

Once a monoxenic culture of amoebae had grown in association with another organism under specific conditions for a minimum period of 10 subcultures, extensive sterility tests were undertaken. These have been described in chapter 2b pp 53. When all sterility tests had been shown to be negative the type of growth under these circumstances was analysed. This was done by a count experiment.

Count experiments - short term

It was generally assumed that growth of a subculture of 37°C amoebae need be examined for 10 days whereas subcultures of room temperature amoebae had to be examined for 21 days. As readings were made in duplicate and tubes were never used more than once, a minimum of 20 tubes for 37°C amoebae and 42 tubes for room temperature amoebae were set up.

The medium for these experiments was prepared in bulk, including addition of associates and amoebae. The initial concentrations of associates and amoebae were always recorded. Where necessary the initial concentration of associates was arranged to be 2.0×10^6 /ml. It may be assumed that unless stated otherwise in the appropriate results section all count experiments had an initial associate concentration of 2.0×10^6 /ml. The initial concentration of amoebae was similarly adjusted to 5.0×10^3 amoebae/ml. except where stated otherwise. The completed medium was dispensed in 5 ml. quantities in the allotted 4" x $\frac{1}{2}$ " screw capped tubes. Incubation temperature was at the appropriate temperature.

Daily, 2 tubes were removed from the incubator and chilled for 5 minutes in ice cold water. The tubes were gently inverted 10 times to loosen amoebae from the walls. 2 samples were taken from each tube using capillary pipettes. Haemocytometer (improved Neubauer ruling) counts were made for each sample. The average of the means of counts from two tubes was taken as the reading. Tubes were discarded after counting.

Amoebae were counted over the entire surface of the ruled portion of the haemocytometer. Only live motile amoebae were counted. The calculation for the number/ml. was accordingly $\frac{x}{9} \times 10^4$.

Smaller more numerous organisms counted on the haemocytometer slide were counted within the 25 smaller squares in the central portion of the ruling. The calculation for the number/ml. was accordingly $y \times 10^4$. Motile flagellates were killed before counting by the addition of 1 part Lugol's iodine to 9 parts flagellate sample. Where necessary concentrated samples were diluted with normal saline before counting and the calculation adjusted accordingly.

Daily counts were continued until the number of amoebae was obviously declining. The growth curves of cultures were plotted graphically and may be seen in the results sections.

Differential counts at peak growth

When peak growth appeared to be reached, differential counts of dead:live amoebae were made. As this had to be done during the experiment and not retrospectively it was often necessary to repeat the differential count on the following day.

Equal volumes of amoebic suspension and stain (1% aqueous eosin or 1% trypan blue) were mixed on a slide and then a sample from the mixture counted on a haemocytometer slide. As dead amoebae absorbed the stain it was possible to relate the number of dead amoebae directly to the number of live amoebae.

Phase contrast microscopy

When only a qualitative assessment of dead:live amoebae was required, observation under phase contrast illumination gave a clear indication. The live amoebae were bright and reflected the light. The dead amoebae were dull and absorbed the light.

Count experiments - long term

This type of experiment was considered suitable for assessment of various media and physical conditions in particular. Assessment of amoebic requirements in media, pH and temperature was carried out directly without the establishment period of 10 subcultures under the specific conditions.

This type of experiment was also used for monitoring the initiation period of monoxenisation of strains.

In operation these experiments were basically similar to the short term count experiments but with several notable exceptions. These differences were due to the fact that long term count experiments included subculture during the period of the experiment.

A minimum of 10 tubes were prepared and filled as described for short term count experiments pp. 67 . Daily counts were made as before but sterile conditions were maintained throughout. All the tubes were used for counts before returning to the first counted, so several days elapsed between counts and the culture was allowed to settle undisturbed. When it was time to make a subculture i.e.

3-4 days for 37°C amoebae and 7 days for 25°C amoebae all the tubes were chilled for 5 minutes in ice cold water. They were all inverted gently 10 times. Two tubes were used to provide count samples under sterile conditions. Then all the tubes were centrifuged at 100 x g for 7 minutes. The supernatant was removed with a pipette fitted with a rubber bulb and care was taken not to disturb the sediment. 5ml. of replacement complete medium containing associates of fixed and known concentration was added. The tubes were inverted once to suspend the amoebae from the pellet. Incubation was resumed. Daily counts and subculture continued in this manner until the end of the experiment.

Experiment to demonstrate need for physical contact between amoebae and flagellates in amoeba-trypanosome cultures (Biswas-Kessel)

A picture of the apparatus used for this experiment is shown in figure 1 . The TP-S-1 medium together with the amoebic inoculum was placed in the lower portion of the apparatus by means of a 20 ml. syringe inserted at A. The clip B was opened to allow the release of pressure caused by inoculation of the volume. The Boné's T. cruzi portion was poured into the upper portion of the apparatus through the top C and the bung replaced. The apparatus was held in a retort stand at 37°C as shown and the two media mixed and equilibrated through the cellulose acetate membrane D. The amoebae and flagellates were unable to pass through the membrane. At the end of three days' incubation the apparatus was disassembled and the lower portion thoroughly examined for the presence of amoebae.

Attempts to axenise cultures

These experiments were carried out to demonstrate the ease with which axenic cultures could be established or alternatively the dependance of the amoebae on their associates.

Amoeba-trypanosome and amoeba-crithidia cultures were used, both growing in TP-S-1 medium. Subculture was carried out routinely but the Boné's T. cruzi fraction was replaced with an equal quantity of TP-S-1 medium. This experiment was only done qualitatively so the concentration of amoebae was not observed although a daily assessment of amoebic growth was made using the inverted microscope. E. invadens cultures continued to be maintained axenically for several months alongside their amoeba-trypanosome counterparts. E. histolytica cultures were maintained for two subcultures without the addition of T. cruzi or Crithidia sp. When the cultures were observed to be 1+ the associates were replaced (4.0×10^6) and further growth observed.

Experiments on reassociation of monoxenic amoeba-trypanosome cultures with one strain of bacterium

Tubes of HSre+S medium were conditioned with bacterial growth of Escherichia coli 'A' strain as described previously pp. 43. All the sediment from two 4" x $\frac{1}{2}$ " tubes of amoeba-trypanosome cultures was collected with a capillary pipette and inoculated into the bottom of the HSre+S culture tubes. Subculture was then made twice weekly always using bacterial primed culture medium. Larger inocula of amoebae were used in these cultures than in mixed bacterial cultures.

Experiment to demonstrate effect of Boné's medium on amoeba-trypanosome growth in modified ATPH medium system (Rahman/Kessel)

Culture tubes were set up in a similar manner to that described previously for routine cultivation of amoeba-trypanosome cultures in modified ATPH medium. However various ratios of Boné's medium to ATPH medium were used. The initial concentration of T. cruzi was constant throughout the groups. Twenty tubes per group were set up and daily counts were made as described under short-term count experiments. No prior adaptation to these conditions during 10 subcultures preceeded this test. No subculture took place during the count experiments.

Experiment to demonstrate necessity for presence of various items in TP-S-1 and TTY-SB media

Two experiments were carried out. One using TP-S-1 medium and one TTY-SB medium. The media constituents were omitted in a stepwise sequence. They and also the complete medium were used in the preparation of a long term count experiment see pp 69 . Subculture by medium replacement took place during the experiment. The initial concentration of T. cruzi epimastigotes was constant throughout the various groups at initiation and subculture. Counts were made daily as described on pp 69.

Assessment of growth of amoeba-trypanosome cultures in TP-S-1 medium supplemented with various animal sera

The sera from a variety of animal sources have been listed in table 1 . They were added to TP-S-1 as a 10% v/v final concentration. A short term count experiment was set up as

Table 1 Various Sera used in supplementation of TP-S-1 medium

<u>Animal source</u>	<u>Method of preparation</u>	<u>Special treatment</u>
Horse	Natural clot pooled Bought in from Wellcome	Inactivated at 56°C
Calf	" " "	"
Bovine	" " "	"
Calf - colostrum deprived	" " "	"
Chicken	Bought in from Flow Laboratories	"
Guinea pig	} Heart puncture, natural clot pooled	"
Rat		"
Rhesus monkey		"

described in chapter 2c pp. 67 . This experiment was not preceded by prior adaptation during a period of ten subcultures. Daily counts and growth assessment was made as described on pp. 67 .

Assessment of amoeba-trypanosome cultures in TP-S-1 medium supplemented with various concentrations of horse serum

Horse serum was added to TP-S-1 medium to give media containing various concentrations of horse serum. A short term count experiment was conducted to assess amoebic growth under such conditions. Methods used were similar to those described above for assessment of different animal sera.

Assessment of amoeba-trypanosome cultures in TP-S-1 medium of varying pH

Samples of TP-S-1 medium were used at varying stages during its preparation for pH assessment on a Pye pH meter.

TP-S-1 media were prepared of various pHs. The pH was adjusted using 1N sodium hydroxide for alkaline pHs and 1N hydrochloric acid for acid pHs. Extra medium was prepared so that the precise pH could be measured before use for amoeba-trypanosome cultivation.

A long term count experiment was set up as described on pp. 69 using the media of various pHs. Counts were made daily and subculture by replacement of medium was made as described on pp. 47.

Preparation of TP-S-1 medium deficient in L-cysteine hydrochloride

TP-S-1 media were prepared using $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ and $\frac{1}{32}$ the normal quantity of L-cysteine HCl. A short term count experiment was set up using these media as described on pp.67.

The pO_2 of the above media together with samples of TP-S-1 medium during various stages of preparation were measured. pO_2 measurements were made using an Astrup Micro Tonometer, Amt 1, Radiometer, Copenhagen. Tap water was taken as 100% oxygen content and the machine set to read pO_2 in relation to this standard.

Growth of amoeba-trypanosome cultures in various type of culture vessels

Culture vessels were selected which could be sterilised by autoclaving and which were sealed with screw caps. The capacity was measured using a measuring cylinder. The internal demensions were measured with a ruler. Some degree of estimation had to be made in the latter measurements and therefore are to be taken as approximate.

A short term count experiment (pp.67) was set up from TP-S-1 Boné's T. cruzi and amoeba inoculum mixed and then distributed to the various culture vessels. Because of the large quantity of materials required, vessels were duplicated and daily counts were made under sterile conditions.

An attempt to establish the peak yield of amoebae from an amoeba-trypanosome culture in TP-S-1 medium from a specific culture vessel

4" x $\frac{1}{2}$ " screw capped tubes were used. A short term count experiment (pp.67) was set up using a large amoeba inoculum,

$5.0 \times 10^4/\text{ml.}$, and a $2.5 \times 10^7/\text{ml.}$ concentration of epimastigotes to give optimum epimastigote:amoeba ratio (see chapter 3c).

A second short term count experiment was set up using twice the size amoeba inoculum of that used in the previous experiment. The epimastigote concentration was similar.

Inactivation of *T. cruzi* epimastigotes

1. Heat

T. cruzi epimastigotes from a 7 day old Boné's culture were counted and diluted to give 6ml. of normal associate inoculum. They were then subjected to 3 hours heat treatment. These associates were used in the preparation of a short term count experiment. The count experiment continued as described previously pp. 67.

2. Formalin treatment

A 1% solution of formalin was prepared and sterilised by autoclaving at 112°C for 15 minutes. *T. cruzi* epimastigotes were prepared as normal amoeba culture associates and prior to use 0.1ml. of formalin solution was added to 10ml. of *T. cruzi* suspension. Short term count experiments were set up using these formalised epimastigotes.

3. Lysis by water

T. cruzi epimastigotes from a 7 day Boné's culture were counted and diluted to give 10ml. of normal associate inoculum. This was centrifuged at $750 \times g$. for 10 minutes. The supernatant Boné's medium was poured off and replaced with 5ml. of sterile distilled water. The epimastigotes were resuspended using a capillary

pipette. The suspension was allowed to stand at 4°C for a minimum of 2 hours. Thereafter the water soluble fraction was separated from the water insoluble fraction by centrifugation at 750 x g. for 10 minutes. The clear supernatant was removed and stored. The sediment was resuspended in 5ml. PBS. Both fractions were stored at 4°C. When used in amoeba-trypanosome cultures only half the normal volume of inoculum was used.

Attempts were made to establish amoebic growth in association with both these fractions individually.

10 subcultures of amoebic growth was followed by a short term count experiment (pp.67).

d) Materials and methods used in virulence tests

Rat caecum inoculation test

Techniques used for cultivation of large numbers of amoebae have been dealt with in chapter 2b pp 43 and pp 47. The amoeba concentrate was counted using a haemocytometer and diluted to give a concentration of 1.25×10^5 amoebae/ml. The amoeba inoculum was kept at 37°C until used for caecal inoculation.

Weanling rats of mixed sexes, weighing 25-35 gm. each, were shaved on the ventral surface. Rats were anaesthetised individually in ether vapour. The ventral surface of the rat was swabbed with Cetrimide solution. Using sterile instruments throughout, the operation was performed. A small longitudinal incision (3mm long) was made in the skin and muscle layer of the lower abdominal region midway between the central line and the side of the animal. The caecum was located using curved blunt forceps and exposed. Using a 2 ml. glass syringe fitted with a wide bore needle (gauge 204), 0.4 ml. of amoeba inoculum was injected through the caecal wall towards the blind end. Injection was carried out slowly and only 0.3 ml. was given if the caecum was particularly small. The needle was withdrawn carefully and at the same time the point of injection was pinched gently with the forceps in order to prevent seepage of the inoculum. The swollen caecum was very gently returned into the abdominal cavity. The incision in the body wall was sown together with cotton. The skin incision was clipped together with a Michel clip. The wound was dusted with 50% sulphanilamide : 50% mephenide powder. The rats were placed in a warmed cardboard box until they

had recovered from the anaesthetic. Rats were grouped randomly in cages of 5 and fed Coward's rat diet and water ad libitum, 2 cages comprised each group.

The rats were killed and autopsied 7 days after inoculation. The caecum was exposed, examined externally, and then cut open and examined thoroughly macroscopically. Each caecum was allotted a score as follows:-

<u>Caecum</u>	<u>Score</u>
Normal	0
Slight thickening; contents less solid	1
Marked local thickening and contraction; contents slightly mucoid	2
Extensive thickening and contraction; contents mucoid and some solid matter	3
Caecum shapeless; contents mucus only	4

A small scraping of the interior of the caecal wall was made and a smear in a drop of normal saline made. This was examined microscopically for the presence of amoebae. If no amoebae were seen a culture was made in HSre+S medium from some of the caecal contents. Cultures were incubated at 37°C.

Cultures were examined after 3 days incubation. A drop of sediment was removed with a capillary pipette and examined microscopically.

The average caecal score of infected rats for each group was calculated and the number of infected and invaded rats noted.

Hamster - liver inoculation test

Weanling golden hamsters of mixed sexes were used. The hamsters were given 2 immunising doses of bacteria from amoeba-bacteria cultures when polyxenic cultures were to be used for liver inoculation. The immunising mixture was prepared by growing several drops of supernatant from a stock amoebic culture in nutrient broth at 37°C. When the bacterial culture was grown 0.05 ml. of this culture was injected by the intraperitoneal route into each hamster.

The hamsters were given a second immunising dose from the same bacterial culture after 3 days. Hamsters which were to be inoculated with monoxenic amoebae not associated with bacteria were not given immunising doses as described above.

Amoebae were grown in large quantities as described in chapter 2b pp 43 and pp 47. The cultures were inoculated so that peak growth was attained 3 days after the second immunising dose. Amoebae were harvested and an inoculum of 1×10^6 amoebae/ml. prepared.

Hamsters were weighed, caged individually, and anaesthetised with hexabarbitone by intraperitoneal inoculation. The ventral surface of each hamster was shaved. A small longitudinal incision was made in the skin very slightly right of the midline just below the diaphragm. The liver was thus exposed. Using a 1 ml. glass syringe fitted with a fine needle (guage 201) 0.1 ml. of amoebic inoculum was inoculated under the membraneous capsule of the dorsal surface of the cystic liver lobe. The inoculation was carried out very slowly and care taken to preclude excess bleeding. The body

wall incision was sown together with cotton. The skin was sutured with a Michel clip. The wound was dusted with sulphanilamide : mephenide powder. Hamsters were returned to their cages and warmed by bench lights. For four days no further treatment was given and food and water was given ad libitum. Any hamsters that died between days 1 and 3 post operation were autopsied, the liver examined and cultured in HSre+S medium, or TP-S-1 + T. cruzi medium as appropriate.

On day 4 post operation, hamsters were killed and autopsied. The liver was exposed and examined for abscesses. Abscesses were noted if they were bacterial. Bacterial abscesses were characteristically walled and hollow containing mucoid liquid pus. Amoebic abscesses were characteristically solid cream coloured and of a spongy consistency. Amoebic abscesses were excised and weighed. All abscesses were examined for presence of amoebae by mashing a small sample in normal saline and preparing a smear for microscopy. If the presence of amoebae and/or the nature of the abscess was in doubt a culture was set up in appropriate medium from some liver abscesses.

Cultures were incubated at 37°C and examined microscopically after 3 days.

The type of infection was based on the average abscess weight of infected hamsters, number of infected hamsters.

Reassociation of bacteria with monoxenic amoeba-trypanosome and subsequent hamster inoculation

Immunising inoculations were prepared from appropriate bacterial nutrient broth cultures. Injections were given as described for normal hamster liver inoculation pp 81 . Twenty hamsters comprised each group and they were caged individually from the first bacterial inoculation.

Amoebae were grown in bulk cultures as described previously. Two days before hamster inoculation various bacterial cultures in nutrient broth were set up and incubated at 37°C. One day before hamster inoculation some of the amoeba-trypanosome cultures were concentrated by removal of three quarters of the supernatant. Various bacterial cultures were added to the amoeba-trypanosome cultures. Approximately 1×10^{10} bacterial organisms/ml. resulted. Incubation was continued at 37°C.

The same procedure was repeated 18 hours prior to hamster inoculation and at various shorter intervals. So that approximately the same final concentration of bacteria occurred between bacterial groups and between time groups, bacterial cultures were incubated at 37°C also from the occasion of the first amoeba-bacterial combination. Thereafter the bacterial fraction was added in a similar volume to that used in the first addition. Counts of bacterial organisms were not made thereafter. Immediately before hamster inoculation amoeba inocula were prepared in the normal fashion.

The hamster operation and subsequent assessment of infection was carried out as described previously pp.81.

CHAPTER 3 DEVELOPMENT OF MONOXENIC CULTIVATION SYSTEM, USING ENTAMOEBA HISTOLYTICA WITH TRYPANOSOMA CRUZI

a) Original initiation of cultures and their maintenance

Introduction

In order to study the growth of E. histolytica in the special conditions of monoxenic amoeba-trypanosome association, it is necessary first to measure the growth of E. histolytica in conventional bacteria associated cultures (Dobell & Laidlaw, 1926). Thus a comparison is provided of the type of growth resulting in monoxenic cultures.

The methods used for monoxenic amoeba-trypanosome cultivation at the beginning of the study have been described in chapter 2. These original methods were not standardised but must be included since all further work has been based on this system. This was the monoxenic system that first showed success. Attempts of cultivation based on the modified Shaffer-Frye system using heat inactivated bacteria (Shaffer & Frye 1948) and Ehs+S medium had been unsatisfactory. The monoxenic isolation of Biswas strain, often used in later experiments, was carried out using the original system based on Pan (1960).

Amoebic growth in this primary system needs to be assessed to provide a comparison for attempts at improvement of the system.

Experimental results of preliminary studies

The methods used for routine maintenance of stock E. histolytica cultures in HSre+S medium have been described in chapter 2 pp 42 together with methods of assessment of amoebic growth. Results of

amoebic growth of Biswas strain in this medium, associated with an unknown bacterial flora are described in figure 1. The growth curve shows the presence of a marked lag phase of two days duration. Thereafter logarithmic multiplication of amoebae took place resulting in peak numbers at day 3. The decline in amoebic numbers that followed was rapid. By day 5 the amoebic concentration was similar to that resulting from the original inoculum.

After the monoxenic isolated Biswas strain had been maintained for over three months (44 subcultures), further measurements of amoebic growth were made. The methods used have been described in chapter 2. Representative samples have been plotted in figure 2. The most striking feature of these results is their inconsistency. However the basic pattern of amoebic growth as shown in the cultures containing a mixed bacterial flora, was demonstrated by all the growth assessments. There was a lag phase, followed by logarithmic growth, followed by a rapid decline in numbers of amoebae.

Discussion

The lag phase of amoebic growth in both culture media is due to the adaptation of the medium that is necessary before it is entirely suitable for the growth of E. histolytica. During this time the death of the amoebae keeps pace with the multiplication that takes place. All the living components of the cultures contribute to this medium adaptation, including the amoebae themselves, by alteration of the pH, redox potential, production of nutrients etc. When the system is suitable for the amoebae they multiply rapidly until the stationary phase is reached.

Figure 1 Growth of E. histolytica (strain Biswas) in HSre+S with unknown mixed bacterial flora

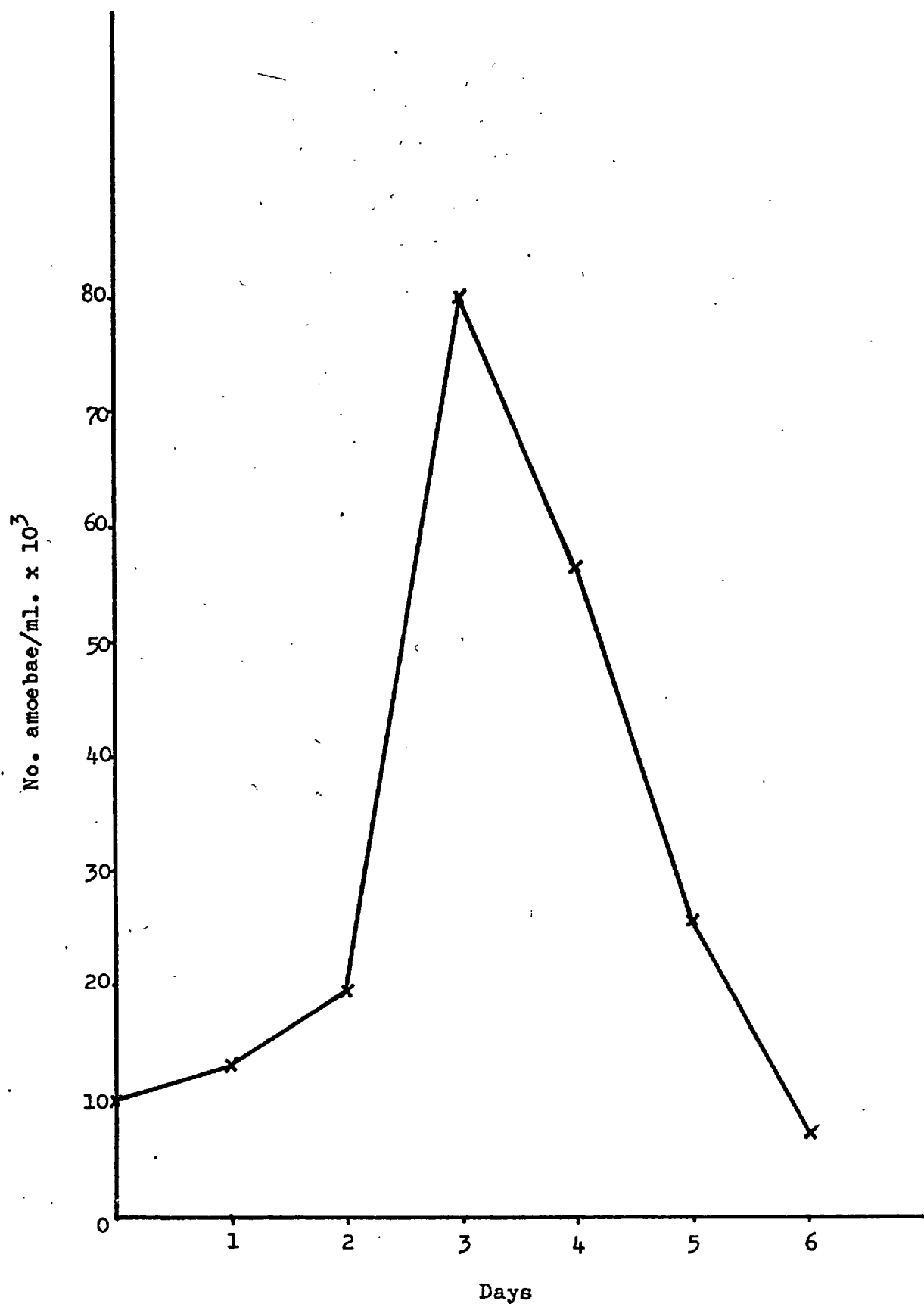
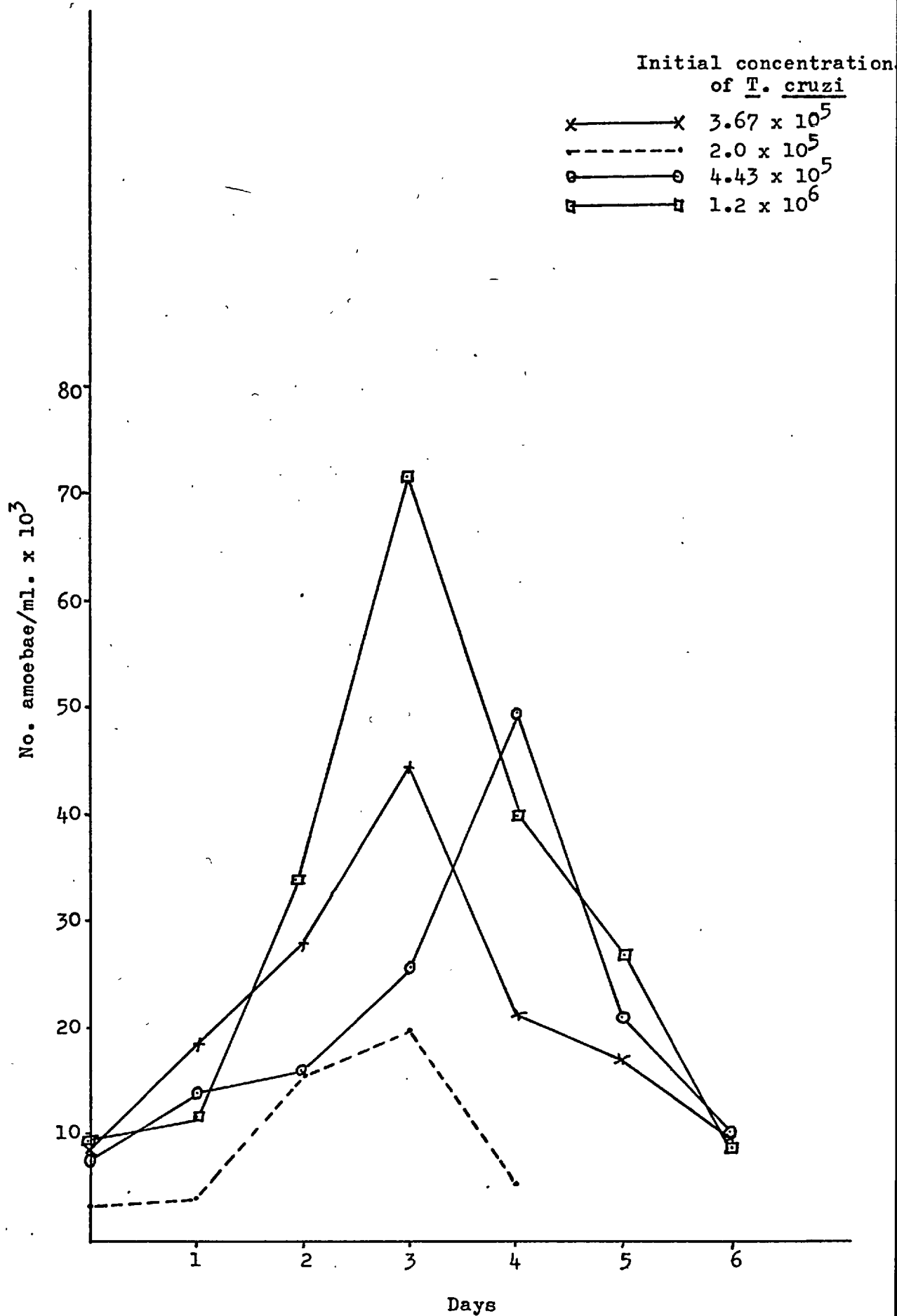


Figure 2 Growth of E. histolytica (strain Biswas) in A-TPH with T. cruzi epimastigotes



It was not surprising that the amoeba-trypanosome cultures provided such inconsistent amoebic growth, in the light of present knowledge. Although the original amoebic and trypanosomal inocula were known, they were not constant for all the experiments. These results indicate two conclusions only. The first, is that routine amoebic cultivation, of a comparable standard to that produced in mixed bacteria associated cultures, was produced in association with T. cruzi epimastigotes. The second conclusion is that the system, as it stood, would have to be developed and modified if any valid quantitative information was required. As there was some slight correlation between the amoebic growth and the initial concentration of the T. cruzi, it would appear that the amoebae were relying directly on the T. cruzi for their nutrient. Possibly further study of this relationship would provide information about the nutrition of E. histolytica.

Although Pan (1960) had measured the initial concentration of both E. histolytica and the hemoflagellates in his experiments, he remarked only on the wide range of amoebic concentrations that could be supported by a small range of hemoflagellates. On the other hand, Phillips (1962) made a study of the effect of different T. cruzi concentrations on amoebic growth. Too high a concentration of T. cruzi (1.6×10^8) had a deleterious effect on amoebic growth, and he suggested that this was due to the rapid depletion of dextrose from the medium.

b) Variation and standardisation of medium; influence of chemical constituents

Introduction

The interacting factors of the amoeba-trypanosome system may be divided into two types. The first set of factors is chemical and physical. The second set is purely biological, viz., the living T. cruzi epimastigotes associated with the amoebae. This section will deal with the chemical and physical aspects, while section 3c deals with the biological aspects.

In considering the chemical and physical influences that affect the amoeba in culture, most influences are determined by the properties of the culture medium. These influences may be categorised. Provision of nutrient, growth factors etc., is one of the most important aspects. Provision of a suitable tonicity, pH, and pO_2 are other aspects. Two other factors that will be considered in this section, which are not governed by the medium, are incubation temperature and the influence of the size and shape of the culture vessel.

All the influencing factors interact with each other, but so that the study was not unnecessarily lengthy, each one has been studied in isolation. However attention will be drawn to instances of marked interaction.

Each factor was not necessarily studied in the chronological order of presentation of results. Therefore some degree of overlap occurred. This was experienced particularly in the use of three different media before their respective merits had been settled.

The study of the chemical and physical influences of the monoxenic cultures system was two-fold in purpose. First it was essential in order to standardise the procedure and materials, so that further results may be valid. Secondly these experiments provide some insight into the specificity of E. histolytica requirements, and the scope of the tolerance of the amoebae. For this reason more than one strain of E. histolytica was employed in most experiments.

Experimental results of chemical and physical variation in amoeba-trypanosome system

1. Medium - nutrient factors

Before dealing with the nutrients present in the medium it is necessary to consider tonicity. No particular experiments were carried out to assess tolerance of the entamoebae in this respect. As the amoebae are dependant on the state of the epimastigotes for their growth, the tonicity of the medium must be at a level that both amoebae and T. cruzi epimastigotes can tolerate. When a series of media were used during the initiation of this work, both organisms were observed to be highly tolerant to changes and extremes of tonicity. T. cruzi epimastigotes were more tolerant of hypotonic extremes than E. histolytica since they continued to flourish in Eagle's BHK tissue culture medium, but E. histolytica amoebae died in 3 - 4 hours in this medium. As this wide tolerance was shown to exist no further attention was paid to the problem of tonicity.

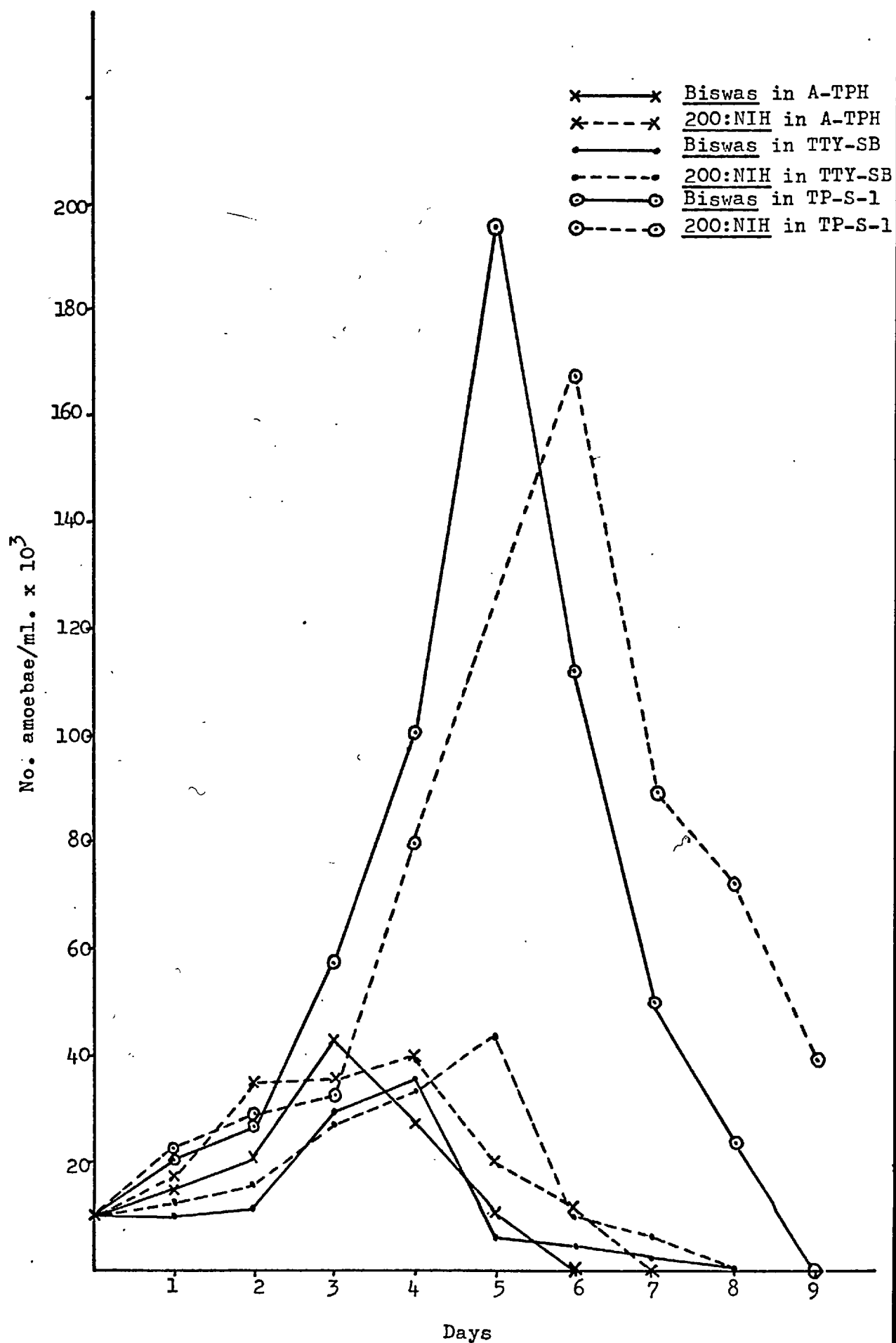
A review of the assessment of various media by other workers has been given (chapter 1). Some of these media which were

considered for use in the amoeba-trypanosome system are listed in chapter 2, pp. 60 together with descriptions of their preparation. The formula for Boné and Parent's medium is also given since this was used for the growth of the T. cruzi epimastigotes and therefore is included in the amoebic culture medium by accompanying the epimastigote inoculum. As described in chapter 3a, the merits of several media for the growth of T. cruzi were considered. The three main types of amoebic culture medium employed were A-TPH, (Pan 1960), TTY-SB and TP-S-1 (Diamond 1968a & b). As one of the ingredients of Pan's medium (Enders' tissue culture fluid as modified by Weller et. al. 1958) could not be obtained easily, a modified version was always used.

Using a constant number and volume of T. cruzi epimastigotes associates and entamoebae these three media were assessed. Not only the yield but also the type of growth varied in each medium, so growth curves have been used to describe the results, see figure 1. Considerable consistency was shown by the results of different strains in the same medium. Also 200:NIH strain was consistent in showing slower growth than Biswas strain. Growth in TP-S-1 medium was outstanding in longevity and yield. TTY-SB and A-TPH media produced similar results but A-TPH maintained poorer longevity.

Although these results alone provided sufficient proof for the greater merits of TP-S-1 medium, it was decided to attempt to modify both A-TPH and TTY-SB media slightly before finally rejecting them.

Figure 1 Growth of E. histolytica with T. cruzi (strain Kessel) in various media



Variations of A-TPH medium concern an investigation of the importance of the Boné's medium which accompanies the T. cruzi inoculum. An experiment described in chapter 2 pp. 73 was carried out on Rahman strain involving growth in a series of media comprising various combinations of ATPH & Boné's media, with a constant initial concentration of T. cruzi. The results plotted in figure 2 show without doubt that the presence of the Boné's medium is detrimental rather than beneficial. Even total exclusion of the Boné's medium did not improve the amoebic growth sufficiently to compare realistically with the growth in TP-S-1 recorded in the last experiment.

Variation of the TTY-SB medium concerned the animal source of blood used to supplement the medium. Horse blood was tested alongside the rabbit blood supplement usually employed. The results, see figure 3, show that this supplement increased the level of amoebic growth considerably. However the problem of bacterial contamination of the horse blood precluded consideration that this medium be used as a routine.

An investigation of the individual constituents of TP-S-1 medium was carried out in case there was a need for simplification, and also to demonstrate the importance of various items. This was done by the systematic omission of ingredients one at a time. No compensation was made for any changes that might have occurred in tonicity as a result of the omission of items from the test media. As described in chapter 2, this experiment was carried out during a period of several weeks. The results are plotted in figures

Figure 2 Growth of *E. histolytica* (Rahman/Kessel) in medium comprised of various combinations of A-TPH and Boné's media

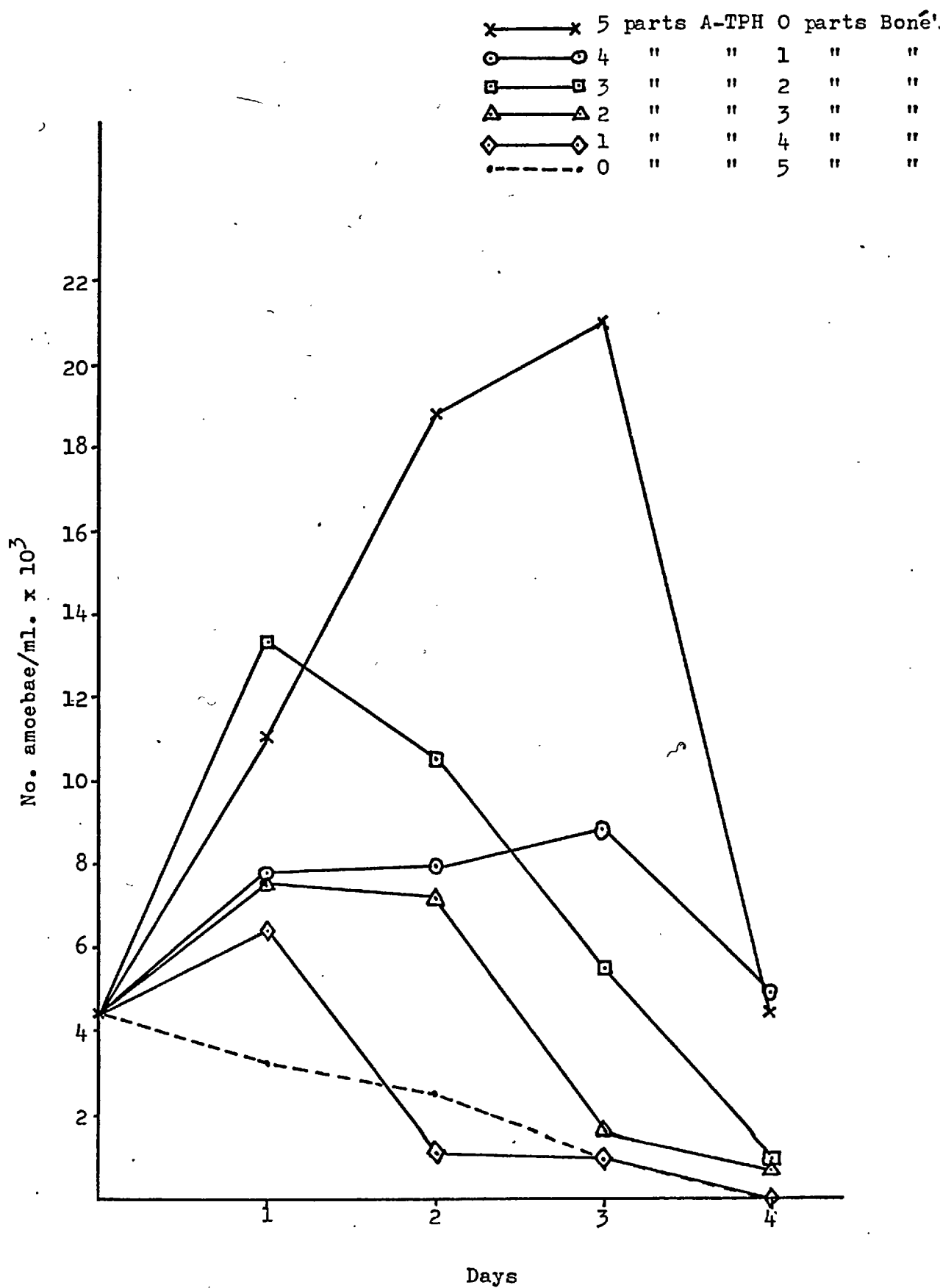
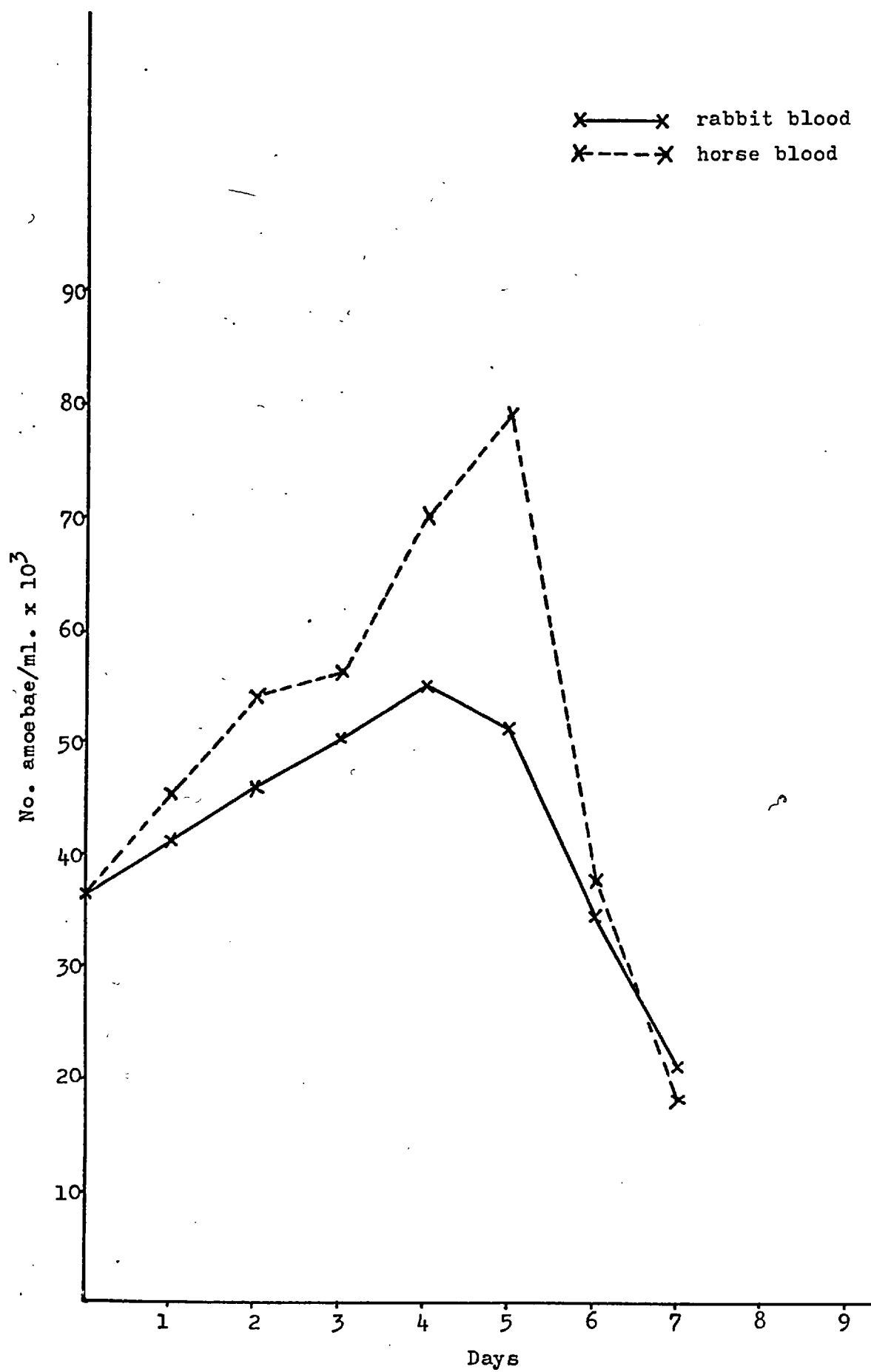


Figure 3 Growth of E. histolytica (strain Rahman) in TTY-SB with rabbit and horse blood supplements



4, 5 and 6. The complete medium was outstanding in its support of amoebic growth compared with all the item deficient media. Only medium with ascorbic acid omitted gave amoebic growth which approached the type of growth produced by the complete medium. As will be shown in the studies on pO_2 this result is not due to the reducing function of ascorbic acid, but rather to a nutritive function. The vitamin mixture NCTC 107 used to supplement the TP-S-1 medium was also shown to be important particularly in long term maintenance. The presence of L-cysteine hydrochloride, was more important than ascorbic acid. The omission of both L-cysteine hydrochloride and ascorbic acid led to amoebic growth which was on the whole rather better than that produced in the absence of L-cysteine hydrochloride it is possible that these items interact. All other constituents of the medium were shown to be essential for any prolonged growth of amoebae. Panmede and trypticase were among the most important.

The presence of the serum was shown to be of considerable importance in the last experiment, and was therefore investigated further. Firstly the animal source of the serum was studied. A supplement of 10% serum was used throughout, and the growth of two strains of E. histolytica was studied. Methods used in these experiments have been described in chapter 2 pp. 73 . The results, depicted by figures 7, 8 and 9, show a complete range of serum preferences by E. histolytica. Only the results of studies on Rahman strain have been presented, since those of 200:NIH

Figure 4 Growth of E. histolytica (strain Rahman) in deficient TP-S-1 media with T. cruzi (strain Y)

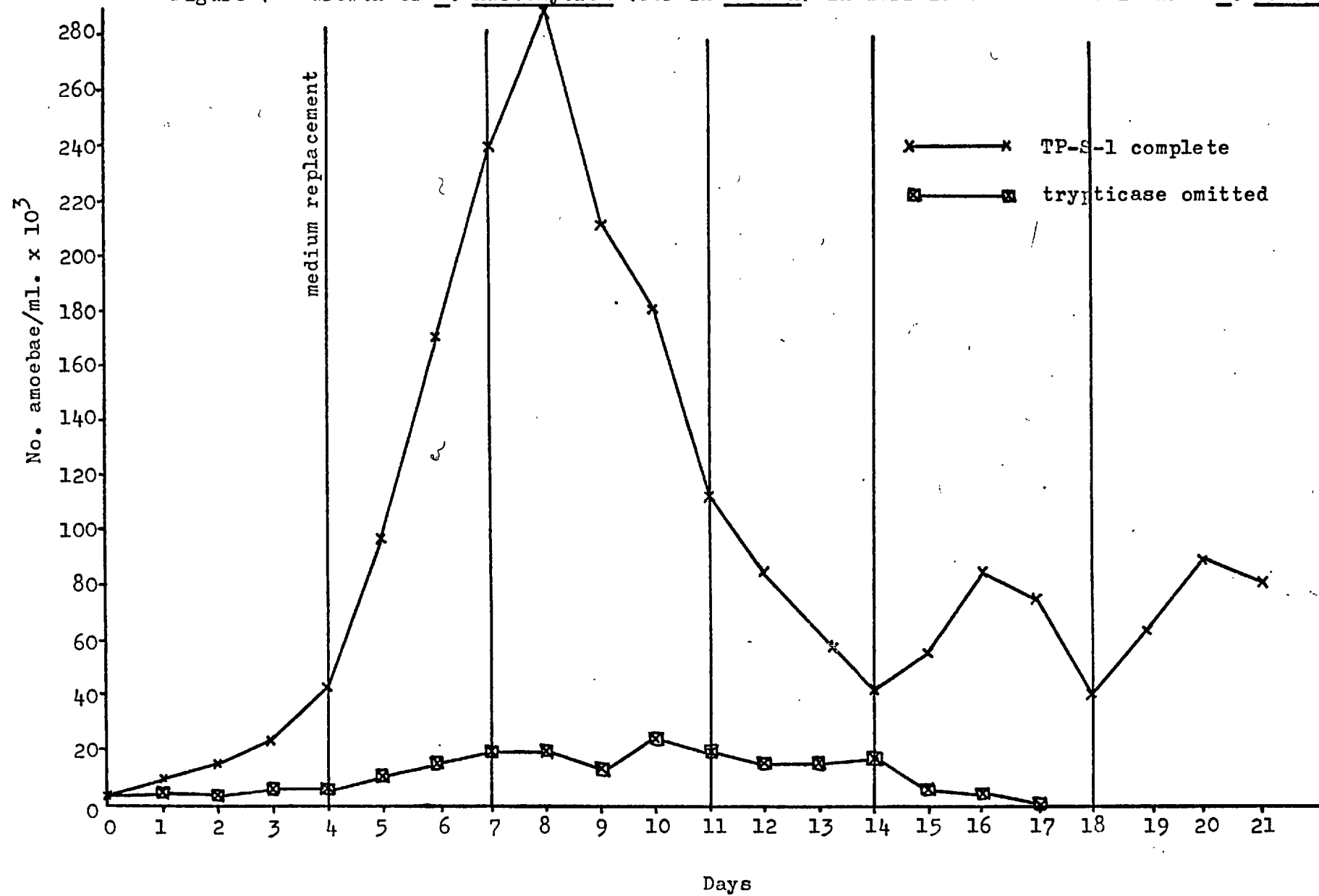


Figure 5 Growth of E. histolytica (strain Rahman) in deficient TP-S-1 media with T. cruzi (strain Y)

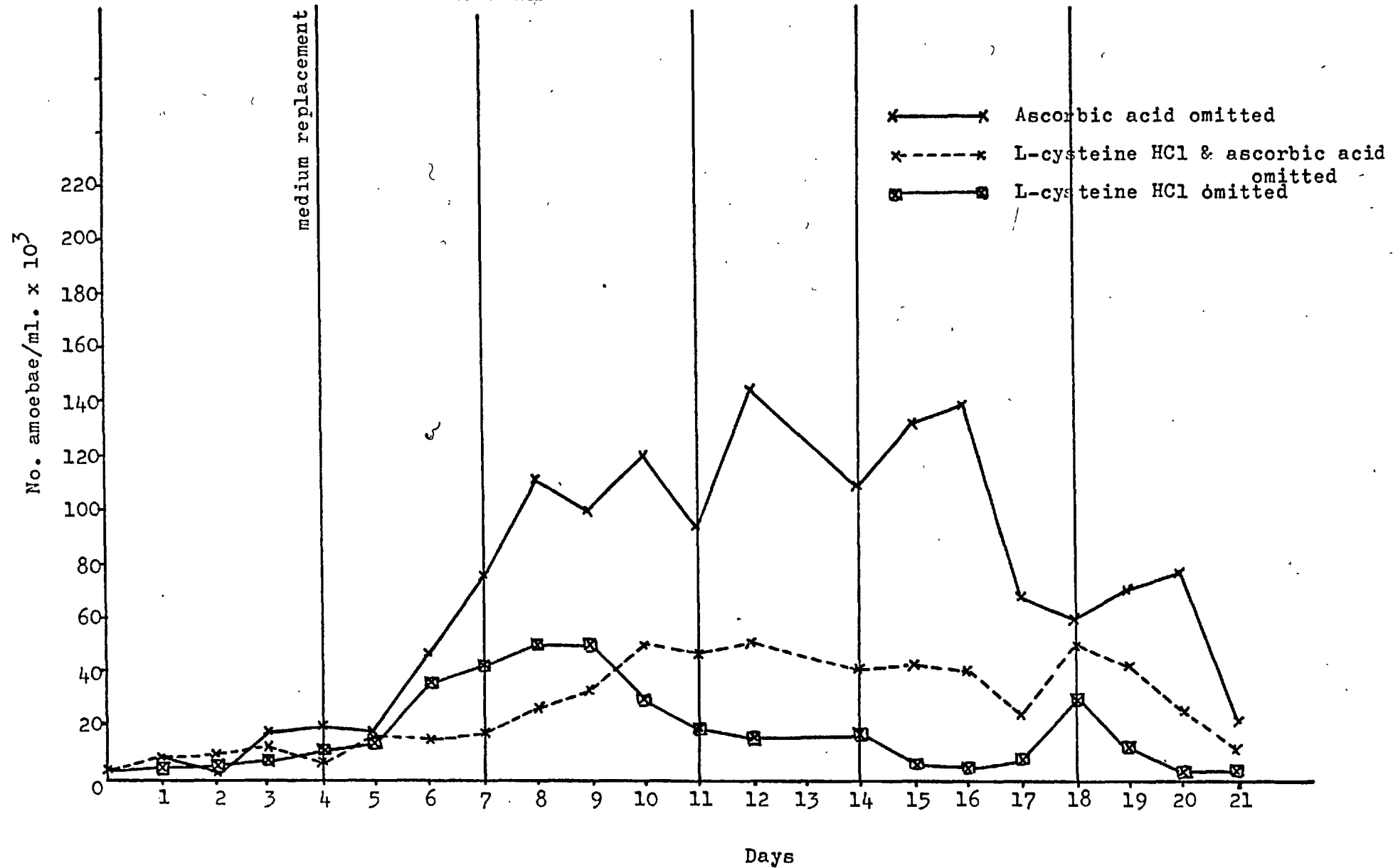


Figure 6 Growth of E. histolytica (strain Rahman) in deficient TP-S-1 media with T. cruzi (strain Y)

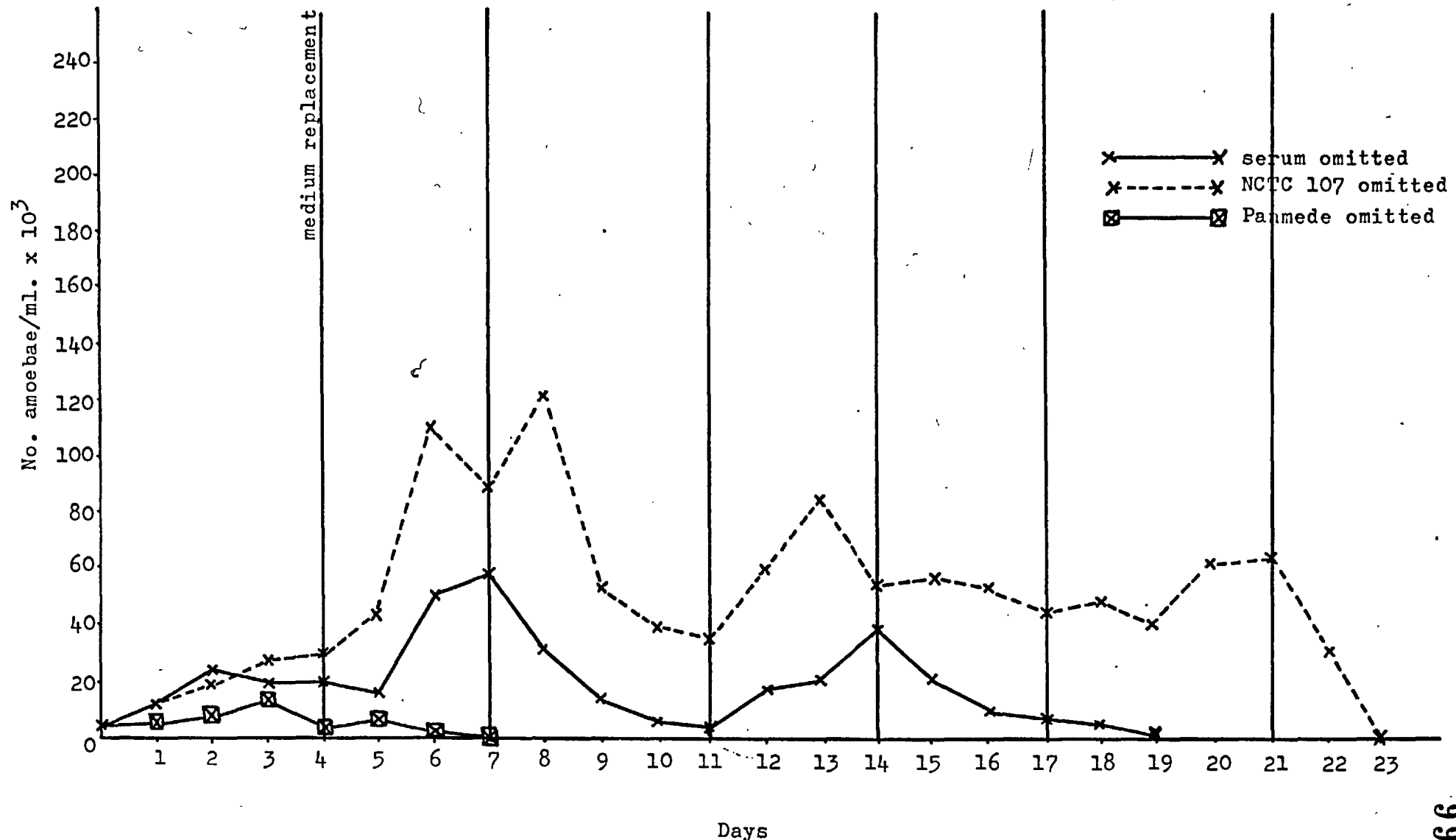


Figure 7 Growth of E. histolytica (strain Rahman) in TP-S-1 supplemented with sera from different animals

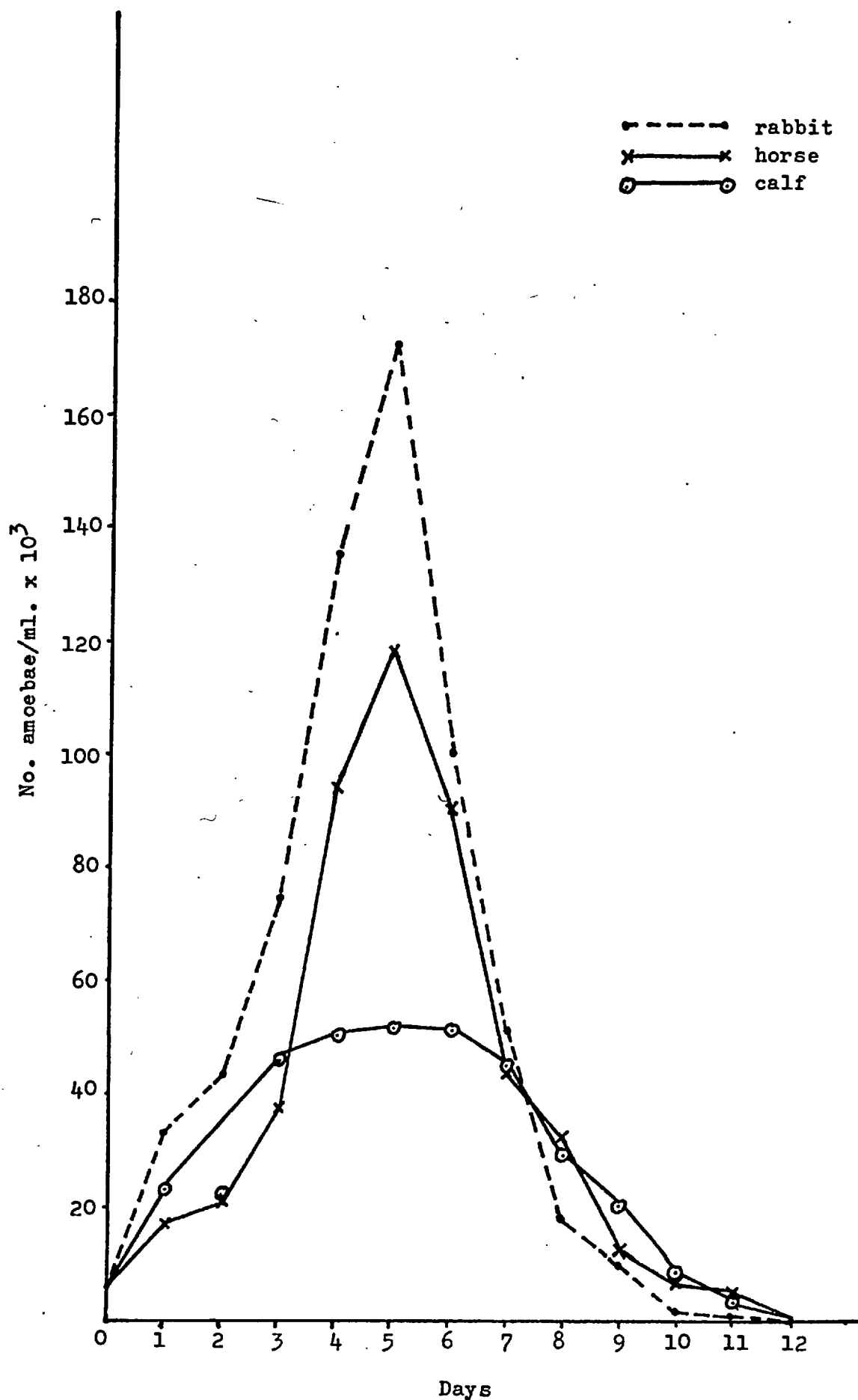


Figure 8 Growth of E. histolytica (strain Rahman) in TP-S-1 supplemented with sera from different animals

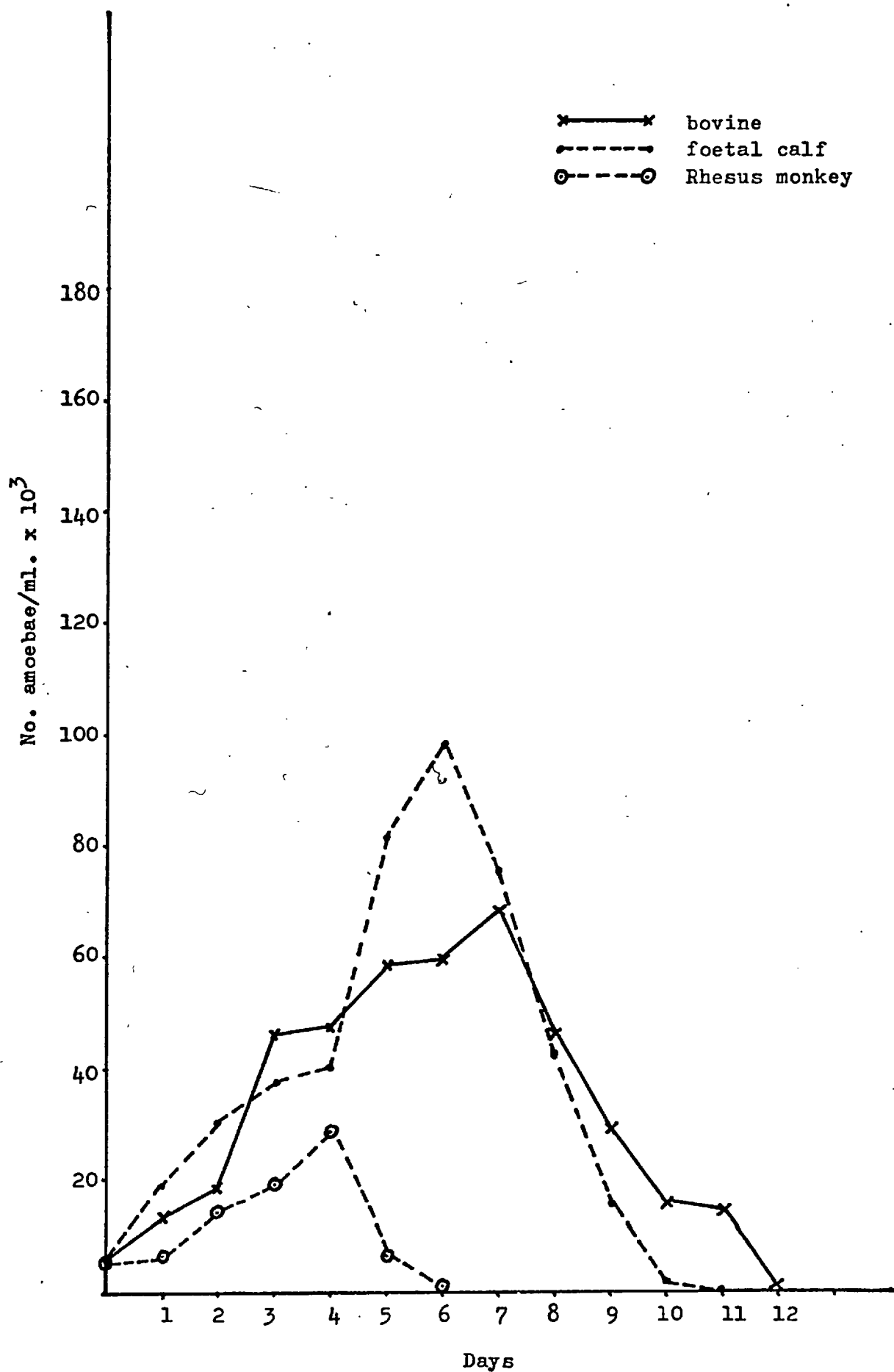
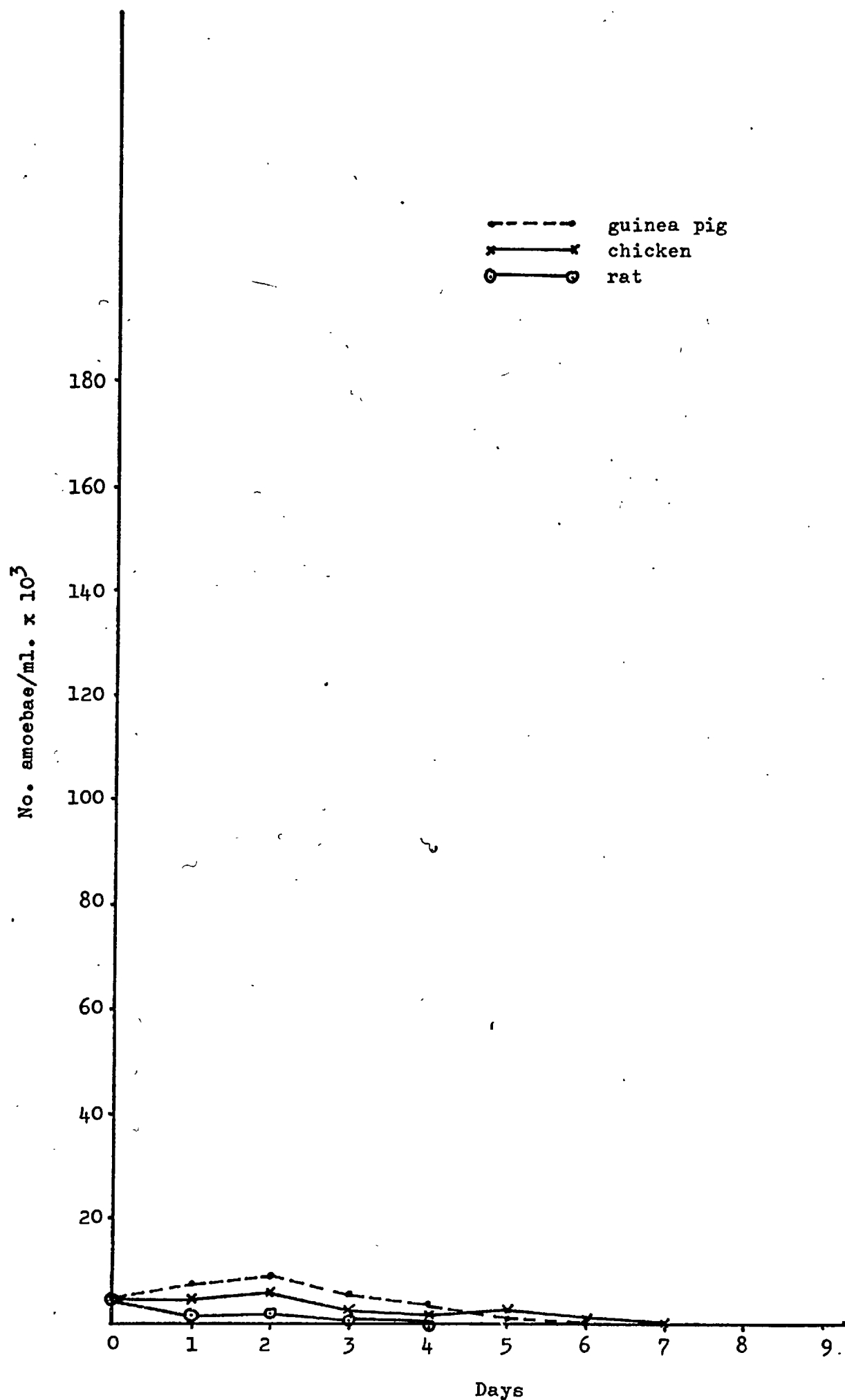


Figure 9 Growth of E. histolytica (strain Rahman) in TP-S-1 supplemented with sera from different animals



essentially duplicated results with Rahman although there were differences in degree. Horse serum which was recommended by Diamond did not support such good growth as rabbit serum. Foetal calf serum (colostrum deprived), produced growth comparable to that of horse serum. Bovine and calf sera supported reasonable growth, but Rhesus monkey, guinea pig, chicken and rat serum did not support growth of any significance. Rat serum was positively detrimental to the amoebae.

The growth of E. histolytica in TP-S-1 supplemented with varying concentrations of horse serum was studied. No adjustment of nutrient concentration was made as this was considered to be in excess, but it should be borne in mind with results of the higher concentrations of serum. These results have been described by table 1. By far the highest yields were obtained from cultures containing 5% - 20% serum, of which 10% was outstanding. A wide degree of serum tolerance was exhibited by the fact that reasonable growth was obtained at both extremes, 40% and 1.25%.

An experiment was carried out to assess possible variation in support provided by three different batches of horse serum. No significant differences in the resulting amoebic growth could be detected.

ii. Medium - pH

This aspect, like others already mentioned is dynamic, being influenced by growth of the associates and the amoebae themselves. Unlike the other aspects discussed so far, it can be measured at any time. Such measurements were made first before any study on amoebic preferences was made. Table 2 describes some of these results. Autoclaving the medium reduced the pH slightly but this was more or less counteracted by the addition of serum. Upon

Table 1 Peak yields of *E. histolytica* produced in medium containing varying concentrations of horse serum

Initial concentration of amoebae : $5.0 \times 10^3/\text{ml}$.

<u>% Concentration of Horse Serum</u>	<u>Peak yield of amoebae $\times 10^3/\text{ml}$.</u>
40	26.1
20	76.1
10	108.8
5	72.0
2.5	30.0
1.25	25.0

Table 2 pH measurements made on the amoeba-trypanosome system using TP-S-1 medium, Biswas strain of E. histolytica & Y strain of T. cruzi

State of System	pH
TP-S-1 in preparation	7.1
TP-S-1 after sterilisation in autoclave	6.7
TP-S-1 after addition of serum and vitamin mixture	6.9
Medium after addition of <u>T. cruzi</u> epimastigotes	6.5
System after one day of amoebic growth	6.3
System after 2 days of amoebic growth	6.3
System after 3 days of amoebic growth	5.9
System after 4 days of amoebic growth	5.7

addition of T. cruzi epimastigotes the pH was again lowered, this time to a greater extent. The greatest lowering of pH was due to the growth of the amoebae themselves and metabolising T. cruzi, the pH reaching a relatively acid level at the end of each subculture.

Experiments were performed to assess the degree of pH tolerance possessed by the entamoebae living in association with T. cruzi. Methods used have been described in chapter 2 pp. 75. Two strains of E. histolytica were used and they were found to react similarly. Results have been recorded in Table 3, which gives the longevity and yields that were produced. A wide degree of tolerance was shown, growth occurring in the range pH 5 - pH 8. pHs 6 and 7 both supported good growth but a slight preference was shown for pH 7, in terms of yield.

iii Medium - pO_2

Chapter 1 has already drawn attention to the differences of opinion held by various workers on the anaerobic/aerobic nature of E. histolytica. A similar approach to that used in the study of pH was employed. The results of the first series are described in table 4. All the media used for amoebic growth that were measured were highly reduced. They became still more reduced by the growth of the E. histolytica.

An attempt was made to grow E. histolytica in the system of TP-S-1 medium with Y strain of T. cruzi in media of a higher oxygen tension. As described in chapter 2 this was accomplished by varying the amount of L-cysteine hydrochloride incorporated into

Table 3 pH tolerance of E. histolytica in amoeba-trypanosome culture

Initial pH of medium	Actual pH	No. days of growth or survival	Peak yield of amoebae x 10 ³ /ml.
3.0	3.27	0	-
4.0	4.11	1	-
5.0	5.1	12 (2 subs)	123.0
6.0	5.95	12 (2 subs)	196.0
7.0	6.73	12 (2 subs)	218.2
8.0	7.2	12 (2 subs)	95.6
9.0	8.5	2	-

Table 4 pO₂ measurements of various E. histolytica culture media

State of System	pO ₂
water - standard	100%
TP-S-1 after addition of serum and vitamin mixture	14%
TP-S-1 after addition of <u>T. cruzi</u> epimastigotes	20%
System after 3 days of amoebic growth	8.2%
HSre+S after 3 days of amoebic growth	15%

the medium. Unfortunately no definite results were obtained from this study other than that L-cysteine hydrochloride must be a growth factor as well as a reducing agent in this system. Even when the concentration of L-cysteine hydrochloride was reduced to 1/32 of its normal concentration in the medium, the oxygen tension was still at a comparable level to normal TP-S-1, 17%. No amoebic growth resulted in cultures containing 1/8 normal concentration of L-cysteine hydrochloride (0.0125g/ml.).

iv. Incubation temperature

Two types of experiment were carried out on temperature tolerance. The first one was to establish thermal death points and growth during a period of ten weeks subculture at varying temperatures. The second measured the growth of test cultures set up from the above established cultures, after the tenth week. This one-step treatment is rather severe but the experiments were designed to give a realistic assessment of tolerance without involving too much adaptation.

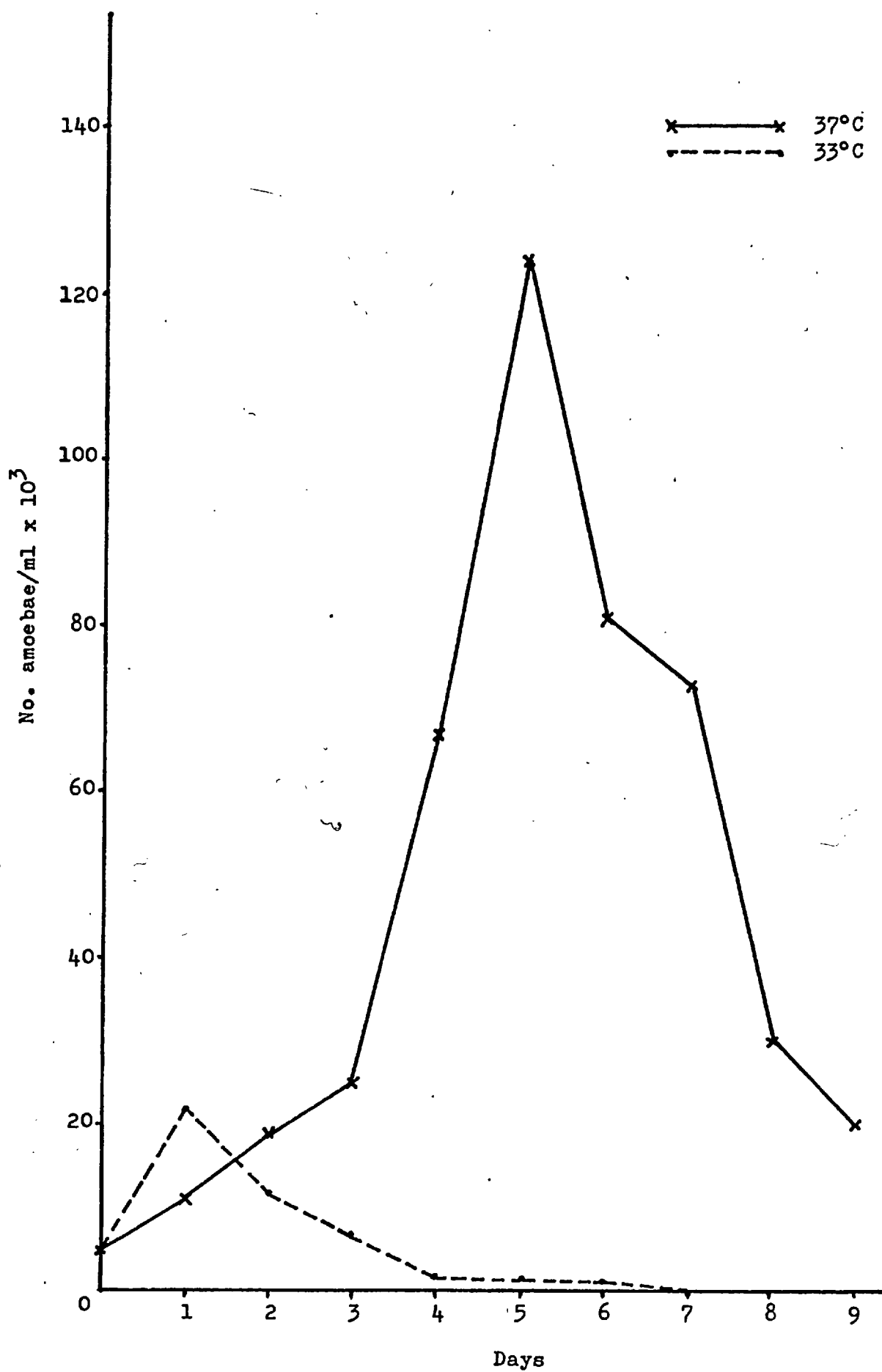
The results of the first experiments are given in table 5. Survival of cultures at suboptimal temperatures was very limited. Apart from the temperatures, 33°C and 37°C, the longest survival at a suboptimal temperature was for one week at 28°C.

The results of the second experiment, growth of E. histolytica at 33°C and 37°C is plotted in figure 10. Growth at 33°C was much poorer than that at 37°C, both in terms of longevity and also yield. Undoubtedly 37°C is the optimal temperature for growth of E. histolytica in the amoeba-trypanosome system.

Table 5 Survival of E. histolytica in amoeba-trypanosome cultures subjected to different temperatures

Temperature °C	Length of survival in days
41°	4
37°	9
33°	7
28°	7
25°	$3\frac{1}{2}$
20°	2

Figure 10 Growth of monoxenic *E. histolytica* (Rahman/Kessel) at varying temperatures



v. Culture vessels

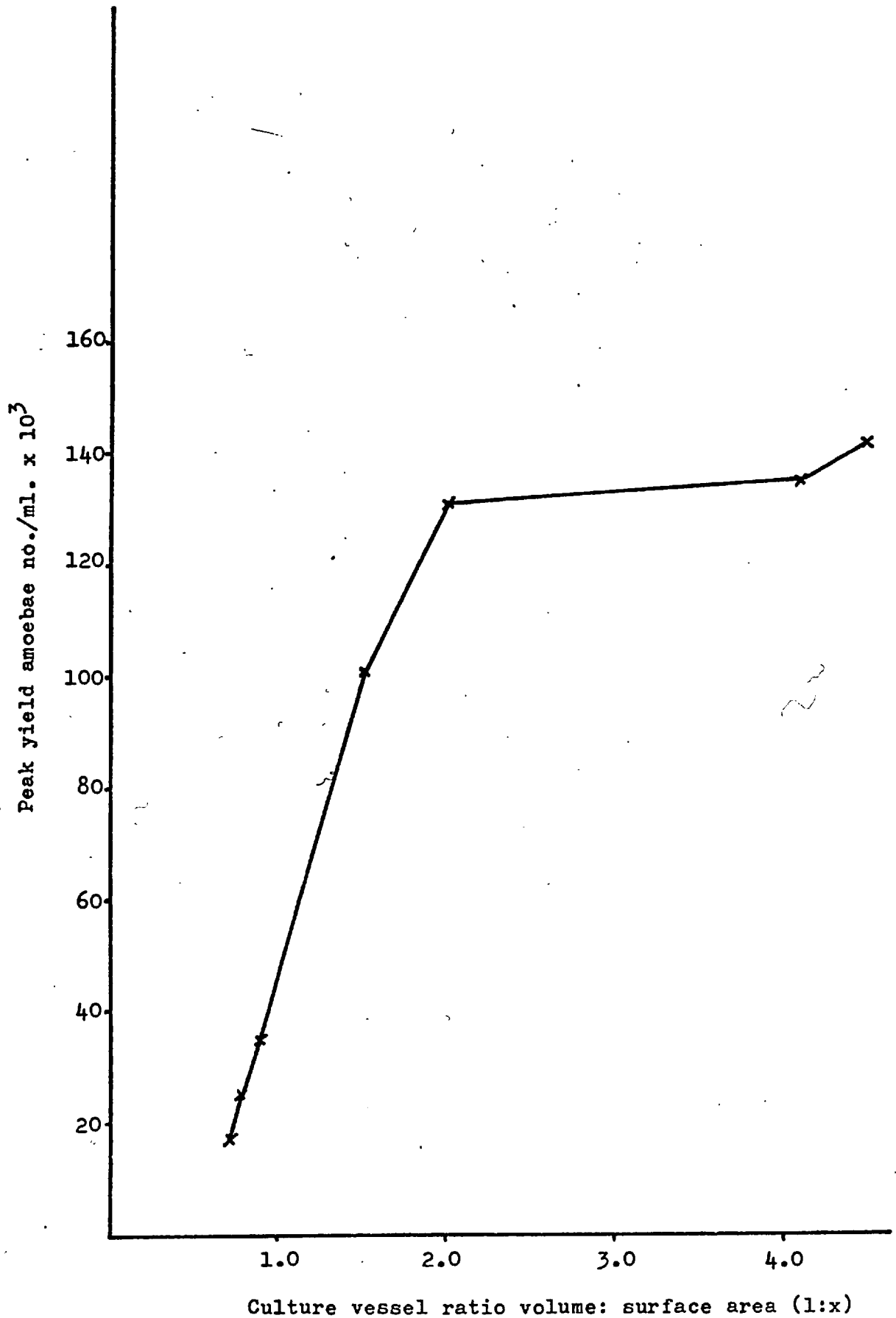
Two experiments were used to investigate the relevance that size and shape of culture vessels might have in relation to amoebic yield from amoeba-trypanosome culture. The first experiment involved the assessment of peak yields of E. histolytica grown under identical conditions of medium, inoculum, associate etc. but in varying culture vessels. Methods used and descriptions of the culture vessels have been given in chapter 2 pp. 76. The results of this experiment are given in table 6 and figure 11. All culture vessels were basically cylindrical. A description of these vessels is given in chapter 2c. Surface areas were calculated from measurements made. Volumes were measured. The results show that there was a direct correlation between the volume (mls.): surface area (mm^2) ratio and the peak yield of amoebae obtained. This increased linearly from a ratio of 1:<1 to a ratio of 1:2. For ratios of 1:2 and greater a plateau was reached with only a slight steady increase.

The second experiment was conducted to find out if there was any limit to the number of amoebae that could be grown in a particular culture vessel. Only one type of culture vessel was investigated and the initial concentrations of E. histolytica and T. cruzi epimastigotes were chosen such that an optimum ratio of epimastigotes : amoebae resulted (see chapter 3c). A high initial concentration of E. histolytica ($5.0 \times 10^4/\text{ml.}$) was used in the small $4" \times \frac{1}{2}"$ culture tubes. Other details of materials and methods have been described in chapter 2. This experiment was

Table 6 Peak yields of E. histolytica grown in association with T. cruzi in varying size culture vessels

Vessel	Vol:surface area	Peak yield amoebae/ml.
Gov ^t . orange juice bottle	1:0.725	1.78×10^4
250ml. round bottle	1:0.818	2.56×10^4
Schweppe's bottle	1:0.939	3.5×10^4
MaCartney bottle	1:1.505	1.05×10^5
6" x $\frac{1}{2}$ " tube (123mm x 15mm)	1:2.00	1.31×10^5
4" x $\frac{1}{2}$ " (large) tube (100mm x 10mm)	1:4.147	1.35×10^5
4" x $\frac{1}{2}$ " (small) tube (100mm x 8mm)	1:4.508	1.42×10^5

Figure 11 Peak yields of E. histolytica grown with T. cruzi (Rahman/Kessel) in culture vessels of various sizes



repeated on three occasions. On all three occasions peak growth was attained at day 5. The highest yield of amoebae per ml. was 3.23×10^5 /ml. and the average yield of amoebae was 3.1×10^5 /ml.

A further experiment using twice the initial concentration of E. histolytica and the same concentration of T. cruzi was carried out. Peak growth of this culture was attained on day 4 and provided a yield of 2.67×10^5 /ml. The fold increase in this experiment was far lower than that in the earlier experiment.

Discussion

Comparison of the media formulae and the results of growth in these media and in the deficient media of TP-S-1 has thrown light on the nutritive requirements of E. histolytica. All three media (A-TPH, TP-S-1 and TTY-SB) contain sodium chloride, A-TPH containing rather more (20g./L extra). Diamond's media employ phosphate buffers whereas Pan's medium employs a bicarbonate buffer. Concerning nutrients present in the media, all contain the same quantity of glucose. The protein nutrients, supplied only by trypticase in A-TPH, is supplied by tryptose and yeast extract in addition in TTY-SB. However TP-S-1 medium, which was outstanding in amoebic growth incorporates the peptone trypticase and Panmede liver extract, both ingredients that were shown to be highly important by the present work. It would appear that most benefit in protein nutrition is derived from Panmede liver extract.

The optimum concentration of serum concentration in TP-S-1 was 10%. The original description of TTY-SB and A-TPH media (appendices 6Q62) only include 5% serum. However both of the latter

media include blood, which enhances growth of the T. cruzi epimastigotes and thereby will benefit amoebic growth indirectly as well as by any direct benefit. Horse blood was more beneficial than rabbit blood in TTY-SB medium.

The presence of the vitamin mixture was more significant than was expected. Some of these elusive substances might be excluded from the Panmede by virtue of its preparation, although probably present in liver. The egg yolk infusion (chapter 3a) might be beneficial as a result of the presence of cholesterol which has been shown to be of significance in amoebic nutrition. (Cedillos, R.A., Reeves, R.E. & Swartzwelder, J.C. 1961, Sharma 1959).

The reducing agents, ascorbic acid and L-cysteine hydrochloride could be growth factors as well as reducing agents. This was certainly demonstrated for L-cysteine hydrochloride. Possibly some kind of interaction between these substances or counteraction by L-cysteine hydrochloride of an affect by ascorbic acid, could account for these results.

The reason for E. histolytica preference of sera from different animals does not correlate with the susceptibility of the animal as host to amoebic infection. Both rat and rabbit are susceptible to infection with E. histolytica. It is interesting to see that all the serum from cows and calves produced a satisfactory standard of growth. Serum provides a pH buffer and presumably nutrient substances. The optimum concentration of serum in the medium is the resultant of the greatest amount of

nutrient benefit and the toxicity of this or other substances.

E. histolytica showed a wide tolerance for culture in varying pH. However it also showed a complete incompetence of dealing with any pH outside this range. In the parasitic habitat the pH is unlikely to vary above pH 8.0 or below pH 4.0, so therefore it is not surprising that in culture it cannot cope with greater extremes of pH. The amoeba would probably only encounter extremes of pH while encysted and therefore would not sustain any damage. In fact the pH treatment of the cyst in the host probably forms an integral part in the hatching process.

In all the media examined for amoeba-trypanosome cultivation, amoebae have been observed to grow the whole way up the tube to the air/liquid interface. More trophozoites are seen at the bottom of the tube but this is due to the slowness to move up the glass side of the test tube from the bottom. TP-S-1 medium was shown to be highly reduced but the amoebae themselves reduced the medium further which would suggest that they require or can utilise oxygen to some extent (Wittner 1968). L-cysteine hydrochloride and ascorbic acid have already been mentioned in their nutritive role as well as their³ reducing role. The addition of the T. cruzi epimastigotes to TP-S-1 medium oxidised the medium to some extent but probably no more than would be expected by addition of such a volume of non-reduced medium.

The temperature range of tolerance demonstrated for E. histolytica in culture in the amoeba-trypanosome system was very

limited and agrees with previous studies (see chapter 1 pp17). This is to be expected from a parasite of a homeothermic host whose temperature varies only fractionally except during illness. The enzyme systems and lipid requirements of E. histolytica have an optimal operational temperature of 37°C, and the need for less specific systems does not arise. However the growth shown at 33°C is probably explained by adaptation, though admittedly of a poor standard.

Studies on the effect of the shape of the culture vessel on amoebic growth has led to two conclusions. The first one is that peak growth attained in cylindrical vessels is dependant upon the volume: surface area ratio. It would seem then that all further quantitative work should be carried out using similar culture vessels throughout. The 4" x $\frac{1}{2}$ " test tubes were chosen as these were most readily available, they require small quantities of materials and have a volume: surface area ratio suitable for high amoebic yields. Although the culture vessels examined were not directly proportional to each other, they were more or less similar in the volume of air space proportional to the volume of the culture. The only one that was markedly different in this respect, the Schweppe's bottle, did not produce results significantly different to indicate further investigation. The second conclusion, reached as a result of the second experiment, is that there is a limit on the maximum yield of amoebae from a culture vessel. The initial concentrations of amoebae and T. cruzi epimastigotes were chosen such that a very good yield might be

expected (see chapter 3c). An excellent fold increase was achieved but this was more than halved if a higher initial amoebic concentration was used. There was no need to increase the epimastigote concentration as the amoeba: epimastigote ratio was still well within the optimum range (see chapter 3c). It would appear, then, that although amoebic growth is dependant on many factors a crowding effect prevents the growth of more than a certain number of amoebae in a culture vessel.

c) Variation and standardisation of medium; influence of biological constituents - flagellates

Introduction

For survey of the literature on the topic of T. cruzi associates for E. histolytica see chapter 1 pp.14 and chapter 3 pp. 88 . It shows that a number of aspects have been studied while other problems remain apparently untouched. A thorough investigation of the role of the T. cruzi associate in the amoeba-trypanosome system not only sheds light on the nutritional requirements of E. histolytica, but also provides basic knowledge needed to produce subsequent consistency in methods. As the chemical and physical conditions of culture were investigated and standardised (see chapter 3b) so also the biological components needed to be investigated and standardised. Obviously this problem is far more complicated since the biological component is still living and metabolising in its own right within the system.

First the cultivation of T. cruzi epimastigotes must be investigated so that the exact phase in growth and decline of the population can be forecast at any time. Thereafter this particular associate characteristic may be kept as consistent as possible by using similar techniques, media, inocula etc., each week. The only variation still likely to occur will be due to variation in batches of media constituents and sera. However growth assessments of different batches of horse serum over a period of time showed these to be reasonably consistent (see chapter 3b). Temperature

variation occurs rarely and then is due to the ambient temperature being higher than that of the incubator room.

The study of the growth of the epimastigote population, continued within the amoeba-trypanosome system, helps to establish the precise role of the epimastigotes in providing nutrient and their interrelationship with the amoebae.

An obvious source of variation within the system is the concentration of epimastigotes. Diamond (1970) recommends $1.5 - 2.0 \times 10^6$ initial concentrations for T. cruzi and $2.0 - 3.0 \times 10^6$ for Crithidial associates. Reeves (1964) found indications that the number of associate bacterial cells reflected a nutritional limit for amoebic growth, using the modified Shaffer-Frye system. Since the associate concentration problem is most probably a case of the ratio, amoeba:epimastigotes, a series of experiments have to be planned accordingly.

Another possible source of variation and a method of investigating the specificity of amoebic nutritional requirements is the variation of support between different strains of T. cruzi.

In investigation of the precise role of the epimastigotes several methods may be used. For instance, the feeding mechanism used by the amoebae on the epimastigotes gives rise to consideration of inducement of phagocytosis. This may shed light on the nutritional function of the epimastigotes.

It is insufficient to consider only the healthy living associate. It is quite conceivable that some benefit, albeit suboptimal, might be derived from dead or dying associates. In fact, studies on the effects of heat and fixation are needed in any consideration of the nutritional function of the epimastigotes. Further elucidation might yet be provided by the use of acellular fractions of the

epimastigote.

Experimental results of studies on *T. cruzi* epimastigote associates

The Kessel strain of *T. cruzi* epimastigotes was maintained in Boné's medium with 5% rabbit serum as described above (chapter 2 pp. 27). An analysis of these procedures, in particular the initial inoculum and serum concentration, was made. The first factor was studied over a period of weeks and the results are given in table 1. Growth curves have not been presented as they were all similar apart from the lowest inoculum and this difference is born out by the late attainment of peak growth. Reference to figure 1 gives the type of growth curve that was found with all inocula of approximately 10^6 and one much lower. Figure 1 also demonstrates the various types of growth that resulted from identical inocula in varying concentrations of serum.

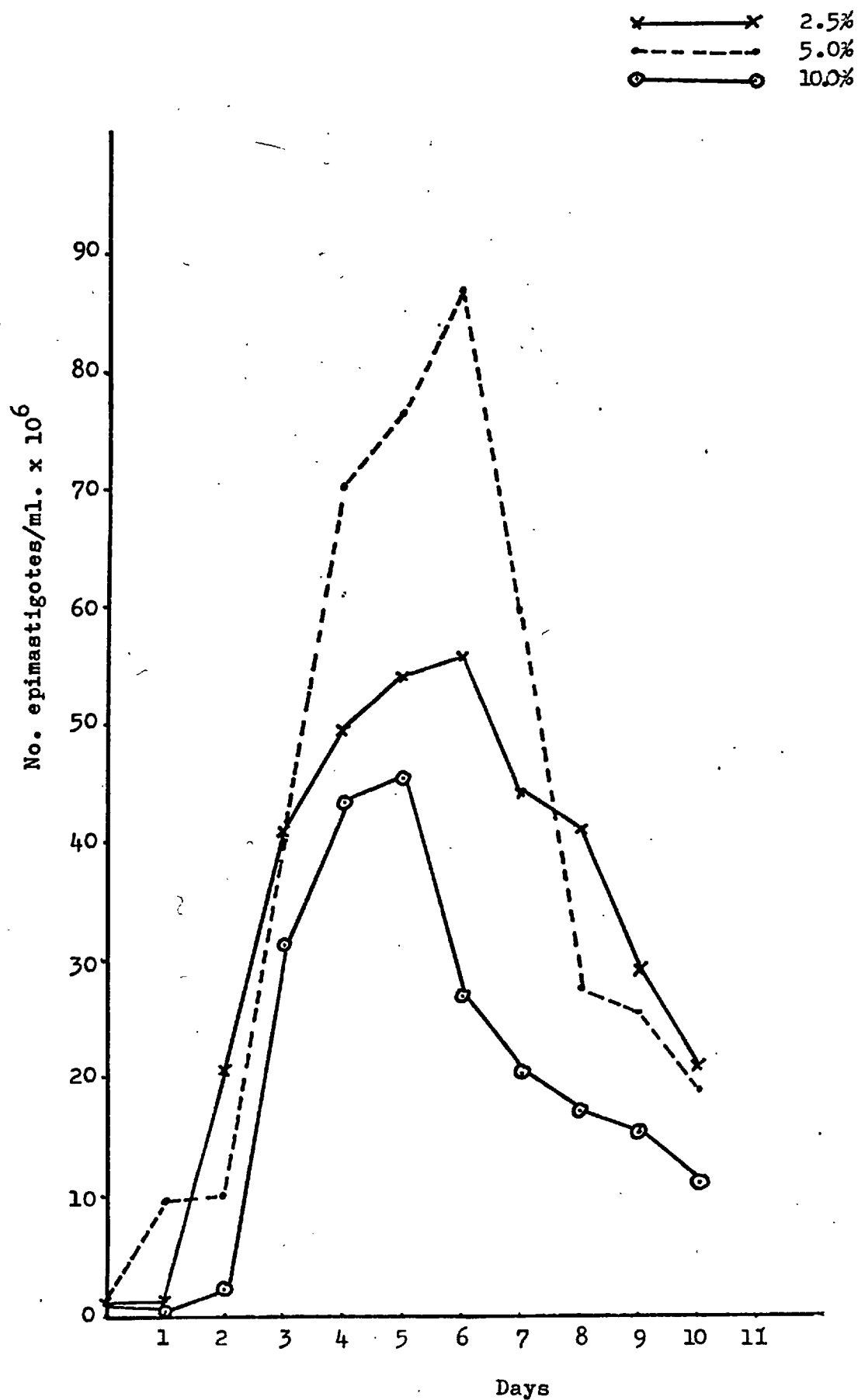
The above experiments were carried out in order to confirm that optimal conditions were being used in the maintenance and preparation of the associates. They also demonstrated that the state of the epimastigote population when it was used for amoeba-trypanosome association, was one having attained peak growth and entering a decline phase. At this stage almost all epimastigotes were still actively motile but some more sluggish fatter forms were seen.

Experiments studying the state of the epimastigotes within the amoeba-trypanosome system were carried out using an inoculum prepared from a seven day old Boné's culture. (For methods see chapter 2 pp 47, 67). For these experiments no amoebae were included

Table 1 Growth of T. cruzi epimastigotes (strain Kessel) in Boné's medium

Expt.	Flagellates/ml. Initial inoculum (theoretical)	Days taken to attain peak growth	Maximum concentration of amoebae/ ml.	Fold increase
1	1×10^4	13	2.7×10^6	270
1	1×10^6	6	5.56×10^7	56
2	2×10^6	6	9.94×10^7	50
3	4×10^6	6	1.67×10^8	42
4	8×10^6	5	2.86×10^8	31

Figure 1 Growth of *T. cruzi* epimastigotes with different concentrations of rabbit serum in Boné's medium



in the system. Results have been presented in table 2. The experiment was continued until death of the epimastigotes occurred although the first four days, only, are applicable to the amoeba-trypanosome system. Obviously the presence of the amoebae feeding on the epimastigotes would reduce numbers in the complete system but these experiments were designed to show the potential of the associate.

From these results, it was decided to continue maintenance and preparation of the epimastigotes as described in chapter 2.

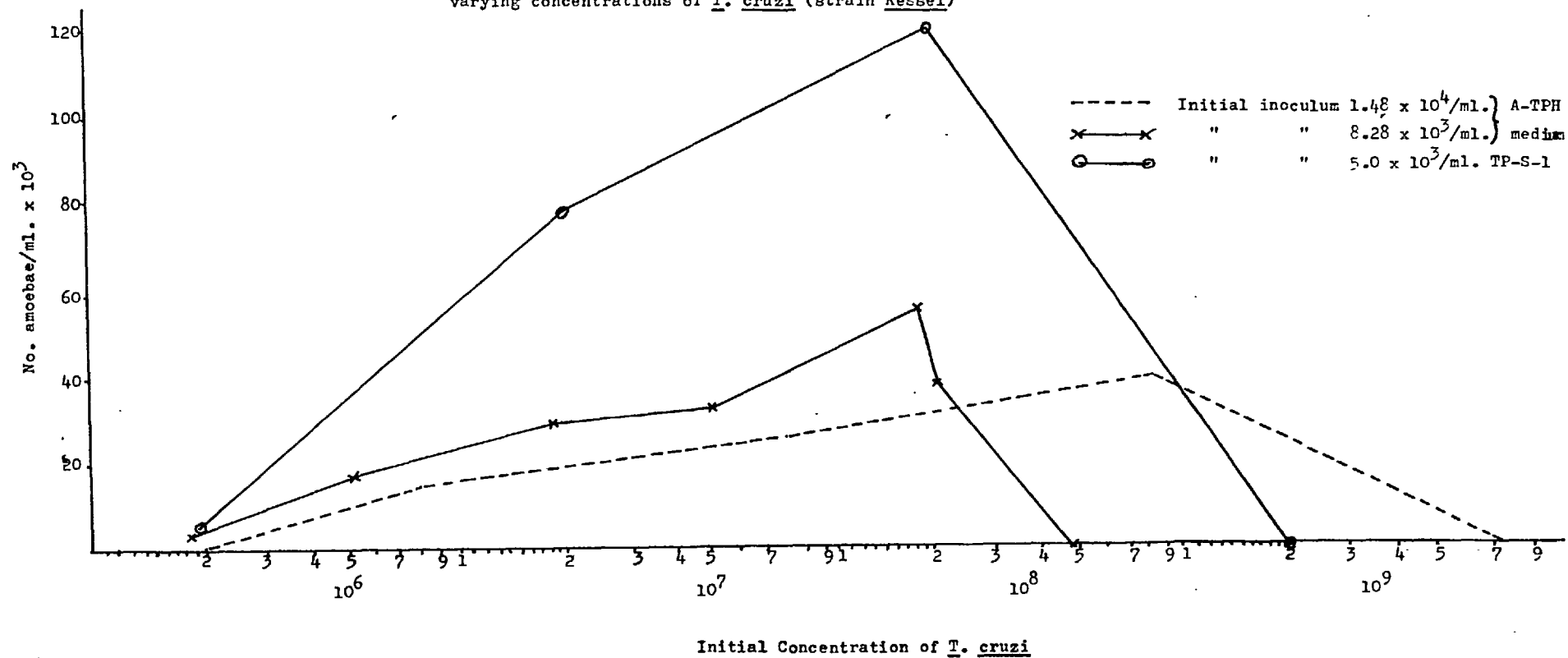
The study of the effect of concentration of epimastigotes in the amoeba-trypanosome system was carried out in two series of experiments. The first series involved variation of the epimastigote numbers while amoebic numbers were kept constant. The second series used converse conditions, varying the amoebic numbers and keeping constant the numbers of epimastigotes. Numbers referred to in this study are initial numbers or concentration, only. Methods for these experiments have been described in chapter 2 pp. 67 . The results of amoebic growth assessment are presented as the yields attained at peak growth. These were compared directly with the initial concentration of T. cruzi epimastigotes in figure 2. Three experiments have been reported in this figure, each using a different initial amoebic inoculum. It must also be noted that the medium used in the two higher inocula of amoebae was ATPH medium. (See chapter 3a).

The results (Figure 2) showed three important points. The first was that no growth at all could be achieved, from the

Table 2 Survival of *T. cruzi* epimastigotes (strain Kessel)
in TP-S-1 medium at 37°C

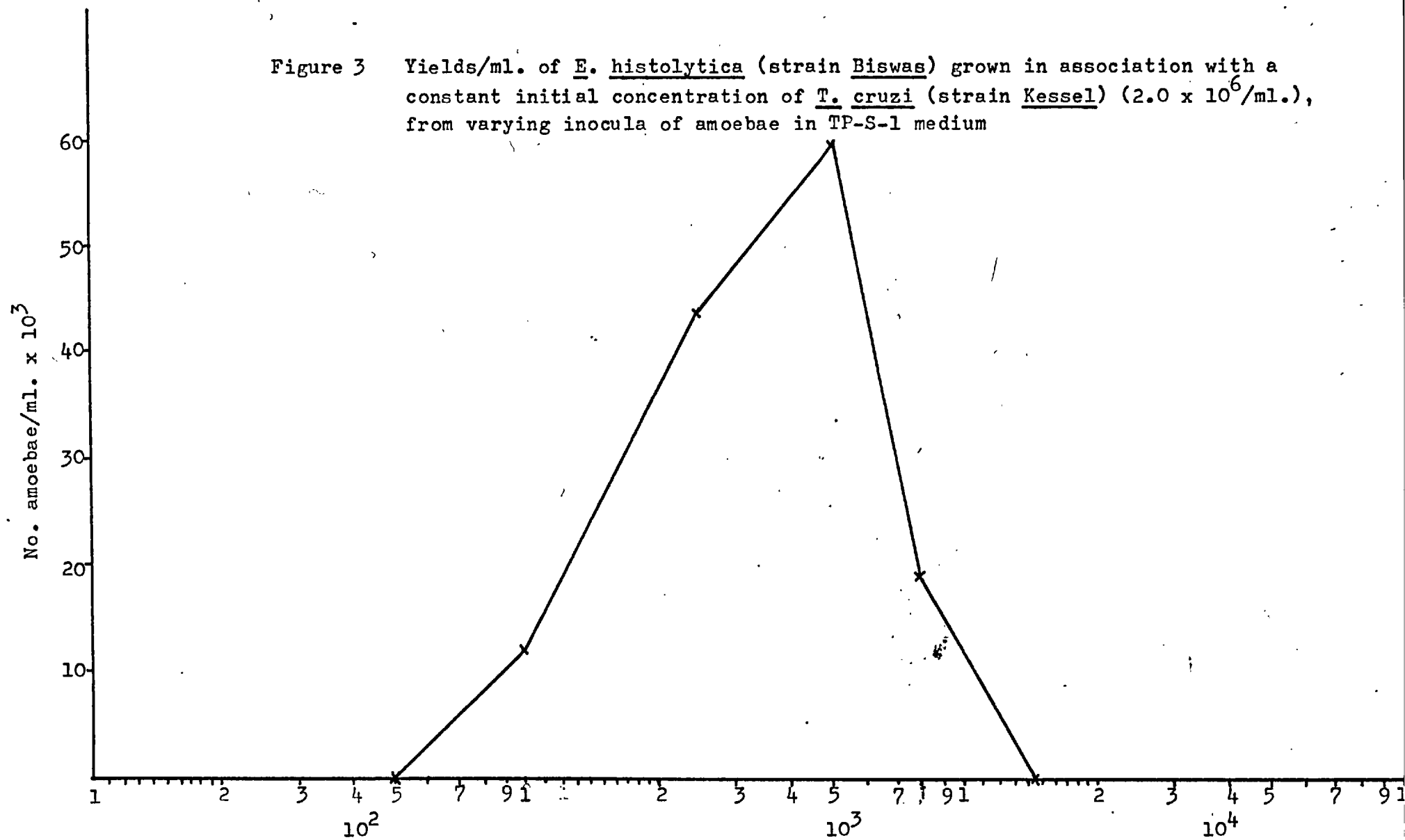
Time in days	No organisms/ml.	Viability	Morphological state of organism
0	2.0×10^6	+	slender, actively motile
1	2.9×10^6	+	slender, actively motile
2	7.94×10^5	-	some stumpy, sluggish
3	3.27×10^5	-	some stumpy, sluggish
4	9.18×10^3	-	mostly stumpy, sluggish
5	5.56×10^3	-	all stumpy, sluggish or dead
6	3.33×10^3	-	all rounded/dead
7	0	-	-

Figure 2 Maximum number/ml. of E. histolytica (strain Biswas) grown in association with varying concentrations of T. cruzi (strain Kessel)



various amoebic inocula tried, using an initial T. cruzi concentration of either less than 1×10^6 flagellates/ml. or more than 1×10^{10} flagellates/ml. Secondly within this range, the relationship between amoebic yield and log initial T. cruzi concentration was linear until maximal growth at optimal concentration was attained. Thereafter a rapid decline followed. The third factor apparent from these results was that the slope of line (maximum amoebic concentration v. initial T. cruzi concentration) varied according to the initial amoebic inoculum. Yields of the higher initial concentration of amoebae produced a markedly different slope than that of the lower initial amoebic concentration. This point was studied further in the second series of experiments, where the initial concentration of T. cruzi was constant. For the purpose of this study it was decided to use a T. cruzi concentration at the lower end of the linear range. It was hoped that, in this way, the limiting effect of the culture vessel size would not influence results. (see chapter 3b). The results have been plotted in figure 3. Two factors were apparent. First, no growth using this T. cruzi concentration could be obtained from initial amoebic concentrations of less than 5×10^2 amoebae/ml. or more than 1.48×10^4 amoebae/ml. Together with the findings from the earlier experiments this indicated that all amoeba-trypanosome cultures would not grow with an amoebic inoculum of less than 5×10^2 amoebae/ml. However, in contrast an inoculum of 1.48×10^4 amoebae/ml., which produced no growth in the latter experiment, produced quite extensive growth in the earlier experiment

Figure 3 Yields/ml. of E. histolytica (strain Biswas) grown in association with a constant initial concentration of T. cruzi (strain Kessel) (2.0×10^6 /ml.), from varying inocula of amoebae in TP-S-1 medium



Initial concentration of E. histolytica

using higher concentrations of T. cruzi. The second factor from the results was that amoebic yield was in linear relationship to the logarithmic concentration, of amoebae present initially, until maximum yield was obtained and thereafter there was a rapid decline. This was confirmation of the pattern that was indicated by the first series of experiments. An important corollary of all these results is that within the bounds of shape of the culture vessel limitations the relationship between the yield of amoebae and initial concentration of T. cruzi could be a case of ratios.

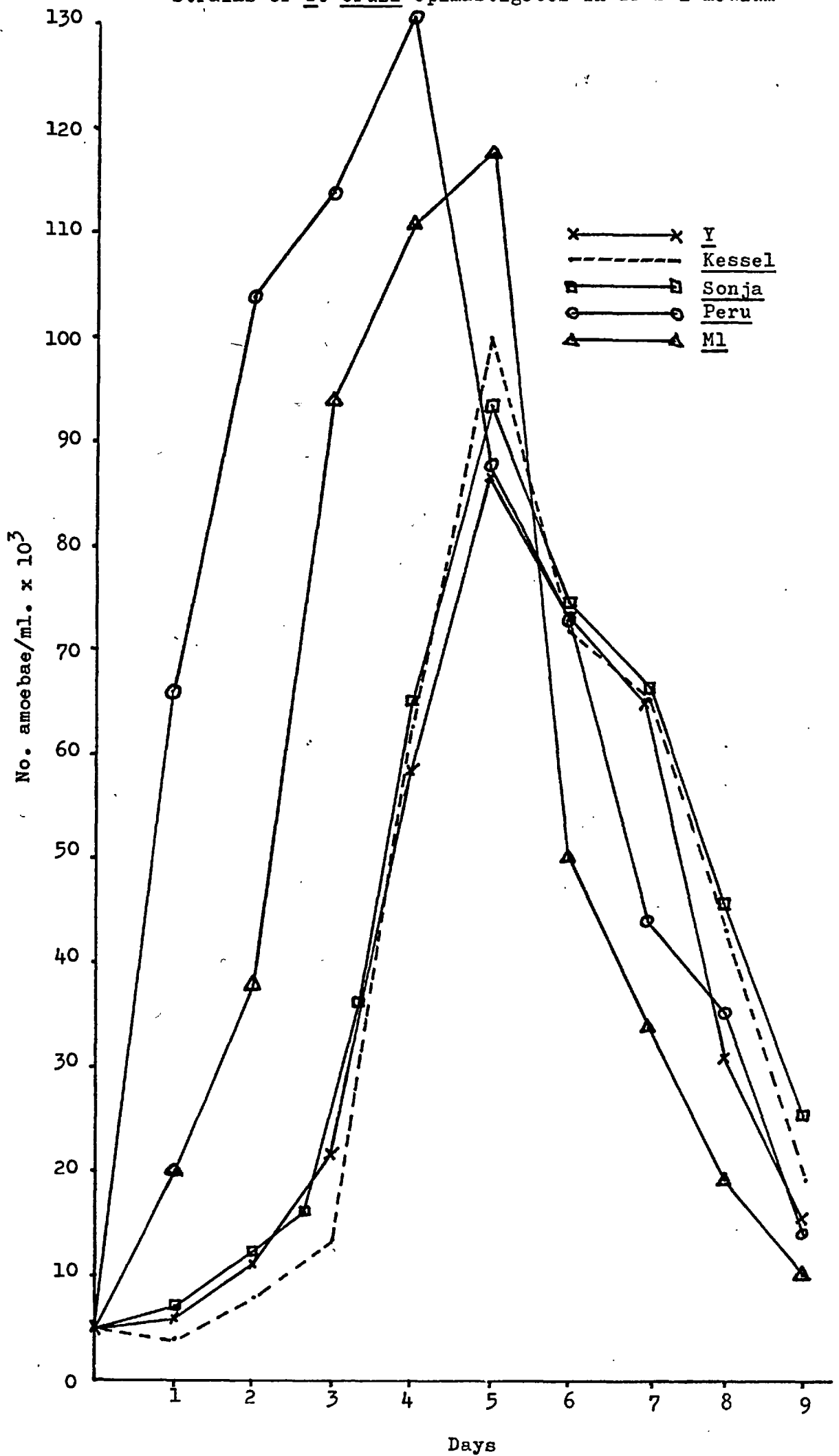
For the purpose of elucidating this problem the results already presented graphically have been presented as the ratio, number amoebae:number T. cruzi, in table 3. This showed there to be a remarkable similarity between experiments when examined in this aspect. The only markedly different ratios were those for the upper limit of the growth range and the upper unsuitable concentration for the last experiment. These results would be influenced by the minimum concentration of amoebae capable of producing growth. This factor has already been commented on using the graphical presentation of results.

The ability of different isolates of T. cruzi to support the growth of E. histolytica was carried out using a constant initial concentration of (figure 3) T. cruzi. The methods employed have been described in chapter 2 pp. 67 . Two strains of E. histolytica were used to ensure that results pertained to the species rather than to a particular strain. Results for strain Rahman have been presented in figure 4. Results for strain 200:NIH were found to be ver

Table 3 Relationships between amoebic growth and initial concentrations of amoebae and trypanosomes

Ratios of amoebae:trypanosomes			
Initial amoebic concentration	Insufficient to support growth	Range in which growth obtained	Upper concentration of <u>T. cruzi</u> preventing growth
<u>Series I</u>			
5.0×10^3	$1:4 \times 10$	$1:4 \times 10^2 - 1:4 \times 10^4$	$1:4 \times 10^5$
8.283×10^3	$1:2.3 \times 10^2$	$1:6.4 \times 10^2 - 1:2.5 \times 10^4$	$1:6.4 \times 10^4$
1.48×10^4	$1:5.3 \times 10$	$1:5.3 \times 10^2 - 1:5.3 \times 10^4$	$1:5.3 \times 10^5$
<u>Series II</u>			
Various	$1:1.3 \times 10^2$	$1:2.5 \times 10^2 - 1:2 \times 10^3$	$1:4 \times 10^3$

Figure 4

Growth of *E. histolytica* (strain Rahman) with different strains of *T. cruzi* epimastigotes in TP-S-1 medium

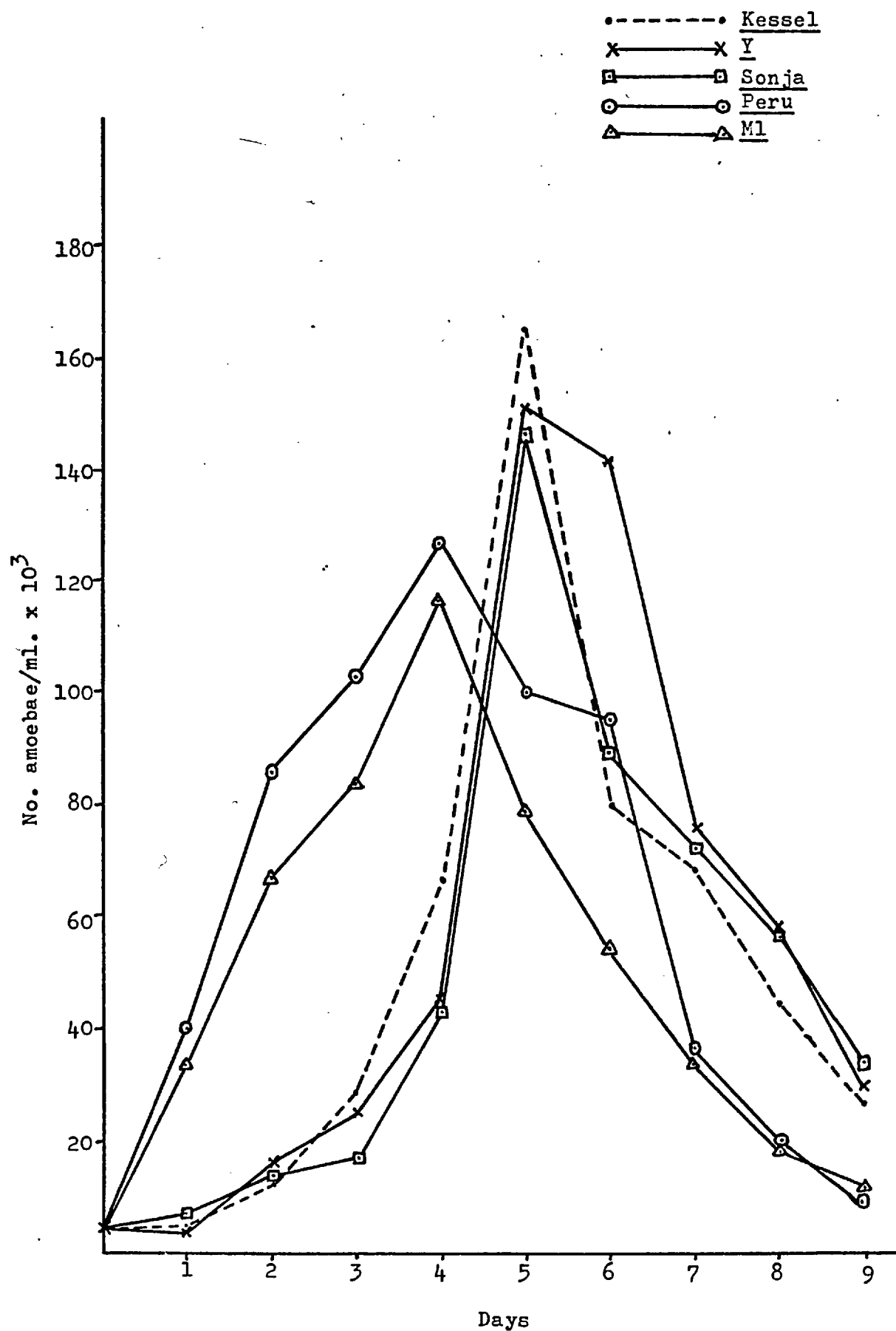
similar and are given in figure 5. Three strains of T. cruzi, Kessel, Y and Sonja gave very similar amoebic growth with both strains of E. histolytica. M1 and Peru strains were rather different from the other strains in their support. With both experiments peak growth of amoebae was reached one day earlier with Peru strain. This also occurred with 200:NIH/M1. An obvious difference in support by the M1 and Peru strains was the greatly reduced lag phase. It is clear from this series of experiments, that superiority of one isolate of T. cruzi to support growth of E. histolytica was not demonstrated

The remaining results pertain more directly to the kind of support afforded to the amoebae by the trypanosomes and some attempt to elucidate the nutritive factors involved.

Observations were frequently made on the living organisms using phase contrast microscopy. Regardless of strains of amoebae or flagellate associate, a similar pattern of feeding was seen. The feeding process appeared to take place in two stages. The first occurred regardless, even when the amoeba was inactive at room temperature and also when the amoeba had fed recently. The second stage only took place if the amoeba had not fed recently and when it was actively motile at 37°C. The last time of feeding could only be assumed by the absence of food vacuoles.

During the first stage the trypanosomes swam freely in the medium and were not attracted to the amoebae. If a trypanosome happened to touch an amoeba it was immediately stuck onto the amoeba surface. Once the trypanosome became attached to the amoeba

Figure 5 Growth of *E. histolytica* (strain 200:NIH) with different strains of *T. cruzi* epimastigotes in TP-S-1 medium



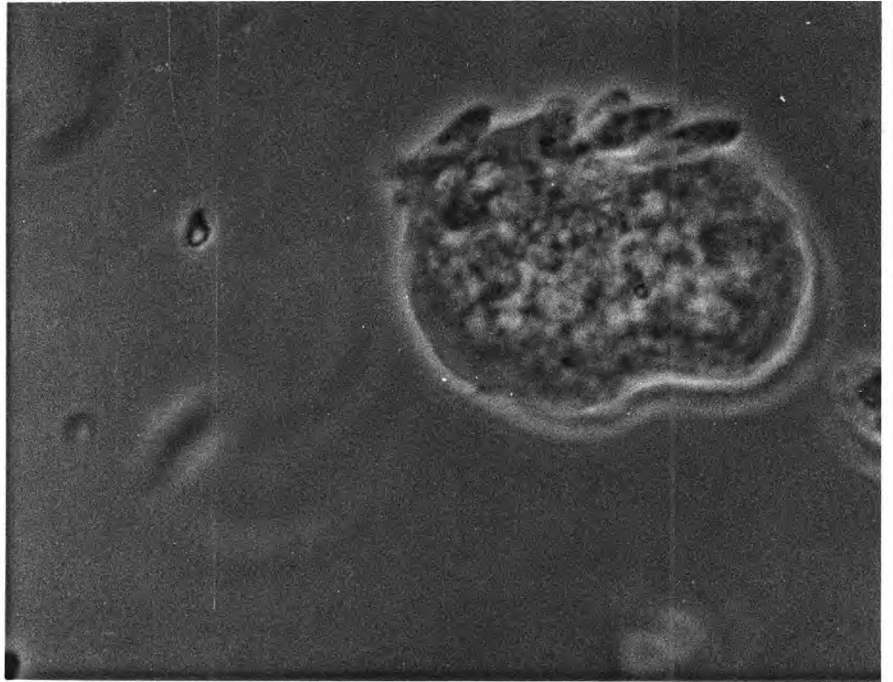
other trypanosomes would adhere to the first trypanosome. See figure 6a. There did not appear to be any particular point of attachment on the amoeba, but attachment never took place at the uroid region. Generally the trypanosome was attached by the tip of the flagellum initially, but later the entire length of the body might become attached. There was apparently no limit to the number of trypanosomes that could be attached to an amoeba; sometimes an amoeba was obscured by 30-40 trypanosomes. If the amoeba was inactive the trypanosomes would free themselves by very active wriggling movements. Sometimes they were swollen at the point of attachment but otherwise they behaved normally.

If the amoeba was active it would gradually change shape making a pocket on the surface, in which the trypanosome body lay, the flagella still free in the medium. The pocket deepened and the amoeba quickly engulfed the trypanosome including the flagellum. From initial attachment to ingestion took 5 to 7 minutes. Figure 6b shows a Crithidium within the food vacuole. For 15-20 minutes after ingestion the trypanosome was still motile within the amoeba but at the end of this period there were only spasmodic jerks. Thirty minutes after ingestion very fine fibrils emerged from the uroid region of the amoeba. As they were wafted about in the medium they appeared to be branched. These could be the flagella fibrils of ingested trypanosomes, which were not digested by the amoeba. Twisting of these would give a branched effect.

An experiment was carried out to investigate the necessity for

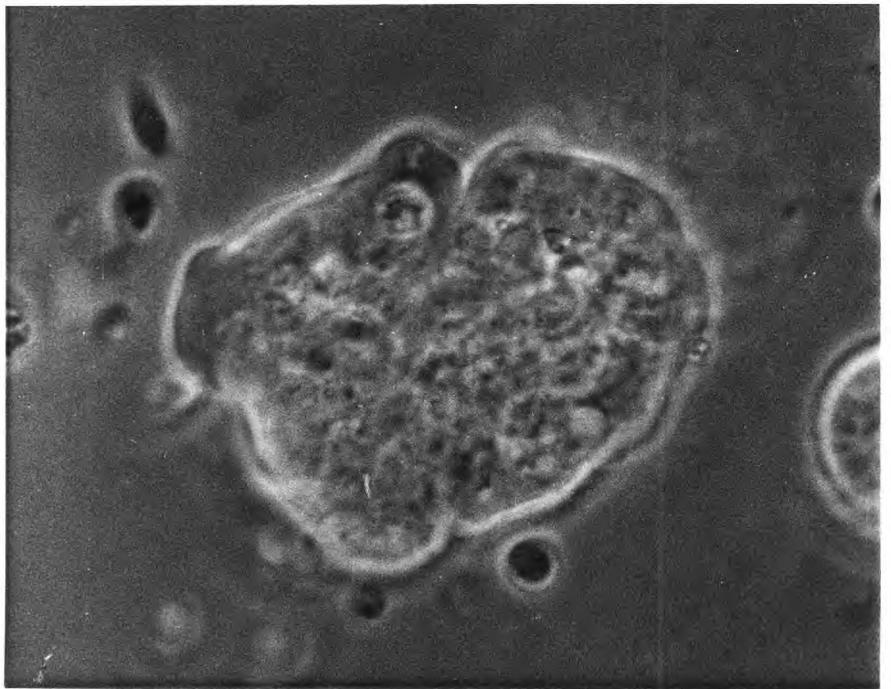
Figure 6

a



x 1,200

b



1,200

physical contact between amoebae and trypanosomes. The apparatus and method used has been described in chapter 2 pp70& figure 1 . Amoebae that were separated from the trypanosomes by a cellulose acetate membrane filter, failed to survive for a week. Some amoebae were present at three days and a subculture was made using all the amoebae but no growth was obtained.

In another experiment, amoebae were cultured using medium in which trypanosomes had been incubated for 24 hours at 37°C. No trypanosomes were included with the amoebae in the inoculum. Amoebae survived for 7-10 days (2 subcultures), but no growth occurred. Amoebae in cultures prepared with the trypanosomes of the above treatment grew excellently. After 10 weeks (20 subcultures) of this cultivation yields of amoebae were only marginally less than those of normal cultures.

A further experiment was carried out to test a corollary to the latter one described. This was simple axenisation from an amoeba-trypanosome culture as described in chapter 2 pp. 72 followed by addition of trypanosomes. After 10 days without associates, when most of the active amoebae had disappeared from the culture (1+ assessment see chapter 2 pp 46), 4.0×10^6 trypanosomes were added to the culture. Within three days excellent 3+ growth was attained.

As phagocytosis was an essential aspect of the amoeba-trypanosome cultures some attempts at induced phagocytosis were made. Charcoal particles, charcoal particles soaked in egg yolk infusion, rice starch grains and M:N carboxymethylcellulose were added to the medium for this purpose. All were ingested except

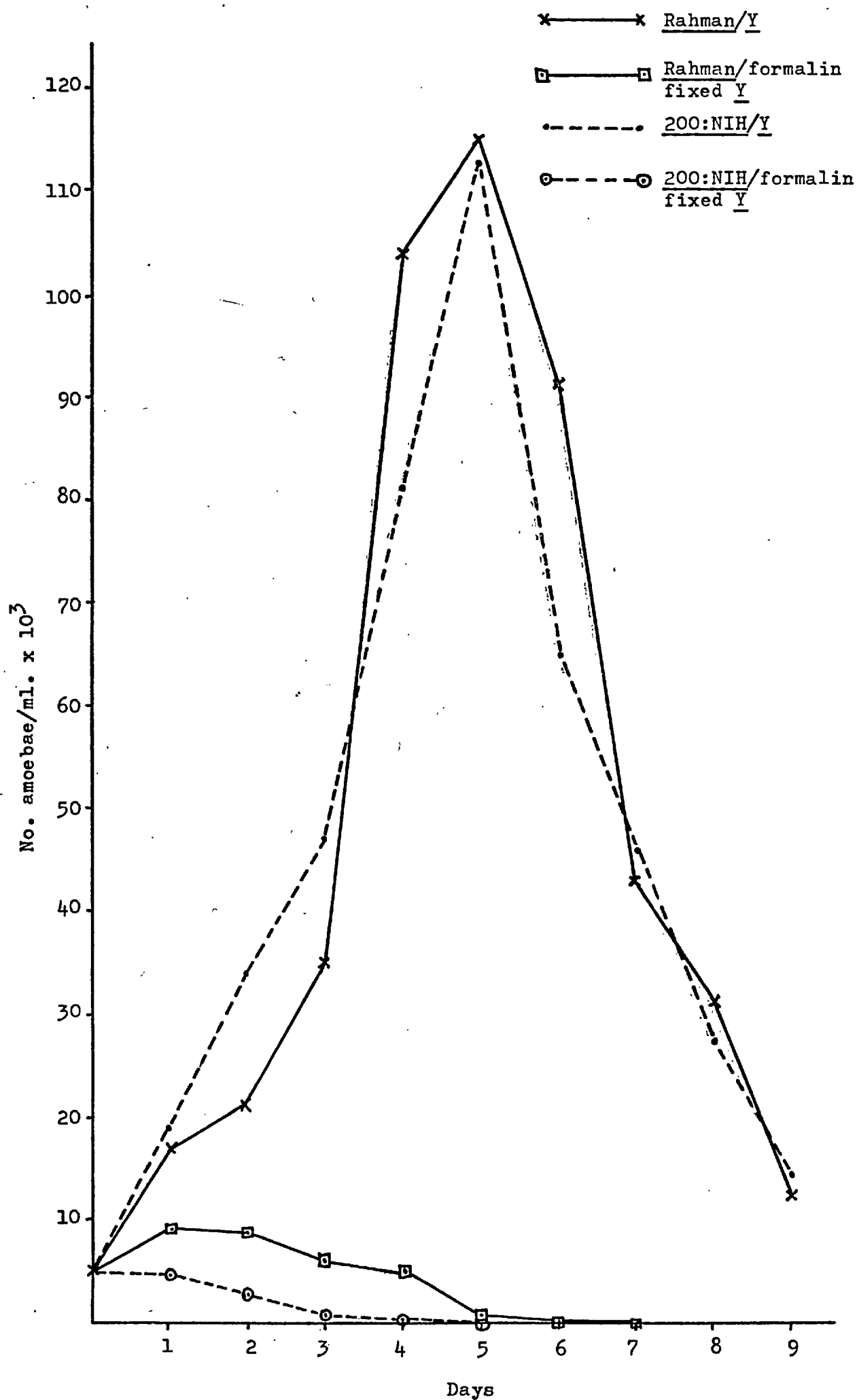
the plain charcoal particles. However the charcoal particles soaked in egg yolk infusion and the carboxymethyl cellulose were voided rapidly within 5-10 minutes. Starch grains remained within the amoebae during one hour's observation and some amoebae still contained them three days later. Cultures of amoebae set up using no flagellate but starch instead, survived for five days only and no multiplication occurred.

The effect of a pinocytosis inducer was also investigated. Aqueous alcian blue was added to the culture medium such that the resultant concentration was 1:10,000. Amoebae kept in this with no trypanosomes failed to survive for more than ten days. No growth occurred. Amoebae were observed to be highly vacuolated which showed that pinocytosis had taken place.

The effect of heat treatment on trypanosome support of amoebic growth involved pretreatment for three hours at various temperatures (see chapter 2 pp. 77 for details). Normal amoebic growth was obtained using trypanosomes pretreated at temperatures up to 41°C. However trypanosomes pretreated at 45°C would only support growth of amoebae for three weeks (6 subcultures). Amoebae associated with heat inactivated trypanosomes (56°C) survived for one week only (1 subculture).

Trypanosomes killed by treatment with 0.01% formalin did not affect their capacity for amoebic support so adversely. Cultures using such associates were maintained for 10 weeks (20 subcultures) and thereafter detailed examination of growth was made. The results have been plotted in figure 7. Growth with normal trypanosomes has

Figure 7 Growth of E. histolytica with live and formalin fixed T. cruzi (strain Y)



been included for comparison. With both strains of E. histolytica tested, growth was extremely poor. Growth was shortlived and the total yield was minimal.

No prolonged growth could be obtained using the water insoluble portion of water lysed trypanosomes. However in contrast, amoebae grown in TP-S-1 medium containing 20% of the water soluble fraction of T. cruzi grew for ten weeks and thereafter were assessed and discarded. Growth was of a minimal standard, peak growth being obtained on day 1 with an increase of only 20%. Amoebae were still present at day 5. It is unlikely that the culture could have been maintained much longer.

Discussion

Although conventional laboratory techniques are inevitably based on long years of experience it was necessary to confirm that the cultivation techniques employed for the T. cruzi epimastigotes were the optimal in most respects for their use in amoeba-trypanosome cultures and their own maintenance. Assessment showed that providing the inoculum was kept as close to 5.0×10^6 /ml. and 5% rabbit serum was used, good, regular growth would result. It will be demonstrated later (chapter 5 pp.265) that the epimastigotes would actually multiply in TP-S-1 at 25°C. As it was found that the trypanosomes remained healthy and viable for as long as the period between amoeba-trypanosome subcultures it was most sensible to continue preparation and maintenance of the epimastigotes as originally postulated. The population entering decline phase is equally appropriate for subcultivation and for

use as amoebic associate.

The concentration of trypanosomes in the amoeba-trypanosome system was a considerable cause of variation. Basically it would appear that the ratio amoeba:trypanosomes is more important than a simple measure of the trypanosome concentration. However other factors must be taken into consideration, in particular amoebic growth in minimal and maximal concentrations of T. cruzi. It has transpired that there is a minimum initial concentration of amoebae that will result in successful growth. Also, as has been mentioned in chapter 3b the size and shape of the culture vessel exerts a limiting influence on the yield that can be produced. However within these limits the amoeba:trypanosome ratio may be used to forecast the resulting type of growth. A direct relationship exists between yield and the logarithm of trypanosome concentration from minimal to optimal growth. Optimal growth is attained by a balance between the greatest amount of nutrient present and the toxic effects of this or other accompanying factors.

The optimal initial T. cruzi concentration, more correctly interpreted as amoeba:trypanosome ratio, is itself influenced by the initial concentration of amoebae. This was shown by the varying steepness of gradients in figure 2. Undoubtedly these differences are real but the steep gradient of the lowest amoebic concentration was probably enhanced by the use of TP-S-1 medium and a longer established amoeba-trypanosome culture. However the experiment using a constant initial T. cruzi concentration confirmed that the

concentration of 5.0×10^3 amoebae/ml. was optimal.

Strain differences of T. cruzi did not appear to be highly significant in amoebic support. The most striking result was the close similarity between Kessel, Y and Sonja strains and the degree of similarity of M1 and Peru strains. Two reasons could explain this. The first is that Kessel, Y and Sonja are of Brazilian origin and M1 and Peru originate from Argentina and Peru respectively. Wide geographical variation could result in strain variation. The second reason is that Kessel, Y and Sonja are considerably longer established in the laboratory compared with M1 and Peru. Cultivation under similar conditions is highly likely to result in similar strains by selection.

Several experiments have demonstrated the essential aspect of the trypanosome presence for amoebic growth. Observation of the amoeba's ingestion of live trypanosomes and voiding of digested remains confirms the nutritive role of the associate. However this, in itself, tells us little of the biochemical basis of this role.

As axenic cultures have been maintained in the same medium as employed in this amoeba-trypanosome system it is conceivable that the trypanosome increases the uptake of medium by the amoeba. Certainly the trypanosome does induce phagocytosis. When inanimate objects were used to mimic the same effect, no growth resulted. Possibly this was due to a reduced volume of medium accompanying the particles due to their nonmotility. Therefore the induction of pinocytosis by chemicals was used to ensure that ample quantities

of medium were taken up. Again although a considerable degree of pinocytosis occurred, no growth resulted. Survival was longer than in the case of induced phagocytosis. This could be interpreted as insufficient medium uptake or adverse effect of the starch in the induced phagocytosis. The induced pinocytosis results imply that some benefit is conferred by ingestion of the trypanosomes.

Therefore the role of the trypanosome is one of providing actual nutrient. The result of heat treatment indicates that the essential nutrients are heat labile, but some motile trypanosomes were still present. All the procedures altering the trypanosome physiological state from one of the healthy active organism were detrimental to amoebic support. It seems unlikely that this is simply a result of reduced supplies of nutrient by prevention of metabolism. Clues as to the nature of nutrient provided by the trypanosome are afforded by the treatments that still allowed growth to continue. Formalin fixation might be considered less damaging to certain proteins than heat fixation. Also lysis by water will not be detrimental to water soluble proteins. Water soluble proteins have already been shown to be significant in amoebic growth using egg yolk infusion in A-TPH medium (see chapter 3b). Certainly some part of the water soluble portion of the trypanosome is beneficial to the amoeba since growth resulted and no enhanced phagocytosis was induced. From the temperature treatment and heat fixation results a non-enzymic protein is implied as the essential factor. The fact that living

trypanosomes support far greater growth shows that either only part of the nutrient is available in formalin fixed trypanosomes and the acellular water soluble portion, or that the essence of the nutrient is present in suboptimal quantities, or that it is presented in a less acceptable form. In any case, as far as the amoeba is concerned, digestion of the living trypanosome is clearly the best method. The more nutrient available the better the amoebic growth that results; viz. amoeba:trypanosome ratio.

d) Different strains of *E. histolytica*

Introduction

Strain variation within the species *Entamoeba histolytica* has been observed in a number of aspects. One of these aspects is virulence, which can vary between strains which are infective but non-invasive, to those which are highly invasive to laboratory animals (Okamoto 1953a & b, Neal 1957, 1971). The virulence of strains isolated from patients with clinical symptoms is higher on average than that of strains isolated from carriers, when inoculated into the caeca of young rats.

Strain variation, expressed in terms of culturability, has already been demonstrated using associates of mixed bacteria and monobacteria in HSre+S medium (see figures 1 & 2).

The "Laredo"-type of strains of *E. histolytica* has been shown to differ considerably from all other *E. histolytica* strains in temperature and tonicity tolerances, pathogenicity and antigenic properties (Neal & Johnson 1966, 1968 and Goldman 1969). These differences are so great that Goldman considers that there could be sufficient justification for creation of a separate species for the "Laredo"-type strains. However he considers that as only 6 strains have been isolated, at present, they might be considered as members of the *Entamoeba moshkovskii* complex. *E. moshkovskii* itself could be found in the free-living condition as a consequence of its wide temperature and tonicity tolerances. In the absence of a categorical recommendation in the literature that these strains comprise a new species, in the present work they are dealt with in this chapter (i.e. as a strain of *E. histolytica*) rather than in the later chapter 5 on other species of amoeba (see p205).

In the present experiments, the ease of establishing strains of E. histolytica and their growth after several weeks of subculturing in the new medium, was studied in relation to their virulence. The term 'virulence' has a variety of definitions. Here it is used to describe the invasiveness and infectivity of an E. histolytica strain when inoculated into caeca of weanling rats (see Chapter 2).

For convenience results with strains of E. histolytica used were divided into 3 groups:-

- i. Invasive strains of E. histolytica
- ii. Non-invasive strains of E. histolytica
- iii. "Laredo"-type strain of E. histolytica

Figure 1 Growth curve of E. histolytica strains in HSre+S medium with mixed bacterial flora

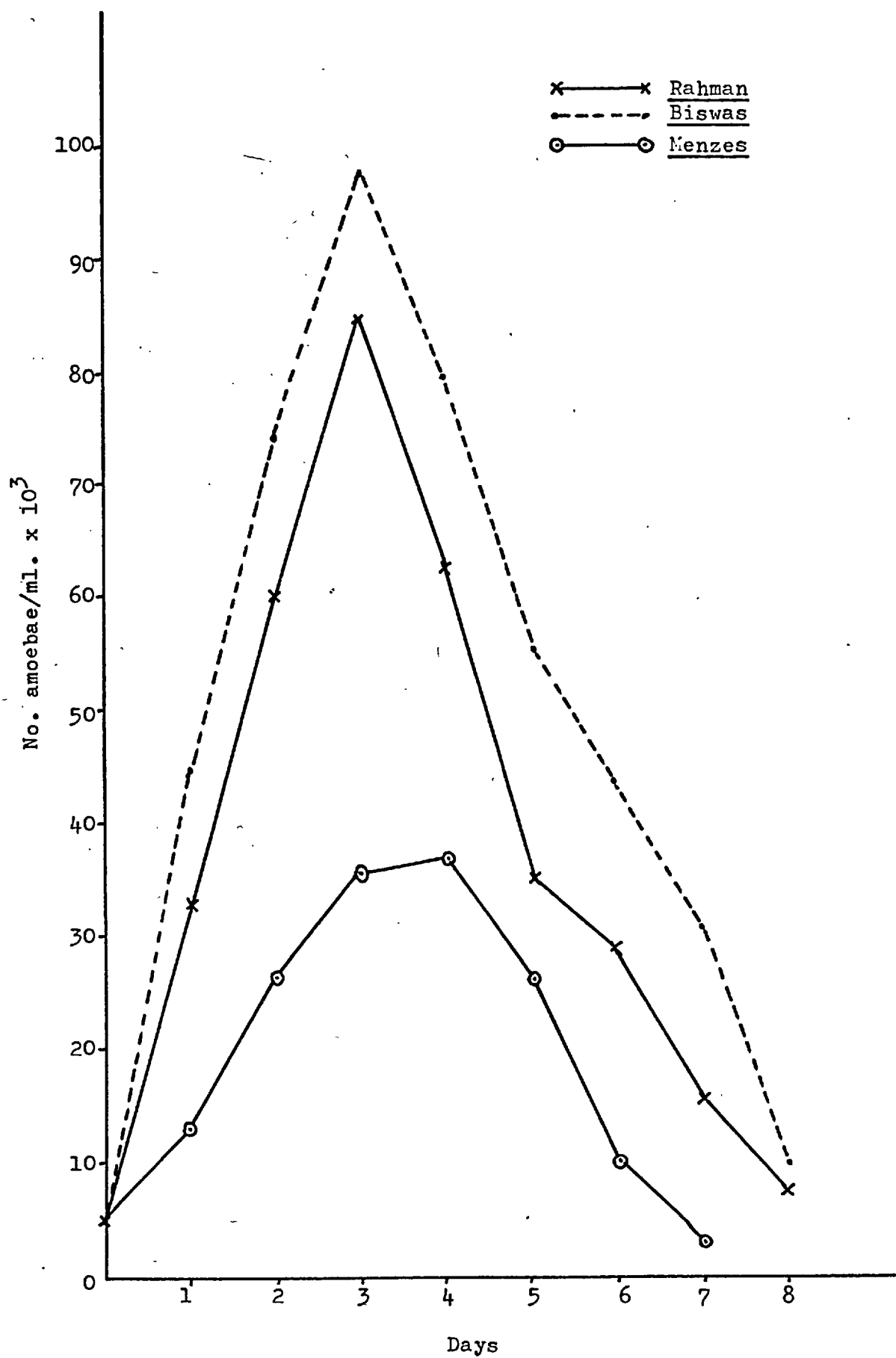
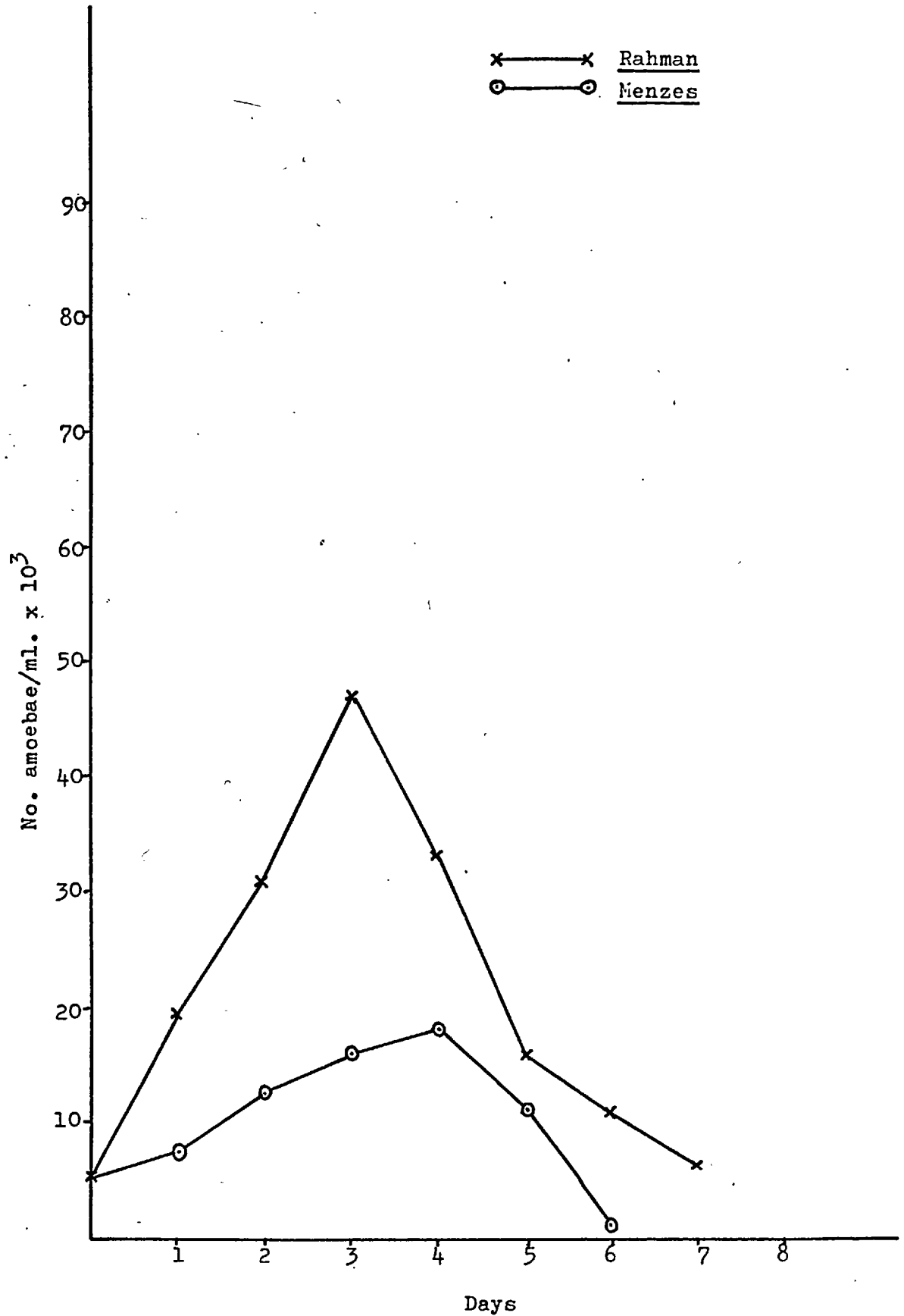


Figure 2 Growth curves of E. histolytica strains in HSre+S medium with Escherichia coli (strain A')



Experimental results of monoxenic cultivation

i Invasive strains

The invasive E. histolytica strains used in this group were Rahman, Biswas, Menzes and 200:NIH. Prior to monoxenisation all strains included in this chapter were known to be pathogenic to rat or guinea-pig caeca on inoculation, from experimental evidence from this or other laboratories.

Initiation of amoeba-trypanosome cultures and their maintenance was carried out as described in chapter 2, pp 48 using epimastigotes of Kessel strain of T. cruzi. All these strains were easily isolated in amoeba-trypanosome association and cultivation was maintained for an apparently indefinite period of time. Biswas was maintained for 2 years 11 months before it was discarded; Menzes for 1 year 8 months, and 200:NIH for 2 years 4 months. Rahman is still being maintained after 3 years 6 months.

The type of growth found during the initiation period is seen in figures 3, 4 and 5. Once the cultures were fully established growth curves were plotted as seen in figure 6. Initiation of the monoxenic culture of 200:NIH was not plotted as the re-association of an axenic culture with trypanosomes presented quite a different problem from the elimination of a mixed bacterial flora and its replacement by trypanosomes. After growth of isolate 200:NIH was established with epimastigotes of T. cruzi, it was observed that the amoebae were dependent on the epimastigote cells. This was shown by the loss of amoebae when the associates were omitted in axenisation experiments described in chapter 2 pp 72.

Menzes strain was initiated in ATPH+2ey medium and was later transferred to TP-S-1 medium once the merits of the different media had been established (see p. 92). Although the circumstances are

Figure 3. Initiation of axenic and monoxenic cultures of *E. histolytica* (strain Biswas) in TP-S-1 with *T. cruzi* (strain Kessel) as monoxenic associate

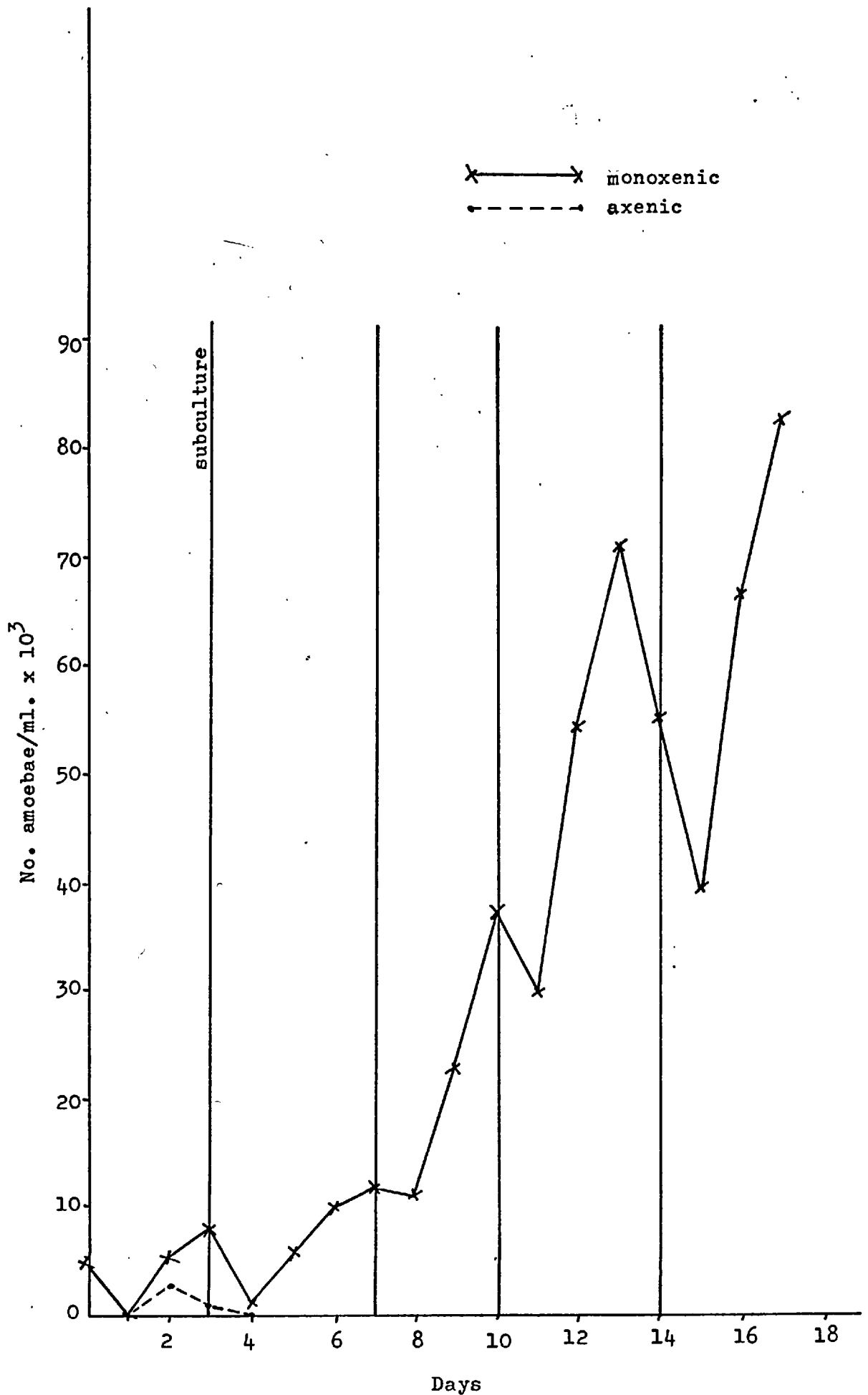


Figure 4 Initiation of axenic and monoxenic cultures of E. histolytica (strain Rahman) in TP-S-1 with T. cruzi (strain Kessel)

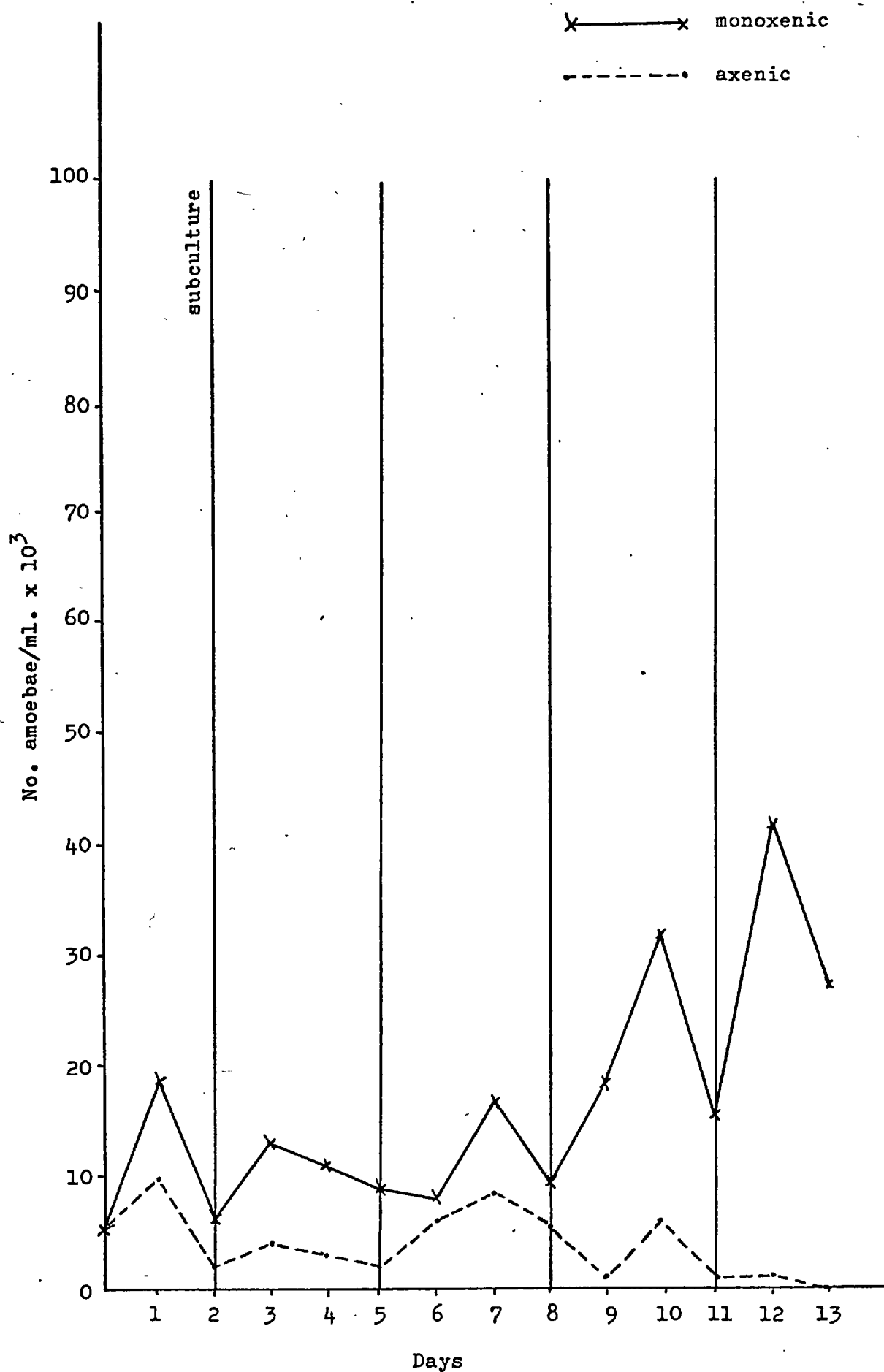


Figure 5 Initiation of monoxenic culture of E. histolytica (strain Menzes) in A-TPH medium with T. cruzi (strain Kessel)

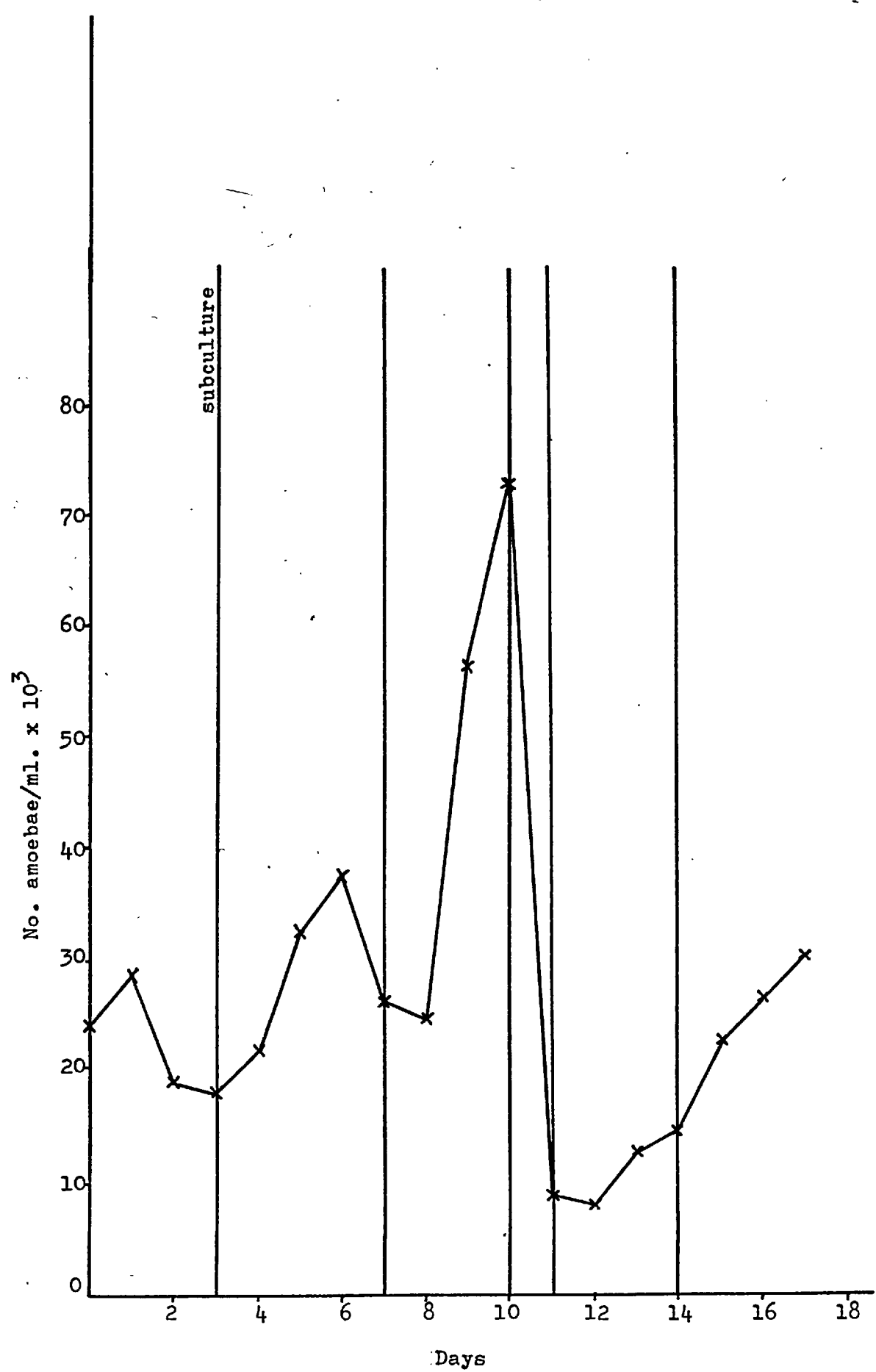
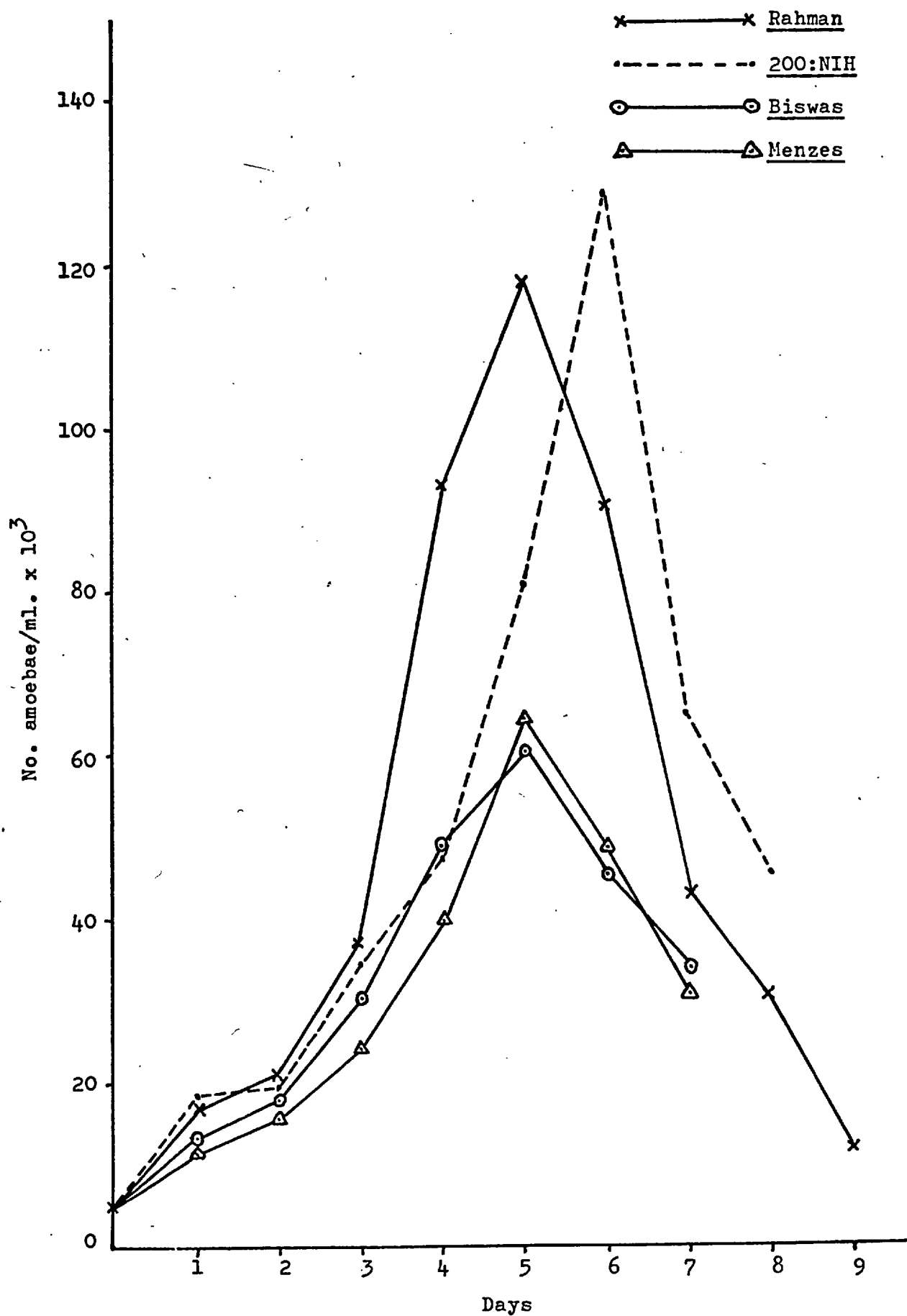


Figure 6 Growth curves of *E. histolytica* strains in TP-S-1 with *T. cruzi* (strain Kessel)



not identical for comparison with the initiation of the other two strains the inclusion of the Menzes strain data here helps to give a fuller picture of the problems involved at this stage.

The size of the original inoculum of amoebae did not appear to be critical and success in establishing amoeba-trypanosome cultures was achieved with the first attempt on each strain. In all strains there was a slight increase in numbers within a short period of isolation. This was also seen in the axenic isolations of Rahman and Biswas strains, which received exactly the same treatment as the monoxenic with the omission of the flagellates. The initial multiplication period was followed by a rapid drop in numbers to fewer amoebae than in the original inoculum. This rapid drop coincided with the time of the first subculture in Rahman and Menzes strains. Thereafter followed slow multiplication which increased until about twice to three times the original concentration of amoebae were present. When the monoxenic system was becoming established a series of cycles of multiplication and death took place. These either coincided with, or were regulated by, time of subcultivation as a consequence of the death of most flagellates within 3 days. Once the regular cycles had been observed daily monitoring of the cultures was discontinued and subcultivation was made in the conventional manner using fresh tubes of medium and only part of the amoebic sediment.

Initiation of Menzes strain was complicated by the presence of bacterial contamination first seen on day 11. As all the culture tubes for this strain contained the contaminant it was most probably introduced during subcultivation on days 7 or 10 rather than during counting procedures. The bacteria could either have been

a slow growing organism introduced on day 7 or a fast growing organism introduced on day 10. As the amoebic growth between days 8 to 10 was somewhat exceptional the bacteria was most likely to have been a slow growing organism introduced on day 7. Presence of a small number of the contaminant could have enhanced amoebic growth. Too many bacteria would destroy the balance of the system and amoebae would be lost in consequence.

Figure 6 describes the growth curves of the established monoxenic cultures of all 4 strains. The most striking feature of the growth curves was the presence of a two day lag phase before logarithmic growth took place contrasting with no pronounced lag phase in mixed bacterial cultures (see Figure 1). Consequently peak growth was attained at least three days later than that seen in the cyclical multiplication during monoxenisation. Biswas was the only strain to show any lag phase during the latter part of monoxenisation and the lag was only 24 hours. A lag phase was undoubtedly present in all culture systems used but often this was not sufficiently pronounced to be seen in the growth curve.

The rapid increase in numbers to peak growth was followed by an equally rapid decrease similar to the pattern seen in the growth curves of amoeba-bacterial culture (see Figure 6).

During successful growth an increase of about twenty-fold was observed with all strains. When counted differentially, using 1% aqueous eosin, only 5% amoebae present at peak growth were found to be dead. (The dead amoebae would not have been included in the growth curves as only motile amoebae were counted.)

From the growth curves it appeared that subcultivation could be made only once weekly. However, when this was attempted success was achieved only with 200:NIH. It may therefore be concluded that, although the amoebae counted during the declining

phase of the growth curve were apparently healthy showing both motility and resistance to staining with eosin, they must have been senescent amoebae.

ii Non-Invasive Strains

When cultures growing in HSre+S medium with mixed bacterial flora were inoculated into rat caecae as described in Chapter 2 the strains were shown to be less virulent than the standard virulent control strains. The results of this virulence assessment may be seen in table I.

Burden and Jawa were shown to be more virulent than Rustom, Sharma and Ambrose.

Table I Virulence assessment

	Strain	Average caecal score	No. rats where caecal tissue invaded	No. rats infected
Virulent	<u>Biswas</u>	2.65	8/9	9/9
	<u>Rahman</u>	3.0	10/10	10/10
Avirulent	<u>Burden</u>	1.5	4/10	10/10
	<u>Jawa</u>	1.4	5/12	12/12
	<u>Ruston</u>	1.2	3/12	12/12
	<u>Sharma</u>	1.17	2/12	12/12
	<u>Ambrose</u>	0.7	2/10	8/10

Scoring system for caecal condition:-

0 - 4

0 normal healthy caecum

4 heavily infected, shapeless
caecum containing mucus only.

Many unsuccessful attempts (41) at monoxenisation of Rustom strain, Sharma and Jawa strains were made and after each occasion it was concluded that insufficient amoebae had been present initially. Therefore on each subsequent occasion the initial concentration of amoebae was increased but without success. Using a very low amoeba inoculum monoxenisation was achieved as seen in figure 8. The isolations of Ambrose and Burden were also initiated with a low amoeba inoculum (see figure 7). The initiation growth curves of the avirulent strains were similar to those describing the virulent strains. However the drop in numbers of amoebae after the first multiplication phase was not so marked in Rustom. Again the cycle of multiplication and death was seen clearly in all strains. It must be emphasised that counts made using a haemocytometer slide are not entirely reliable at such low concentrations as seen in the avirulent initiation curves.

Burden and Jawa strains were maintained in amoeba-trypanosome association without difficulty for a period of two months, when they were discarded. Their growth curves seen in figures 9 and 10 showed the occurrence of 24 and 48 hour lag phases respectively before multiplication to peak growth two days later. With the exception of the lag phase these growth curves were similar to those with mixed bacterial flora in HSre+S (as seen in figures 11 and 12), but the peak was considerably reduced. A 15-fold increase for Burden and a 6-8-fold increase for Jawa were found. Differential counts at 3 days growth seldom revealed more than 5% dead amoebae.

Growth of Ambrose, Rustom and Sharma strains in association with T. cruzi was never very successful. Rustom and Sharma were maintained for twelve subcultures, and Ambrose for only five.

Figure 7 Initiation of monoxenic cultures of E. histolytica strains in TP-S-1 with T. cruzi (strain Kessel)

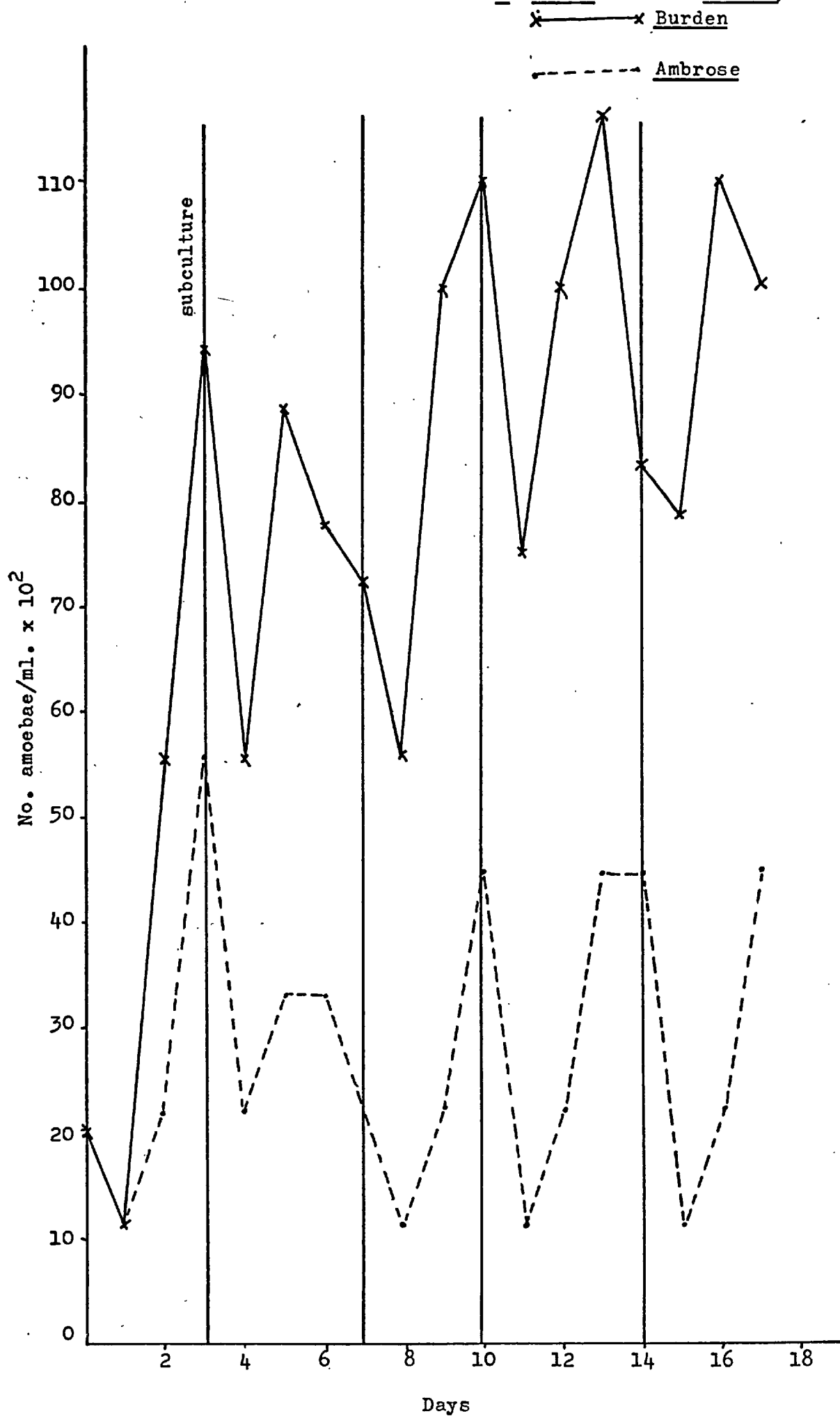


Figure 8 Initiation of monoxenic cultures of *E. histolytica* strains in TP-S-1 with *T. cruzi* (strain Kessel)

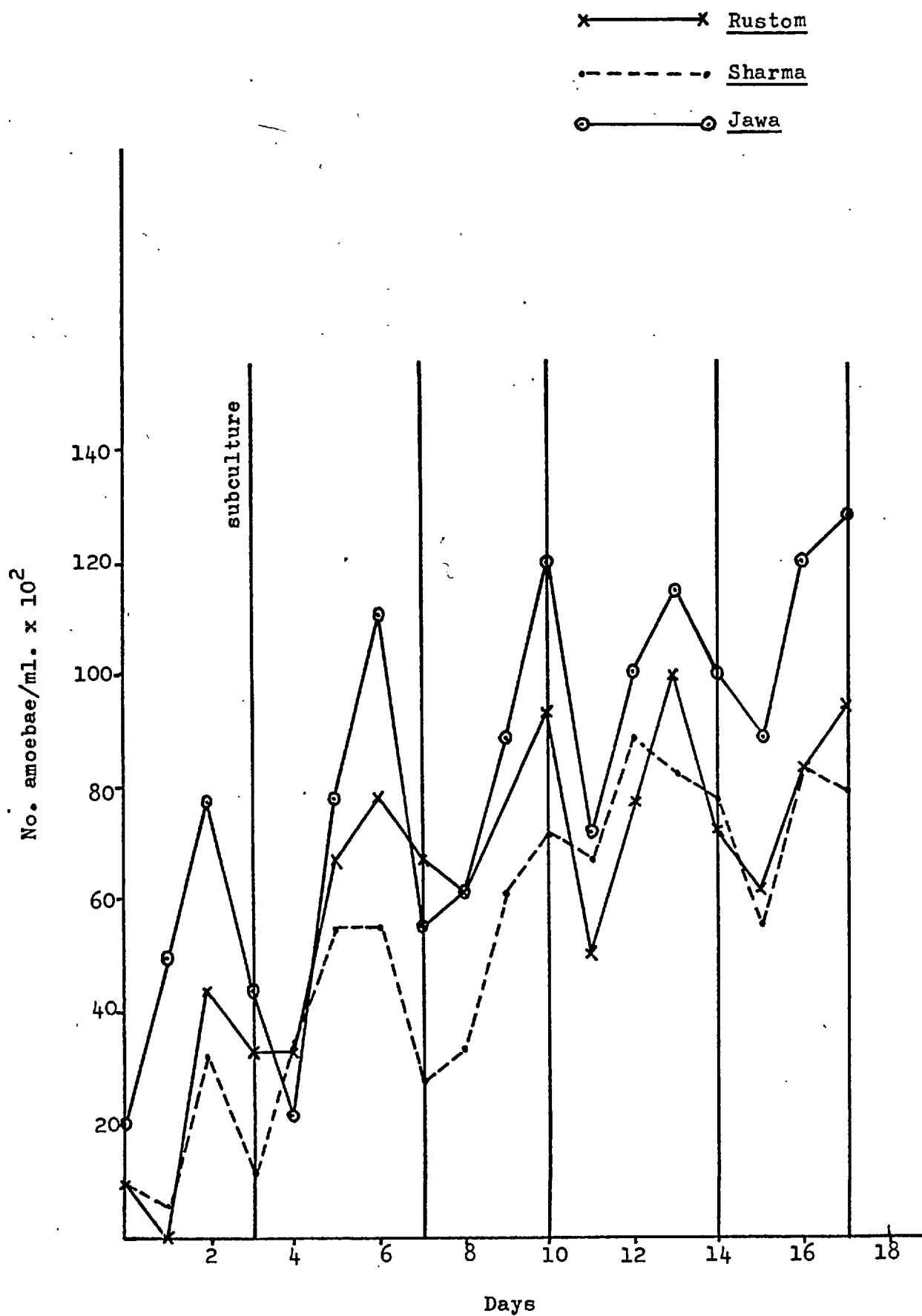


Figure 9 Growth curves of *E. histolytica* strains in TP-S-1 with *T. cruzi* (strain Kessel)

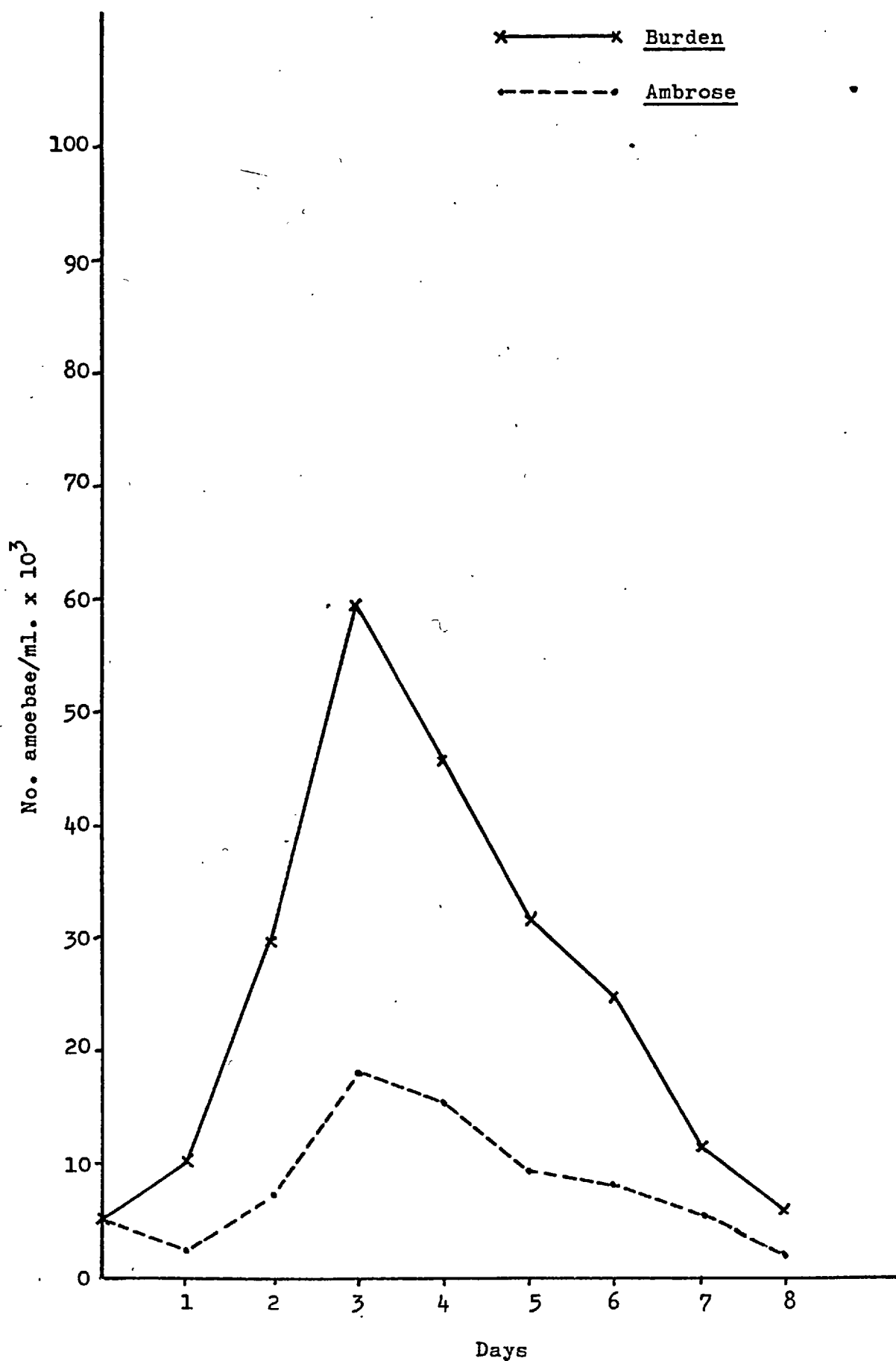


Figure 10 Growth curves of E. histolytica strains in TP-S-1 with T. cruzi (strain Kessel)

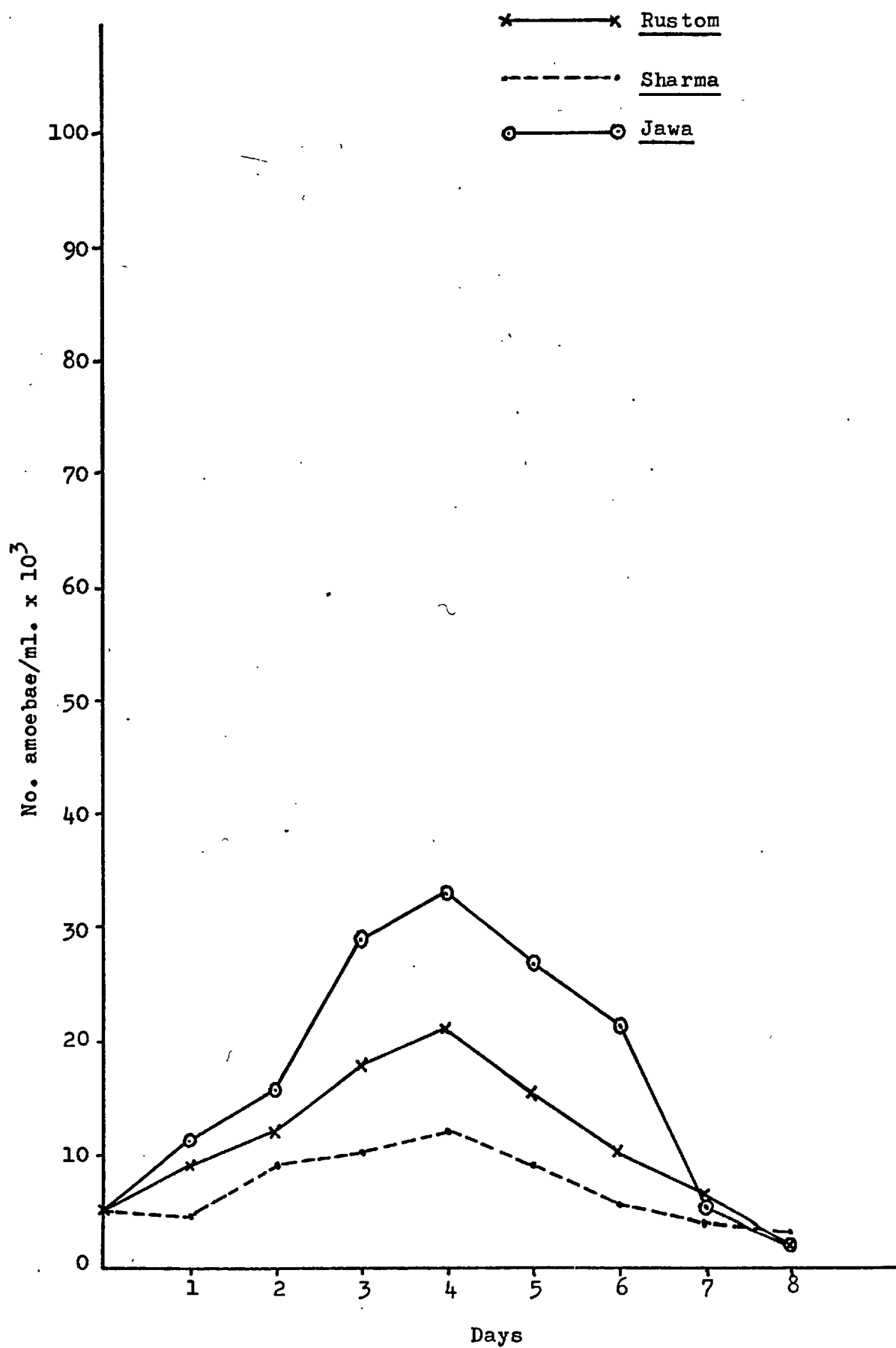


Figure 11. Growth curves of E. histolytica strains in HSre+S with mixed bacterial flora.

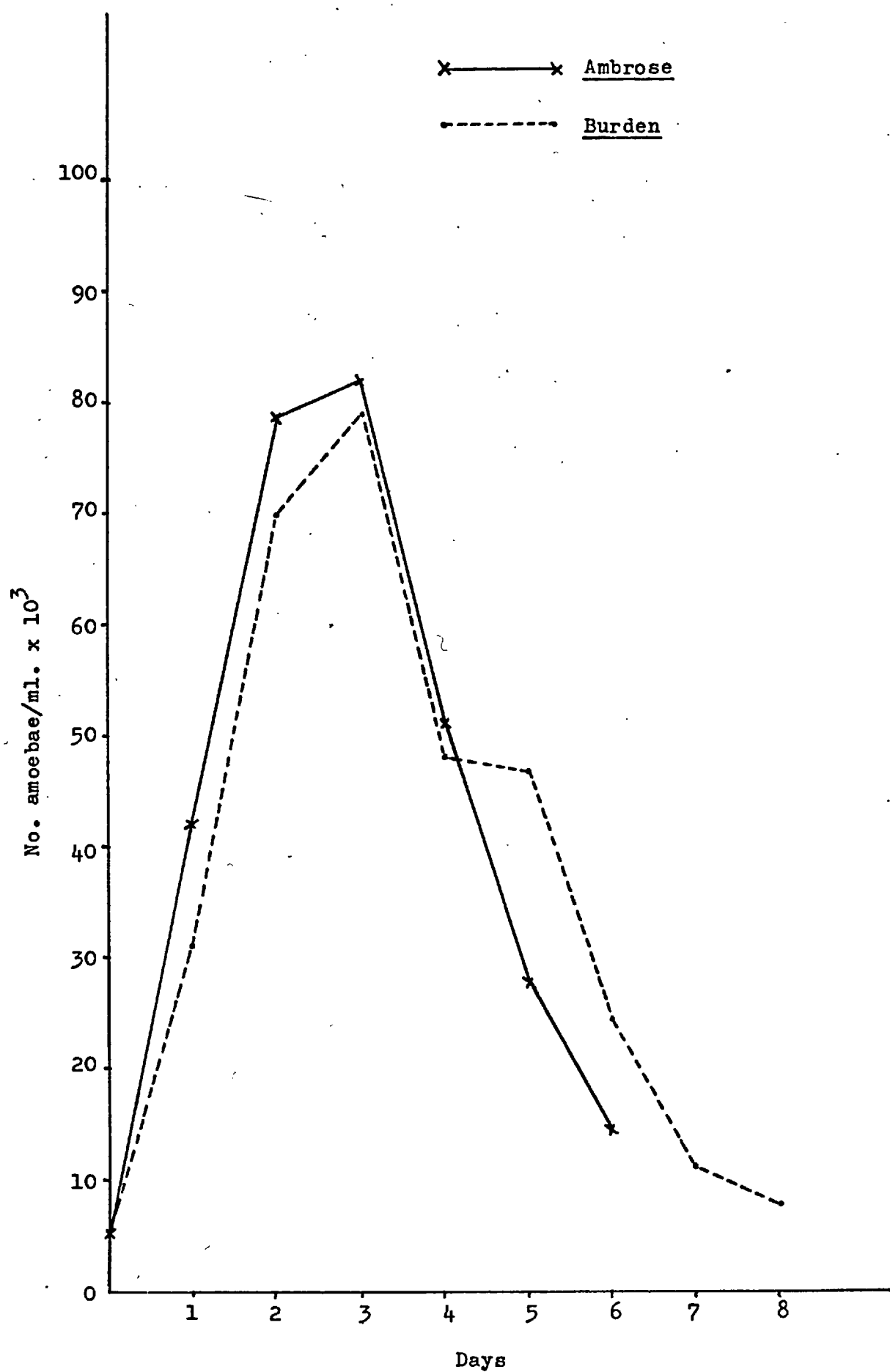
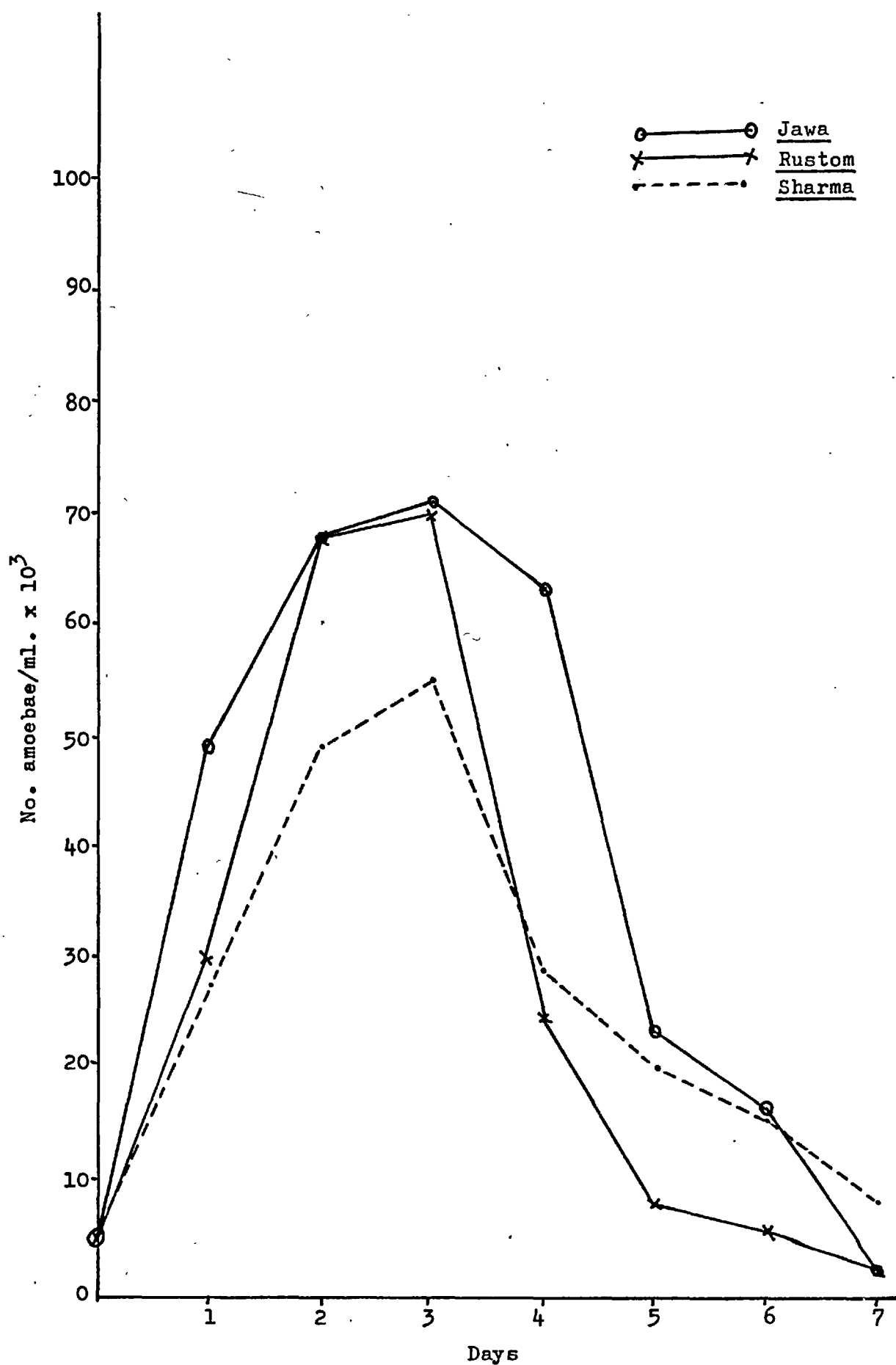


Figure 12 Growth curves of E. histolytica in HSre+S with mixed bacterial flora, (strains Jawa, Rustom & Sharma)



Subcultures always had to be made using all the amoebae present from the old culture.

The growth curves obtained with these strains plotted in figures 9 and 10 show that multiplication was much reduced compared with that obtained with a mixed bacterial flora (figures 11 and 12) especially in the case of Ambrose. A 48 hour lag phase preceded peak growth at 3-4 days. Rustom and Ambrose yielded a 5-fold increase and Sharma only a 2-fold increase. Differential counts revealed a high dead amoeba content of 25%. Attempts to remove dead amoebae resulted in loss of the cultures.

iii "Laredo"-type strains

No growth of Laredo strain could be obtained at 37°C with mixed bacterial flora. This was not surprising since prior to storage in liquid nitrogen for 5 years 10 months cultivation had been at 25°C for 2 months in this laboratory and several years in another laboratory. Therefore all attempts at monoxenisation of Laredo strain were carried out at 25°C.

As may be seen in figure 13 the Laredo strain grew immediately in amoeba-trypanosome association. Growth in the first week was not quite as good as in that following and that seen in the growth curve figure 14. Laredo strain contrasted with the other strains of E. histolytica whose peak growth became delayed by the appearance of a lag phase after continued cultivation in amoeba-trypanosome association. No pronounced lag phase was observed with Laredo strain. The growth curve was very similar to that seen during initiation and also with mixed bacterial flora in Ehs+S medium. Peak growth attained during growth curves at 5 days was in fact 24 hours earlier than that attained during

Figure 13 Initiation of monoxenic cultures of E. histolytica (strain Laredo) in TP-S-1 with T. cruzi (strain Kessel) at 25°C

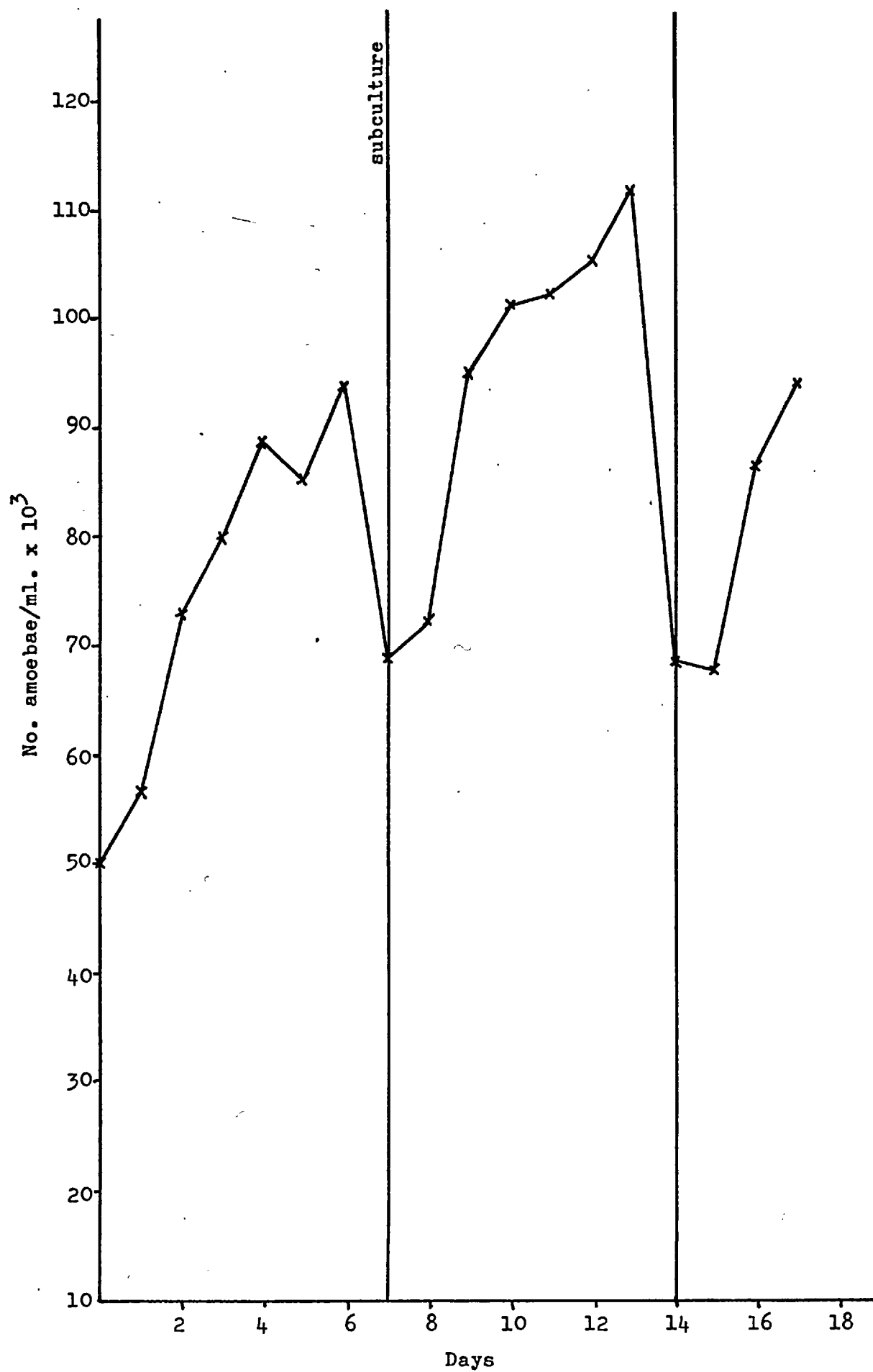
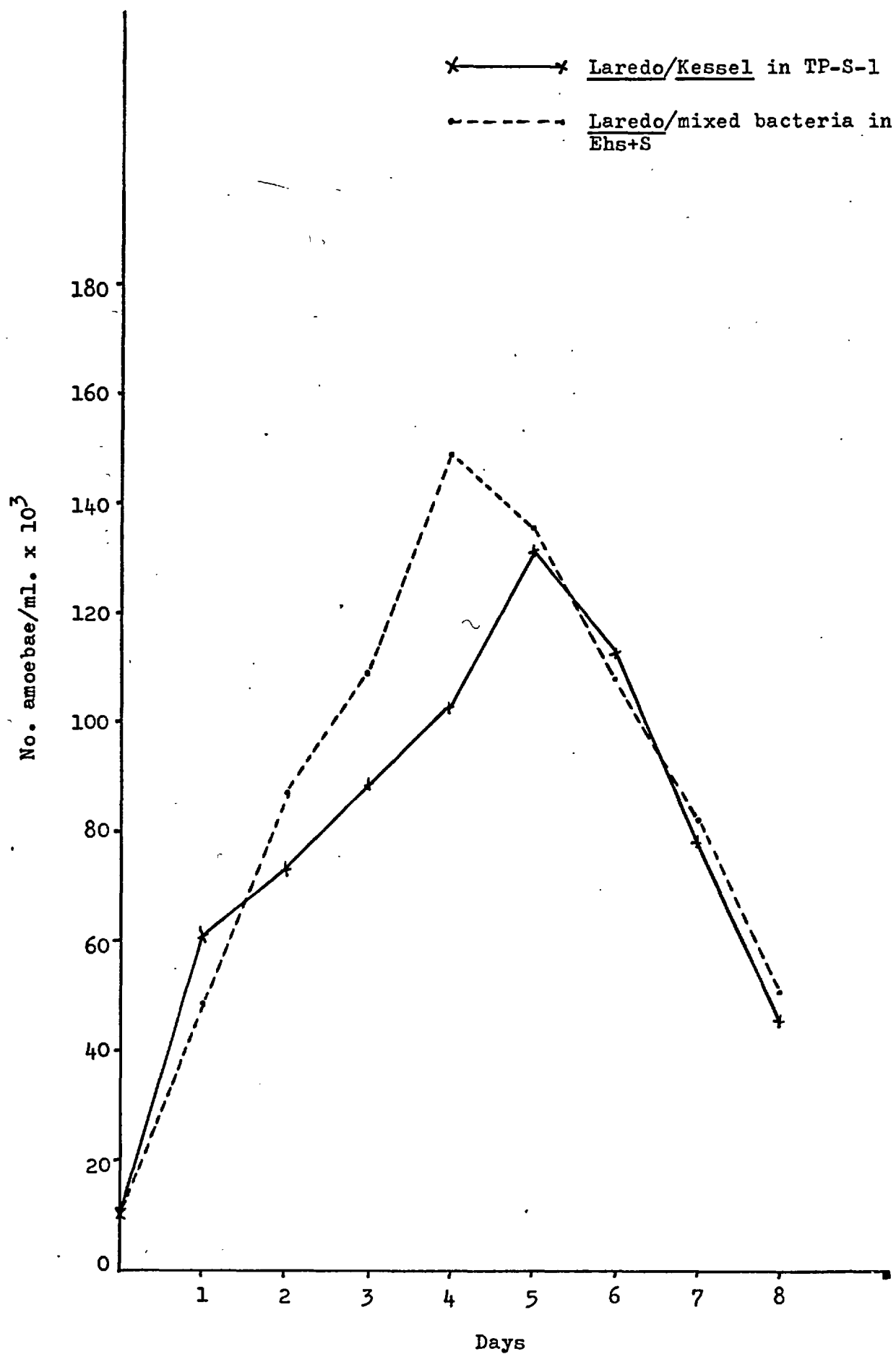


Figure 14 Growth curves of Laredo/Kessel in TP-S-1 and Laredo associated with mixed bacterial flora in Ehs+S



initiation.

Laredo strain was still maintained with weekly subcultures for $3\frac{1}{2}$ months and then discarded. A 25-fold increase was obtained regularly and differential counts at day 5 showed the presence of less than 2.5% dead amoebae.

Discussion

Throughout the period of monoxenic initiation the growth picture is the resultant of interacting factors. Not only are the amoebae having to adapt to new nutrient in the form of an enriched medium and flagellates; but they have lost some of the staple nutrients provided by their original medium and concomitant bacteria. Variation in growth of different strains is due to strain characteristics, some of which can be expressed in terms of virulence. Probably the differences between various isolates shown in acclimatization to the amoeba-trypanosome system, is not only determined by the amoebae but also by the bacterial flora to which they were accustomed. The slight increase in numbers shown initially by all strains is probably the inevitable result of carry over of nutrients within the amoebae and in the suspending medium used for the inoculation. It appears that even within the first 2 days the flagellates play some part in making the situation more favourable for the amoebae as the amoebae increase less in the axenic isolations. As the nutrient substances that are carried over are used up, so the multiplication ceases and rapid drop in numbers probably occurs when essentials have been used. It is probably then that the greatest amount of selection takes place.

It is only the amoebae that are capable of metabolising the appropriate nutrients from the flagellates and the medium that will

survive. Thereafter, the amoebae which grow in this situation will divide giving rise to amoebae which are metabolically capable of growing in the new media. It is conceivable that, initially, there are different amoebae possessing varying capacity for dealing with the new nutrients and thus division would not necessarily be regular. This might explain the very reduced, or non-existent lag phase during the second half of initiation. As cultivation is continued in the same system so finer selection takes place and thus the population becomes more regularised. This must be a somewhat similar situation to that seen in cloned populations.

This could in part explain the unusual pattern of growth as shown by the late peak attained by 200:NIH. As it had already been cultured axenically for several years the strain must have become selected for feeding by absorption and pinocytosis. The reversion to particulate feeding would provide another occasion for selection.

A recent reference to the influence of "parasitic state" of a strain on its successful isolation and maintenance in in vitro culture was made by Abioye (1971). His finding, that it was more difficult to establish strains from symptomatic patients is strange in the light of the experimental evidence presented here. However, he did not use identical conditions of associate and media for every occasion. The various diphasic media and the bacterial associates used provide poorly defined and inconsistent circumstances. The monoxenic amoeba-trypanosome system as described in this work provides conditions which are readily reproducible both qualitatively and quantitatively. Admittedly the medium is only semidefined but slight variation that might be expected from different batches of sera was rarely encountered, (see chapter 3b

pp 103). Also correct assessment of the "parasitic state" of strains is more likely to be obtained from a controlled in vivo experiment than from varying descriptions of patients' symptoms.

The results of attempts at amoeba-trypanosome cultivation of strains in groups i and ii correlate closely with the degree of virulence expressed by the experimental work in rats. The more virulent non-invasive strains multiplied more steadily and to a greater degree than the less virulent strains. The invasive strains (Group i) dealt with earlier, also fit into this pattern. An interesting corollary to this is that a long attenuated strain, 4490/63 has never been successfully isolated in the amoeba-trypanosome system.

Possibly a particular enzyme system enabling invasion of the host tissue by amoebae also facilitates the use of trypanosomes as nutrient.

The ease of monoxenisation and the excellent growth obtained with the Laredo strain present an interesting deviation from the pattern of amoeba-trypanosome cultivation correlated with virulence. The virulent and avirulent strains examined earlier all indicate that good growth in amoeba-trypanosome cultures may be expected from virulent strains and poor or no growth is likely to be obtained from strains of very low virulence. Since all "Laredo"-type strains have been demonstrated to have low infectivity to caeca of weanling rats and none produced lesions (Neal & Johnson 1968) these strains could be classed with the other avirulent strains described previously. However, as already mentioned on p. 7 other characters common to all "Laredo"-type strains distinguish them from normal E. histolytica.

If Laredo was simply another avirulent strain of typical E. histolytica very poor growth or none at all might be expected in amoeba-trypanosome cultures. However, it was noteworthy that the type of growth obtained with Laredo was similar to that of other room temperature Entamoeba species, E. moshkovskii, E. ranarum and E. invadens. (see chapter 5 figures 4, 5, 6 pp. 217-219. Peak growth was preceded by a steady gradual multiplication and then a sharp rise in the rate of growth between days 4 and 5. This type of growth curve might well be the result of the overriding effect of the temperature, on the amoeba-trypanosome association, regardless of the amoeba species differences. This is unlikely as considerable variations have already been observed among different strains grown at one temperature of 37°C. Variation between Entamoeba species would generally be expected to be greater.

The most pertinent observation is not that growth of Laredo was similar to other room temperature strains, but that it was so different from the growth obtained with the avirulent strains. Perhaps ease of monoxenisation might be considered as another parameter in the comparison of typical E. histolytica and E. histolytica-like amoebae.

CHAPTER 4 VARIATION OF THE MONOXENIC SYSTEM: ASSOCIATES

Introduction

It has already been cited in chapter 1 that other workers have attempted monoxenic growth of entamoebae with a variety of organisms. These varied from antibiotic inhibited bacteria (Shaffer & Frye 1948), to flagellates (Pan 1960) to trichomonads (Rees 1949), and growth within the chick embryo (Everitt, Sadun & Carrera 1953). The choice of associate for initial monoxenisation was to some extent governed by the procedure used for elimination of bacterial associates. This problem could be by-passed by using amoebae removed aseptically from human cases of amoebic liver abscess, but such material is not often readily available. The methods for eliminating the concomitant bacteria fall into two groups: first the use of antibiotics and secondly microisolation techniques. The simplest technique is obviously the use of antibiotics but this necessitates the use of an associate unaffected by the antibiotics penicillin and streptomycin. T. cruzi and other flagellates are excellent in this respect. In chemotherapeutic studies, the choice of T. cruzi as associate also offers the advantage that potential amoebicidal compounds are unlikely to be trypanocidal. However, certain objections to the routine use of T. cruzi have been raised on the grounds of safety. Therefore the use of another associate flagellate nonpathogenic to humans is desirable. Diamond (1968a) found Crithidia sp., a parasite of the cog-wheel bug, Arilus cristatus

to be a suitable amoeba associate.

After establishment of a monoxenic strain and the use of antibiotics has been discontinued, the associate organism may be exchanged. Choice of organisms for association at this stage is not governed by their resistance to antibiotic treatment. It was hoped that the investigation of the ability of various flagellates to support amoebic growth would shed some light on the factors required by the amoebae in a monoxenic system.

Only the epimastigote stage in the life cycle of T. cruzi has been used as amoeba associate by other workers. Primarily because it is the most readily available in large quantities. The different stages of the trypanosome life cycle have specific but interrelated metabolic properties. Not all forms of trypanosome need necessarily support amoebic growth. Further investigation is necessary along these lines.

Flagellates have been used most commonly in cultural association with entamoebae, but other unrelated organisms such as Trichomonas foetus and tissue cultures have also been used. (Rees 1949, Reeves et al 1957b). Very little is known of the specificity of amoebic requirements and the role of the associate in supplying these. By examining a wide range of unrelated organisms as potential associates some indication of the respective roles of associates might be achieved. It is also hoped that this might lead to some elucidation of specific amoebic requirements.

Another reason for this investigation was the outcome of several fruitless attempts at monoxenisation of the smaller amoebae,

Dientamoeba fragilis and E. hartmanni and also H. meleagridis, using T. cruzi as associate. As E. histolytica actively ingests the trypanosomes in A-T culture it was considered that the failure experienced with smaller amoebae was due to the size similarity between the amoebae and flagellates. Therefore the discovery of a very small associate capable of supporting E. histolytica growth might provide a lead in the monoxenisation of other species. With the exception of bacteria the smallest acellular organisms which are readily available and cultured in the laboratory were found to be algae and yeasts.

It is generally agreed that tissue culture cells represent an undifferentiated cell system. Their cultivation in defined media, with the addition of serum, has availed the system for considerable research into the basic metabolic processes of mammalian cells. Krupp used tissue fragments from liver in the cultivation of E. histolytica to supplement monobacterial cultures. From the evidence given above it was considered that tissue cell cultures might support the growth of E. histolytica successfully. The essential metabolic properties afforded to the amoebae by the tissue cells might be easier to identify from these "basic" cells than from other successful associates. Therefore a number of established cell lines were investigated as possible amoebic associates.

Lastly in the search for very small associates the use of red blood cells (RBC) was considered. Shaffer & Iralu (1961) showed that once E. histolytica had recovered from the toxicity caused by association with RBC during the first 5 days of culture, growth was

actually enhanced compared with control cultures. They did not maintain cultures in association with RBC and it is probable that the toxicity characteristic would prevent any prolonged cultivation.

Many factors have to be considered in the use of various associates. Practical details have to be taken into consideration, such as availability, lack of pathogenicity and ease of bulk cultivation. Besides the practical details there are many factors that are likely to influence the success of an organism or part thereof in the role of amoeba associate. Unless the associate shares similar properties as entamoebae with regard to temperature, toxicity, pH or other physical and chemical factors the associate will be unlikely to support amoebic growth. However, as will be seen below a constant reference must be made to the state of the associate when studying its role in supporting E. histolytica growth.

As the various organisms used as potential associates are so diverse they will be dealt with under the following subsections:-

- a) Flagellates other than T. cruzi
- b) Various stages in life cycle of T. cruzi
- c) Miscellaneous associates

a) Flagellates other than *T. cruzi*

Introduction

All the work described in this chapter was done using established monoxenic amoeba-trypanosome isolates. The experiments were solely to confirm Pan's (1960) suggestion that *E. histolytica* could be supported by a wide variety of flagellates. The choice of flagellates tested, was primarily determined by their availability and the ease with which they could be cultured. It was naturally desirable to test the *Crithidia* sp. recommended by Diamond (1968a). Use of *Leishmania enriettii* and *Trypanosoma lewisi* was not precluded, although Pan had used them in his studies, because the culture systems were not the same. *L. tarentolae* was also used.

Much work has been done assessing various aspects of *E. histolytica*-*T. cruzi* association, (see chapter 3). It was considered that repetition of a detailed study on each associate was unlikely to yield information of any importance. Therefore the other flagellates used in this chapter, were used only as the cultural form; that is the epimastigote of trypanosomes and *Crithidia* sp., and the promastigote of leishmanias.

Experimental results of monoxenic cultivation with various flagellates

These studies were made by simple replacement of *T. cruzi* by the flagellate to be tested, and subsequent maintenance for a period of at least ten weeks. During this time general observations ($3+ - +$ score see pp 46) were made and detailed growth studies were made at the end of this period. The initiation .

maintenance and assessment of growth methods are described in chapter 2. Growth of amoebae was achieved with every flagellate that was tried. The growth of two strains of E. histolytica with various flagellates is portrayed by figures 1-4. So that comparison may be made with the results of growth with T. cruzi, the initial concentration of the flagellates in the test cultures was $2.0 \times 10^6/\text{ml.}$, as this was frequently used for T. cruzi growth experiments. (see figure 3 chapter 3c). Support given by Crithidia sp. and T. cruzi epimastigotes for E. histolytica was comparable. In both cases better growth was shown by strain 200:NIH than Rahman, cf. Figure 1 with Figure 6 chapter 3d. A similar order of logarithmic multiplication was shown by both associate culture, but there was a longer lag phase in crithidial cultures and greater longevity. Survival of amoebae in association with T. lewisi was good but yield was poor, due to a low level of multiplication. Amoebae growing with L. enriettii survived for only just seven days and an extremely low yield was obtained, particularly with Rahman strain. L. tarentolae supported a reasonable standard of growth of E. histolytica. Although there was a pronounced lag phase logarithmic multiplication was excellent, peak growth being achieved slightly late, as with crithidial associates. Differential counts made at peak growth revealed the presence of 4-5% dead amoebae in crithidial and L. tarentolae associated cultures and 22% - 26% dead amoebae in T. lewisi and L. enriettii associated cultures.

Figure 1 Monoxenic growth of E. histolytica with Crithidia sp
(strain ReF.I:PRR)

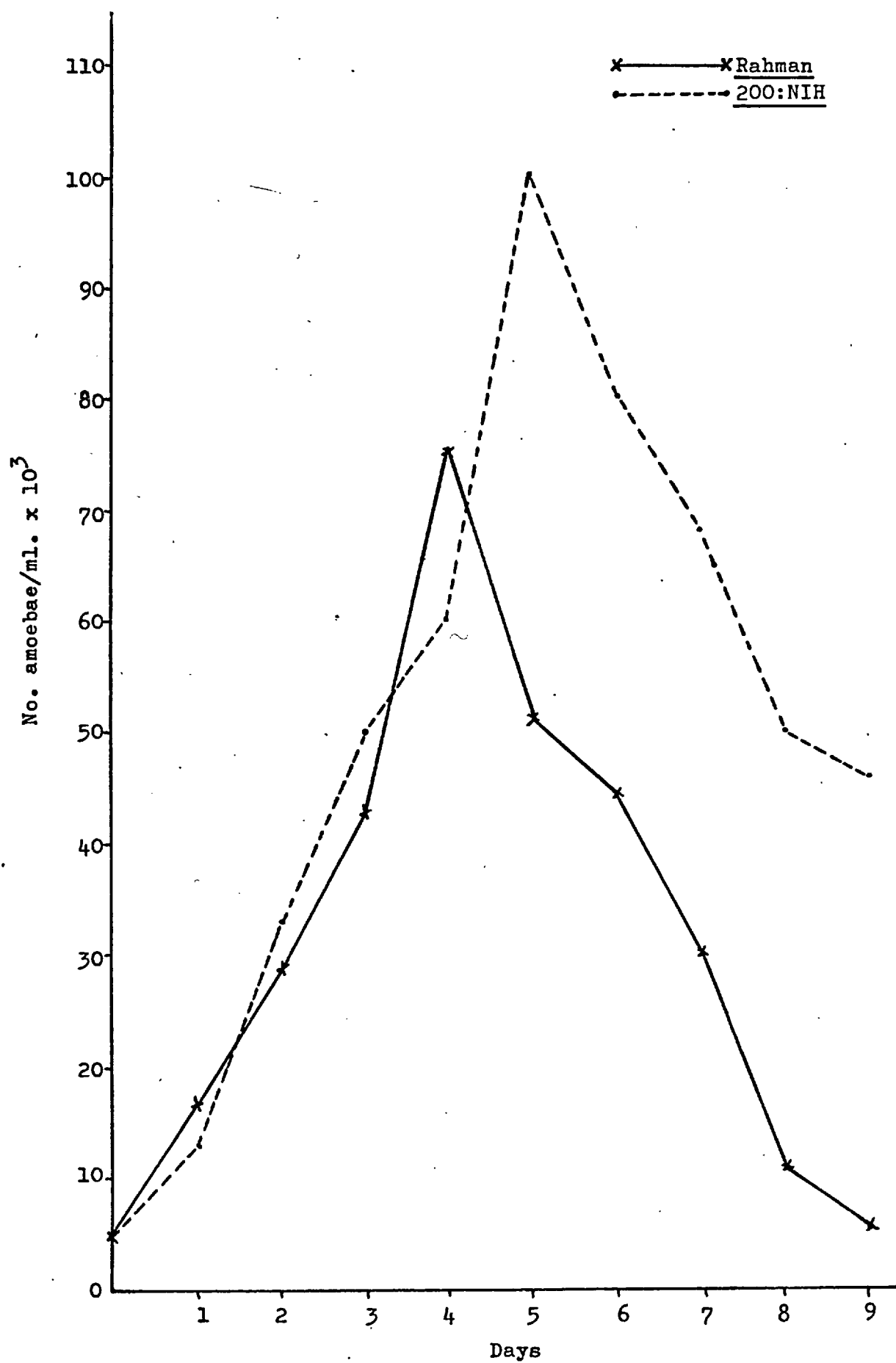


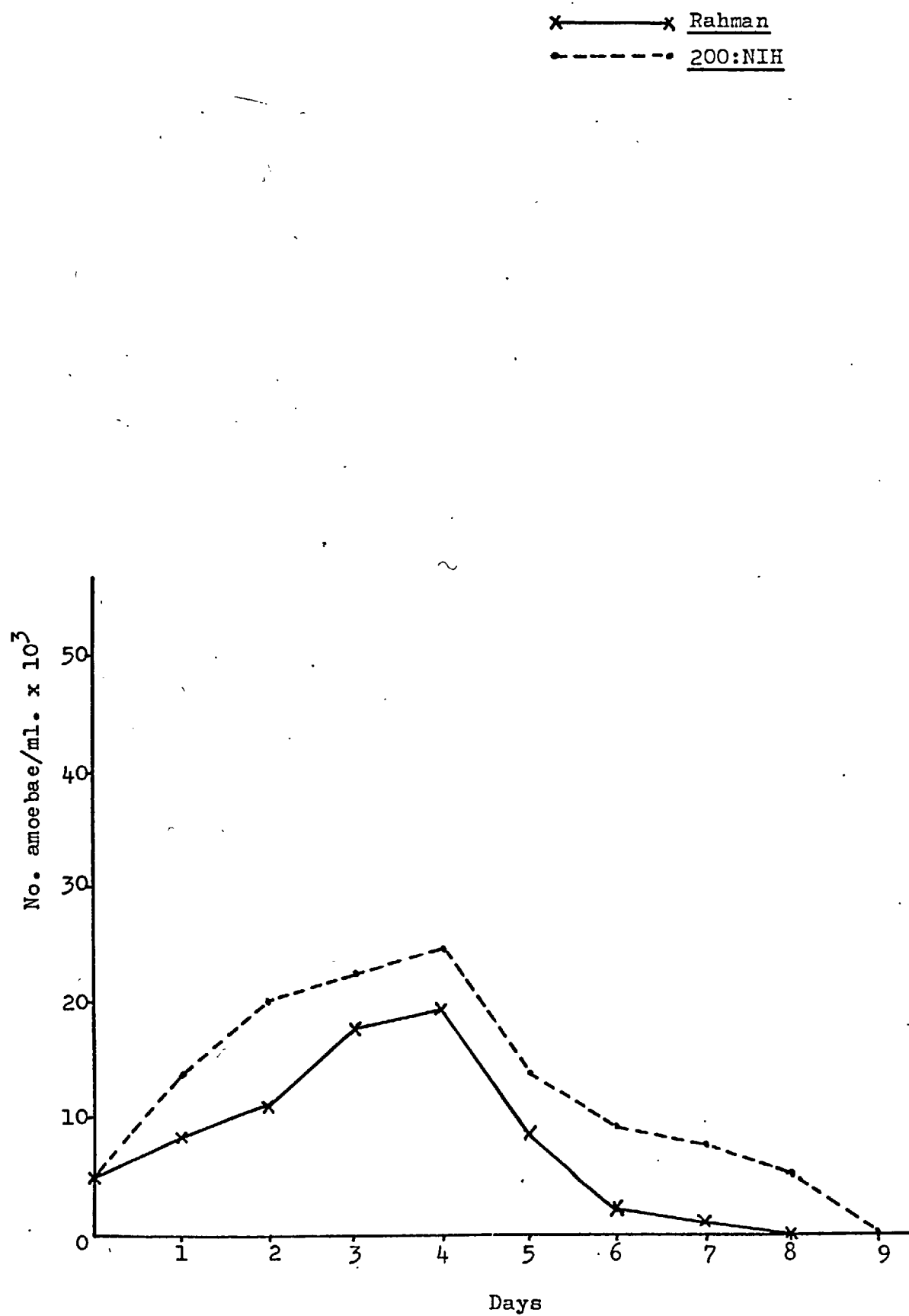
Figure 2 Growth of E. histolytica strains with T. lewisi

Figure 3 Monoxenic growth of E. histolytica with L. enriettii (strain YE)

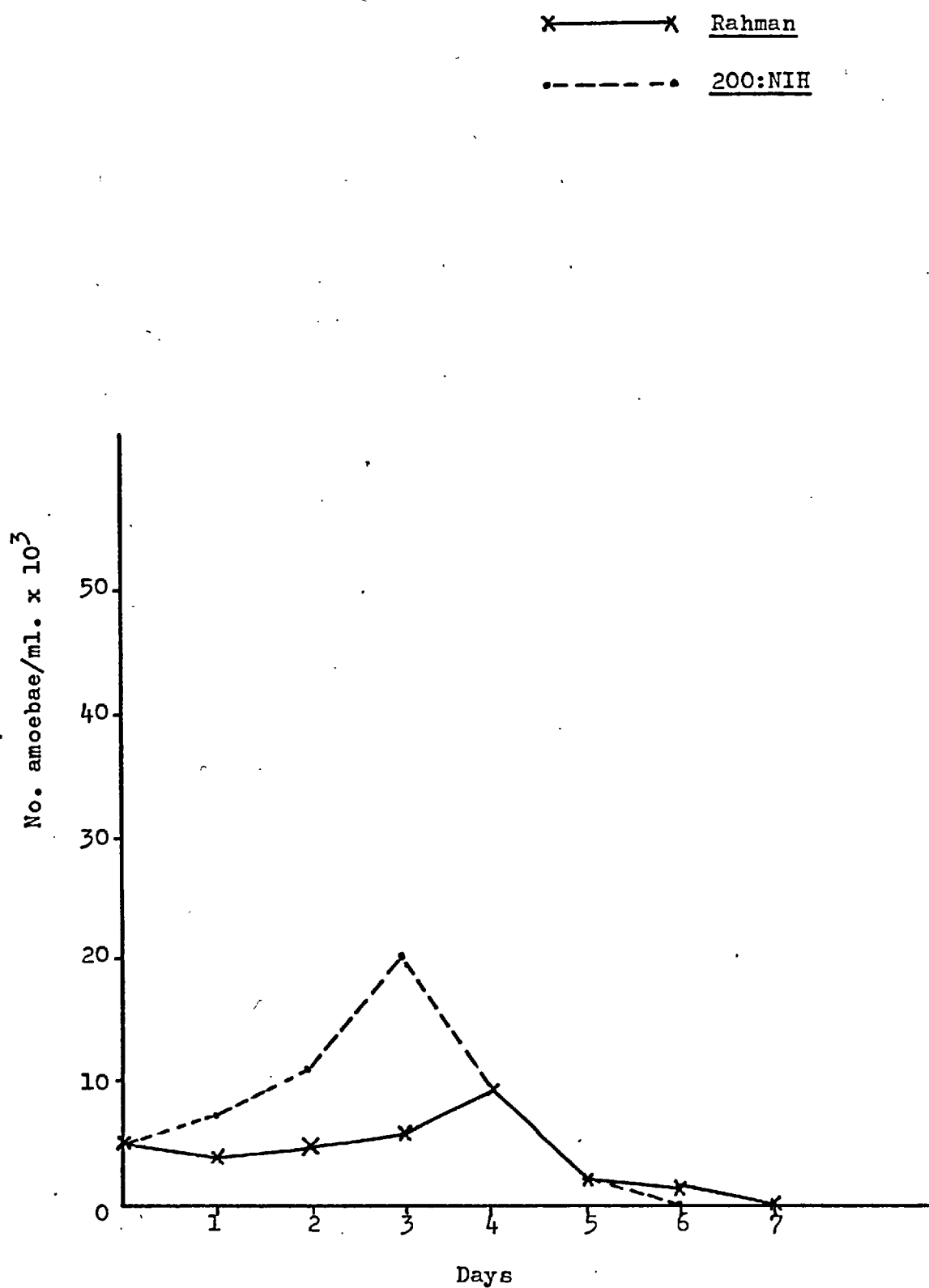
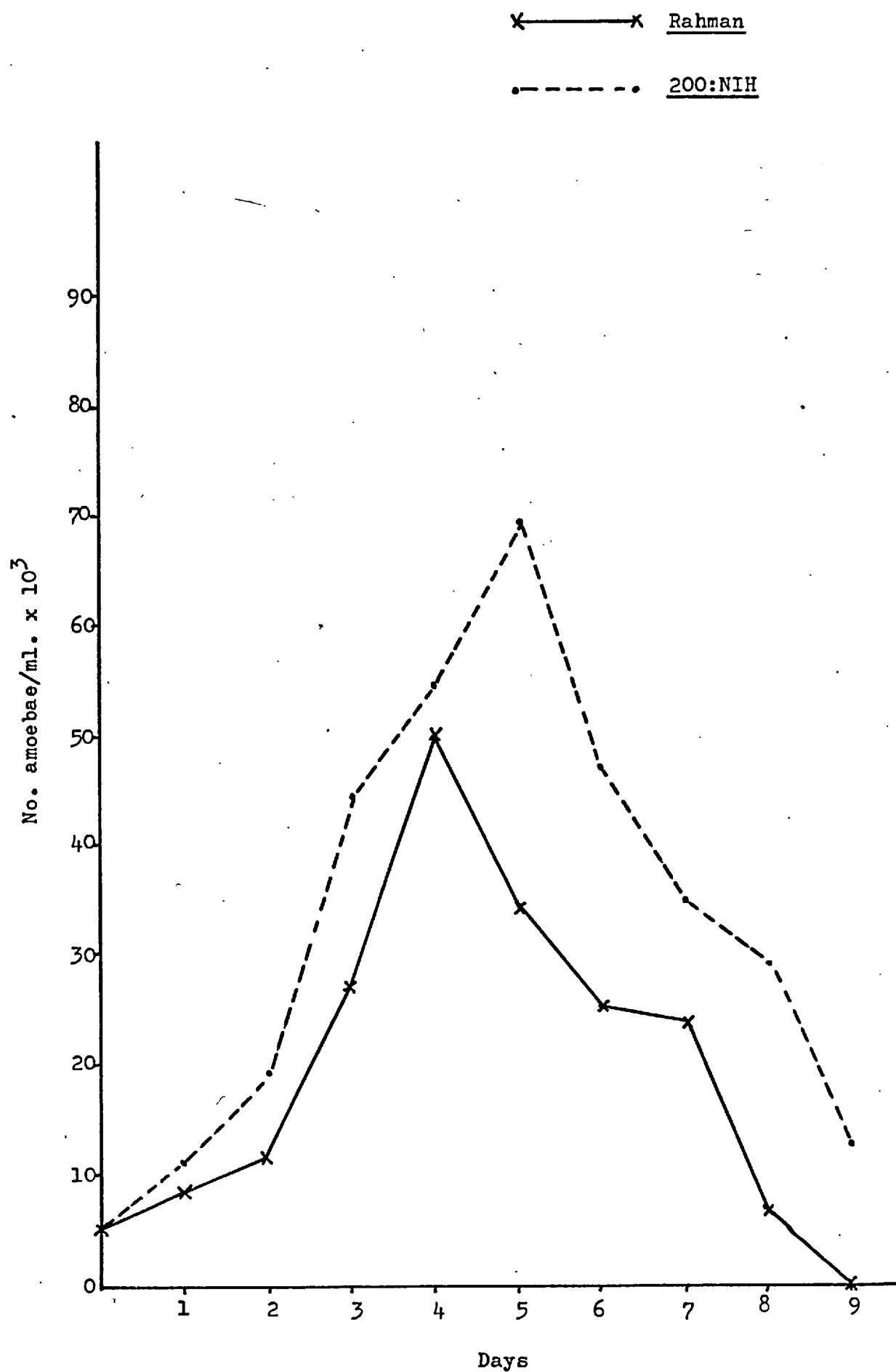


Figure 4 Monoxenic growth of E. histolytica with L. tarentolae (strain tar)



Discussion

Growth of E. histolytica in association with all the flagellate species tried was adequate. Variation between species was quite considerable and greater than that shown in strain variation of T. cruzi epimastigotes. Therefore it may be treated as being significant. Pan (1960) found far better support afforded by L. enriettii at 33°C instead of 35°C. This could be a reflection of temperature tolerance, and so the viability of the associates. Some flagellate associates were observed to be reasonably tolerant of the change in medium and temperature. Crithidia sp. was particularly tolerant and in fact grew excellently in the amoeba system. T. cruzi and L. tarentolae survive for three to four days morphologically normal. On the other hand L. enriettii and T. lewisi became morphologically degenerate within a day. A more detailed study of the precise correlation between amoeba growth and associate conditions has already been expounded in chapter 3 pp140. However these results also agree that E. histolytica requires living metabolising associates for good growth.

b) Various stages in life cycle of *T. cruzi*

Introduction

T. cruzi assumes different morphological forms during its life cycle. All these are more or less readily available in vitro by manipulation of the culture medium. The different stages, metacyclic trypanomastigote and amastigotes were compared with epimastigotes in their ability to support growth of *E. histolytica*. The only stage of *T. cruzi* not available from culture was the trypanomastigote stage and this was obtained from the blood of infected mice.

Experimental results of monoxenic cultivation with stages of the life cycle of *T. cruzi*

The methods used in the preparation of the *T. cruzi* associates and their use in the amoeba-trypanosome cultures are described in chapter 2. Under optimal conditions the same strain of *T. cruzi* would have been used throughout. However much greater ease was found in the development of metacyclic trypanosomes from strain M1 than Y, and was used accordingly, for this stage. So that a proper comparison may be made, results for the two strains of *T. cruzi* have been presented separately and epimastigote support has been included in each set of results. Two strains of *E. histolytica* were used, as in the last chapter, in order to dispel any doubts as to the validity of results being characteristic of *E. histolytica*.

Monoxenic isolates in association with all the various stages of *T. cruzi* were maintained without any difficulty for ten weeks.

Growth was observed to be normal during this time in all cases, but the concentration of amoebae in the cultures growing with blood trypanomastigotes appeared to be low. The growth curves of Rahman strain associated with Y strain T. cruzi epimastigotes, amastigotes and blood trypanomastigotes are plotted in figure 1. Growth with epimastigotes and metacyclic trypanomastigotes of M1 strain is plotted in figure 2. The results of similar experiments using strain 200:NIH of E. histolytica are described in figures 3 and 4. For both strains of E. histolytica very poor support was afforded by the blood trypanomastigotes. The presence of the red blood cells from the infected mouse blood could have had a toxic effect on the amoebae, but great care was taken to eliminate these cells. Growth with blood trypanomastigotes was not only poor in respect of yield but also in longevity, all amoebae being dead by 5 days in both strains. Peak growth at day 2 showed only a 4-5 fold increase in numbers.

Growth with amastigotes was comparable with that with epimastigotes. For strain Rahman better growth was obtained with amastigotes than epimastigotes and the reverse situation was seen for strain 200:NIH. Peak growth reached a day earlier in amastigotes associated cultures of both strains. Apart from the variance of peak growth, growth curves of E. histolytica with epimastigotes and amastigotes varied in Rahman strain by the absence of detectable lag phase, otherwise the growth curves were similar.

The same cannot be said for the growth of E. histolytica with epimastigotes and metacyclic trypanomastigotes of M1 strain. Growth with the metacyclic trypanomastigotes of M1 strain was of short

Figure 1 Monoxenic growth of E. histolytica (strain Rahman) with different morphological forms of T. cruzi (strain Y)

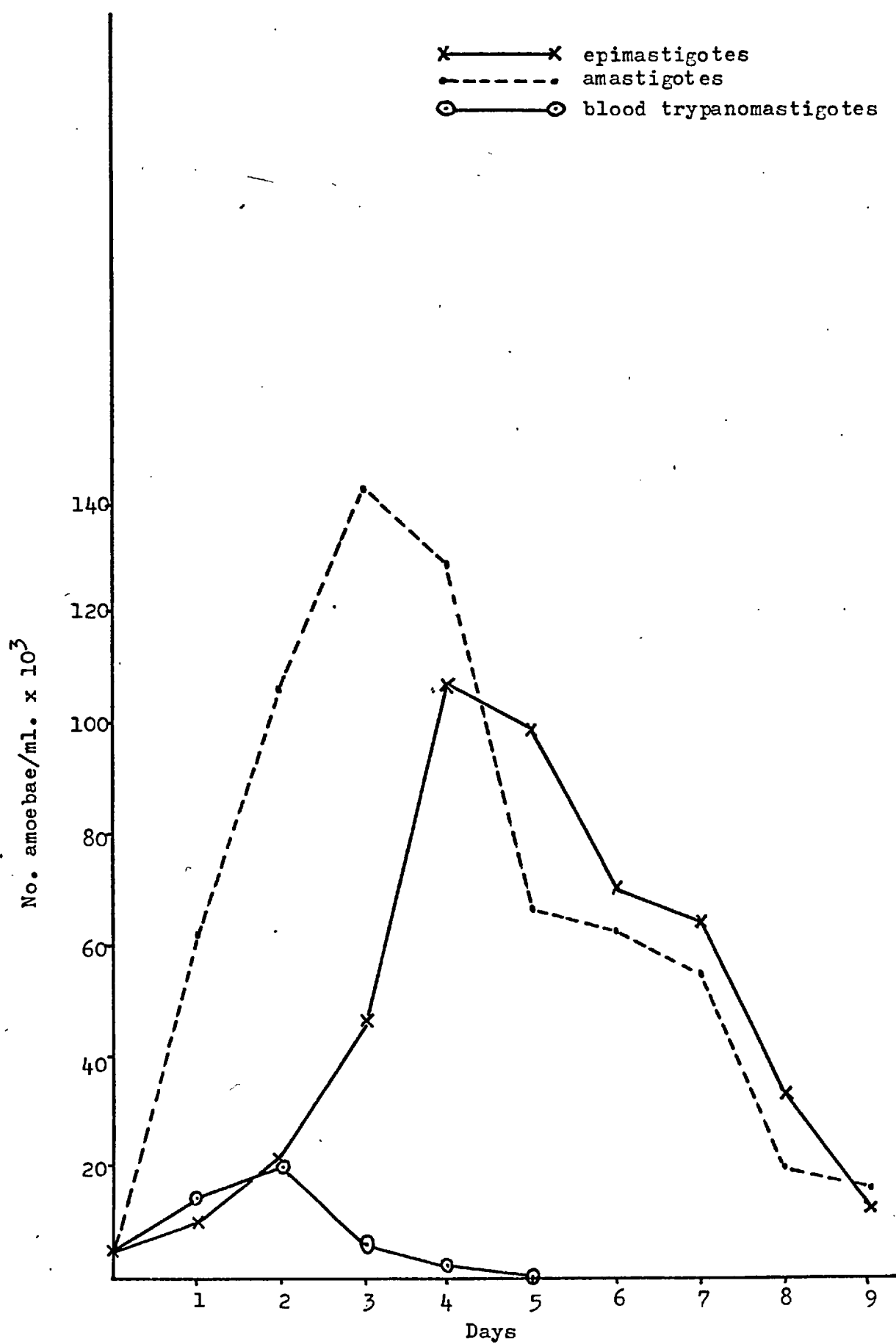


Figure 2 Monoxenic growth of E. histolytica (strain Rahman) with different morphological forms of T. cruzi (strain M1)

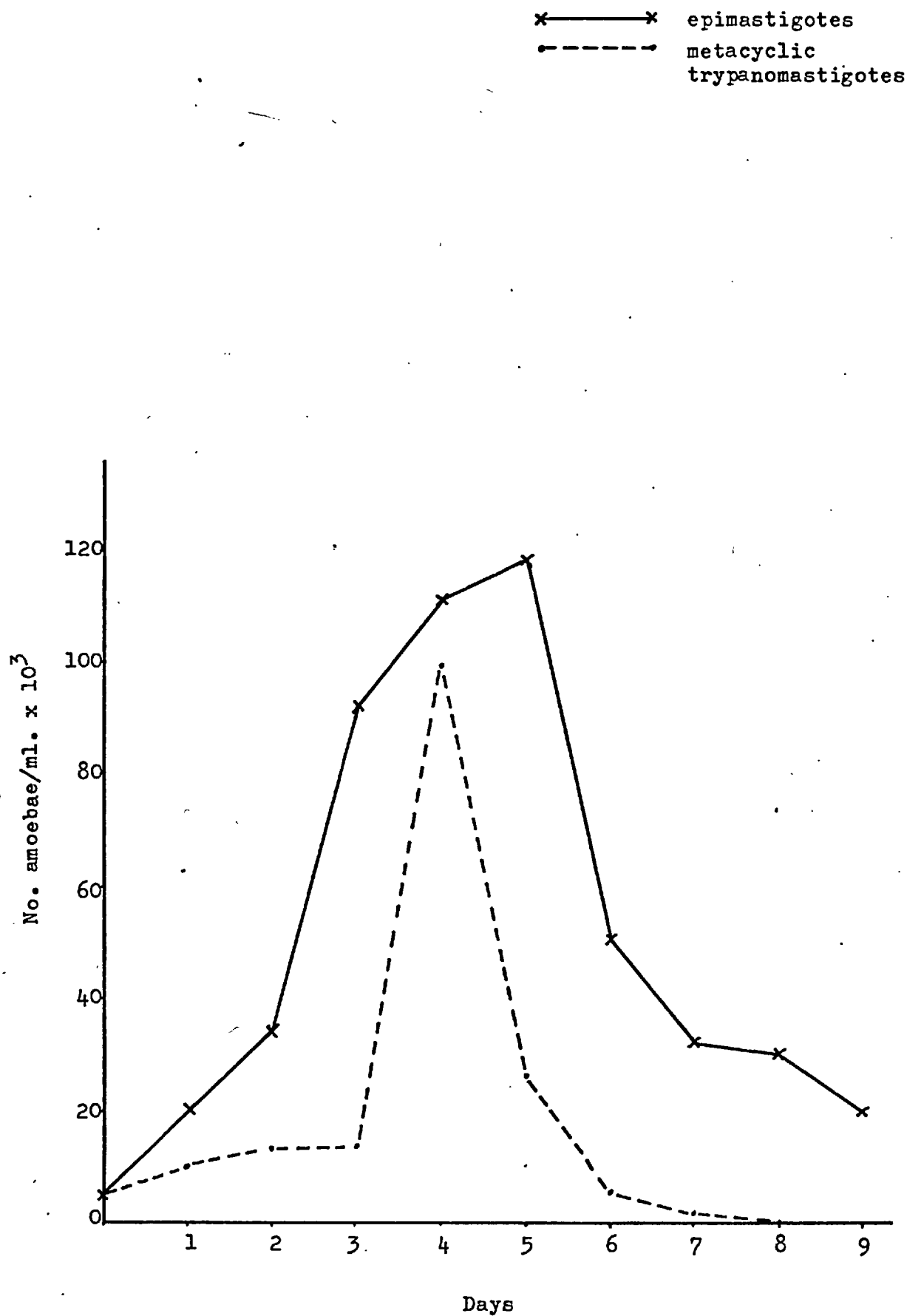


Figure 3 Monoxenic growth of *E. histolytica* (strain 200:NIH) with different morphological forms of *T. cruzi* (strain Y)

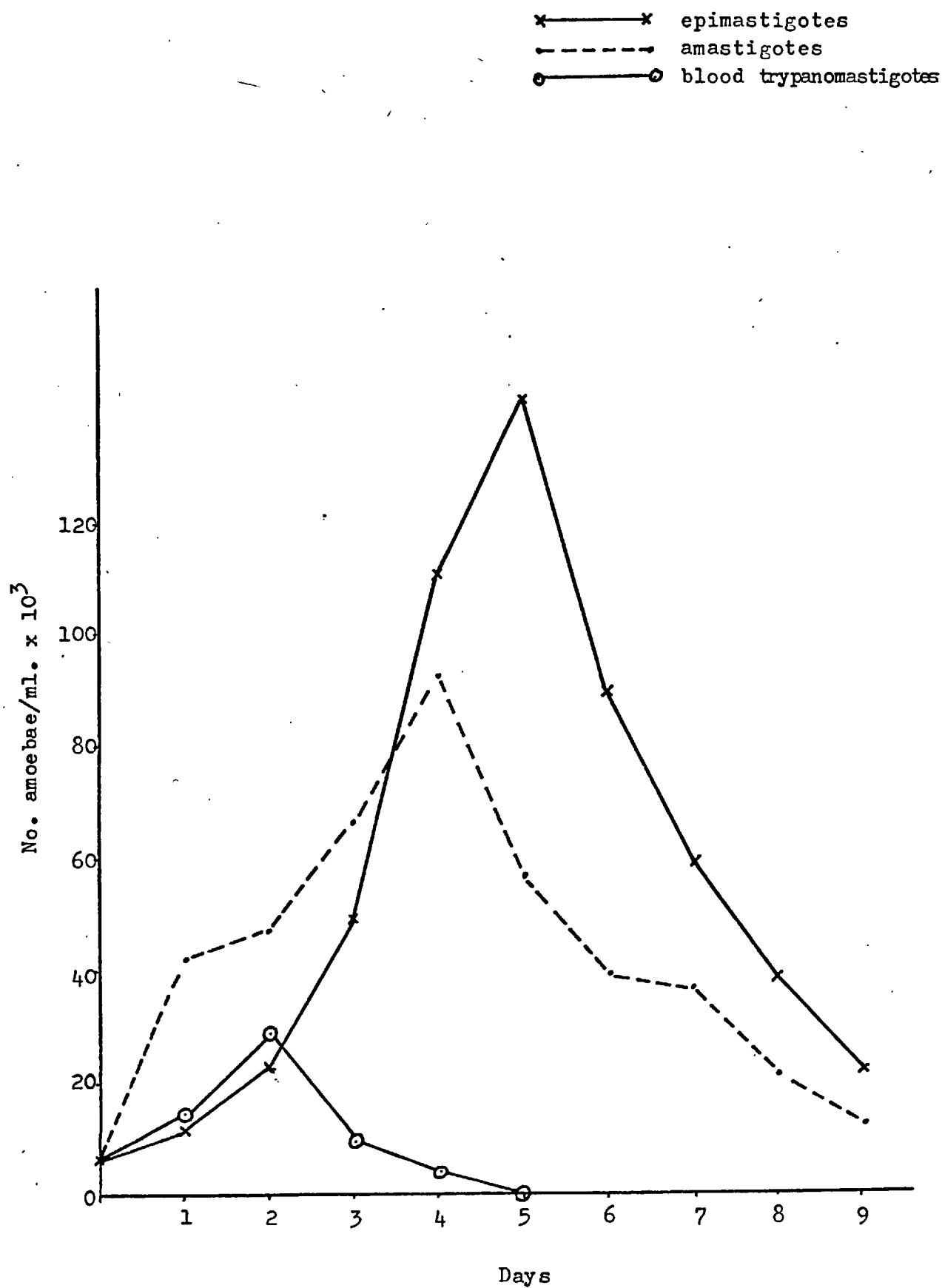
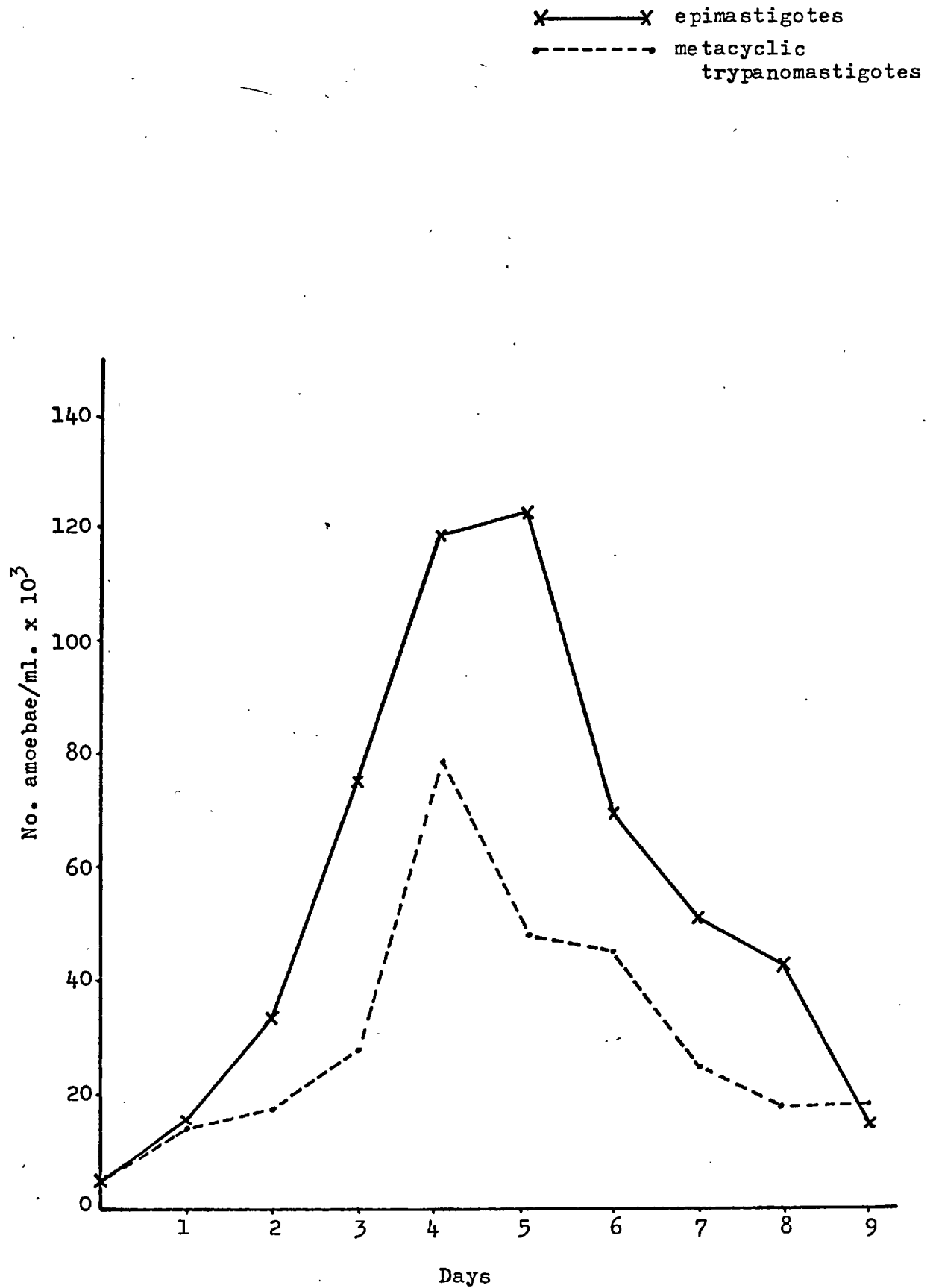


Figure 4 Monoxenic growth of *E. histolytica* (strain 200:NIH) with different morphological forms of *T. cruzi* (strain M1)



duration, particularly in the case of Rahman strain (figure 2). Again peak growth was attained one day early but this was accompanied by a very long lag phase. The decline in amoebic numbers, after peak growth, was also extremely marked, although numbers decreased less rapidly with strain 200:NIH (figure 4) than Rahman strain (figure 2).

In all cultures apart from the trypanomastigote associated cultures the associates were observed to remain active and appear morphologically normal for at least 3 days in the monoxenic system. In contrast, blood trypanomastigotes had degenerated and disappeared from the cultures by 24 hours. The amastigotes were exceptional in their survival in the amoeba system, healthy organisms still being seen 7-10 days after subculture.

Contrary to expectations the amoebae growing in association with the non-motile amastigotes were found in an even distribution along the length of the culture tubes. A degree of disturbance of the amastigotes would occur during the daily examination of the cultures, but this could not have accounted for the large quantities of amastigotes observed within the amoebic food vacuoles. It seemed likely therefore that there was a constant migration of amoebae up and down the walls of the tubes.

Discussion

The biochemical properties of each different morphological stage of the life cycle of T. cruzi will vary according to its function. It is known from electron microscope studies that the proportion of nuclear material is greater in amastigotes than in

trypanomastigotes. Considering that this stage is actively involved in division it is not surprising. The kinetoplast is highly developed in trypanomastigotes as this is an actively motile stage. The results of this work might be indicative of the lack of specificity of E. histolytica in its requirements of its associate. Perhaps the multiplying forms are more suitable because of the biproducts of this activity.

All forms developed by special cultivation techniques should be considered as such. However the metacyclic trypanomastigotes have the property of invasion and may continue their development in the normal fashion. This was shown by infection of tissue cultures of these forms. It was not possible to demonstrate the properties of the cultured amastigotes except by morphological examination. Since these are normally intracellular parasites it would be remarkable if they were exactly similar in culture and inside the host cell.

The results of amoebic growth with these different forms of T. cruzi might be confusing unless close observation of the associate state in the system is made, (see table 1). Both epimastigotes and amastigotes survived well in TP-S-1 medium at 37°C, especially the amastigotes. Both forms were still in an actively multiplying condition for two days. This was shown by haemocytometer slide counts made at the same time as amoeba counts. It is significant that although both of these forms reacted well within the system and had been grown in similar medium they are extremely different morphologically both in size and function. However their support

Table 1 Growth of T. cruzi associates in TP-S-1 at 37°C

Associate	Nos. organisms/ml. over period of days									
	0	1	2	3	4	5	6	7	8	9
Epimastigotes	2,000,000	2,900,000	794,000	327,000	9,180	5,560	3,330	0		
Amastigotes	2,000,000	1,890,000	3,100,000	467,000	211,000	58,900	7,890	5,560	6,670	3,330
Blood trypanomastigotes	2,000,000	13,000	8,890	7,780	0					
Metacyclic trypanomastigotes	2,000,000	2,300,000	1,890,000	16,700	5,560	0				

for E. histolytica was comparable. These results mean that E. histolytica requirements are not peculiar to the physiology of epimastigotes.

The metacyclic trypanomastigotes also survived for 3 days in the amoeba system but showed only degeneration and slight multiplication during this time. By three days they were markedly sluggish and apparently more attracted to the amoebae (see chapter 3 pp. 126). This could account for the prolonged lag phase since the amoebae were feeding later in these cultures and the slightly reduced peak numbers compared with epimastigote associated amoebae. However there is no obvious reason for the extremely rapid decline in numbers.

As has already been explained, the poor growth with blood trypanomastigotes may have been due to contaminating red blood cells. Shaffer and Iralu found that the initial contact of red blood cells with amoebae was toxic to the amoebae. (Shaffer and Iralu 1961).

One can conclude from the above results that E. histolytica growth can be supported by any morphological form of T. cruzi. However the type of growth is determined by the capacity of the associates to live within the system albeit for a short period.

c) Miscellaneous Associates

Introduction

The aim of this investigation was to show the diversity of organisms capable of supporting amoebic growth. Organisms chosen for potential association with E. histolytica, dealt with in this chapter, present a miscellaneous collection.

Some attempt has been made to test as wide a variety of morphological and metabolic properties as was feasible. The selection was limited by the maximum size of organism capable of ingestion by amoebae, and by their ready availability.

For convenience the experimental results will be dealt with under the following headings:-

- i. Algal associates
- ii. Non-flagellate protozoa
- iii. Tissue culture cells
- iv. Red blood cells

Experimental results of monoxenic cultivation with various organisms

i. Algal associates

Attempts were made to associate E. histolytica with four species of alga, which are as follows:-

Euglena gracilis

Ochromonas danica

Nannochloris coccoides

Ankistrodesmus convolutus

The methods used are described in chapter 2 pp.285,167. Growth was not successfully established with the two very small algae, Nannochloris and Ankistrodesmus. Amoebae survived in association with these organisms for only four days. This level of survival was similar

to that of E. histolytica without any associates, i.e. axenic.

In contrast, some degree of growth with Euglena gracilis and excellent growth with Ochromonas danica was obtained. The growth curves of cultures maintained by these associates are shown in figures 1 and 2. The initial concentration of Euglena present in experimental tubes was 2.0×10^6 /ml. These algae died fairly rapidly (2 days) when placed in a dark incubator at 37°C. Some difficulty was experienced during routine cultivation of cultures associated with Ochromonas since these organisms are facultatively photosynthetic and appear to be extremely temperature tolerant. They grow copiously in the dark in TP-S-1 medium at 37°C and obviously had a deleterious affect on the amoebae if they were present in sufficient quantities initially. Therefore the initial concentration of Ochromonas was considerably reduced. Growth of E. histolytica with two concentrations of Ochromonas was assessed in the growth studies, portrayed in figure 2. As seen in figure 1, cultures with Euglena were short lived and showed a very poor yield, which was attained early, on day 1 or 2. Growth with Ochromonas was almost comparable to that of cultures associated with epimastigotes of T. cruzi for both strains of E. histolytica used. Slightly better growth of 200:NIH and much better growth of Rahman strain was obtained with the higher concentration of algae, but as explained above this could not be increased without drastic effects. It is interesting to note that a very late peak growth was attained in most cultures. This characteristic was not observed

Figure 1 Monoxenic growth of E. histolytica strains with Euglena gracilis

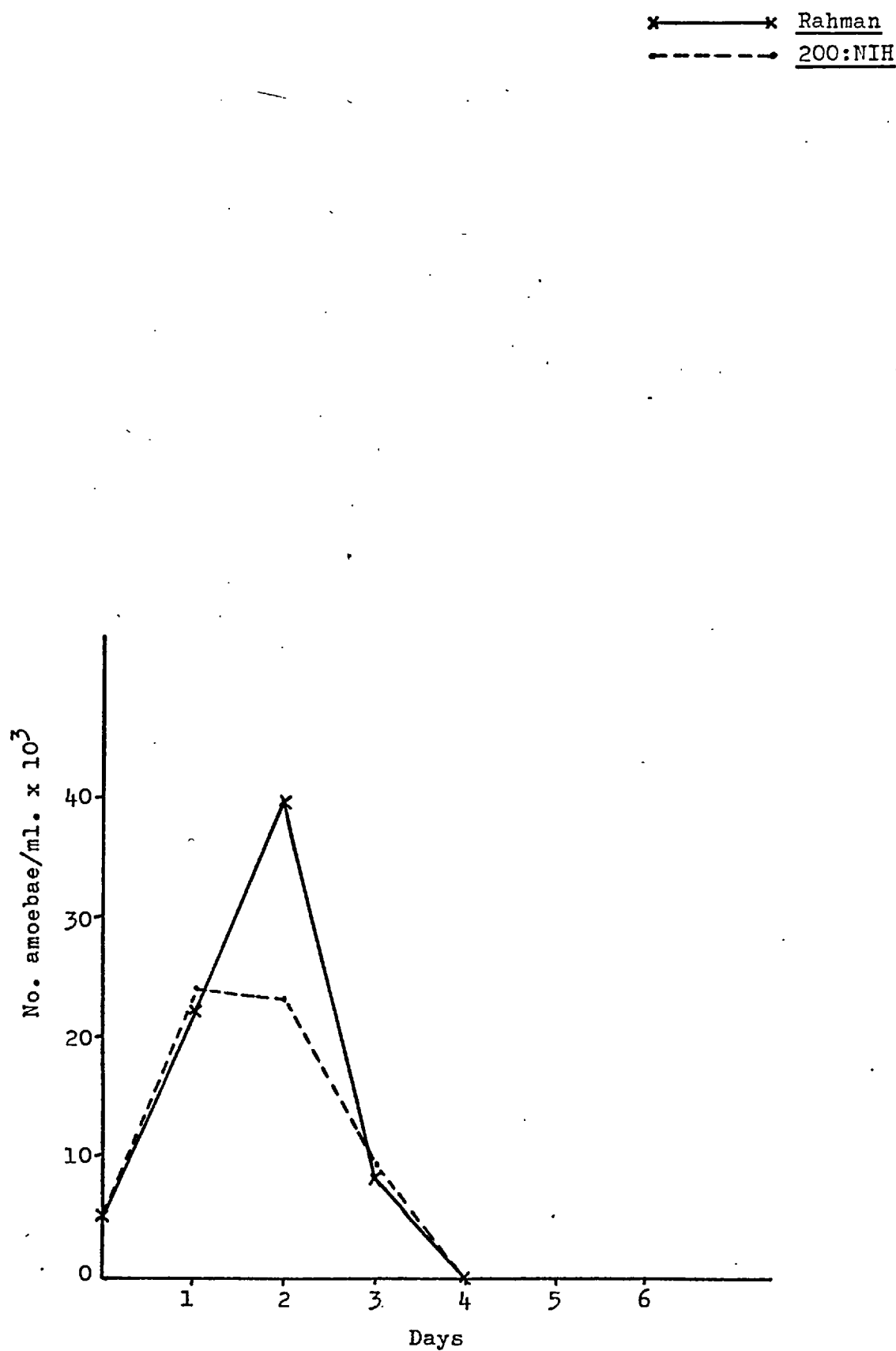
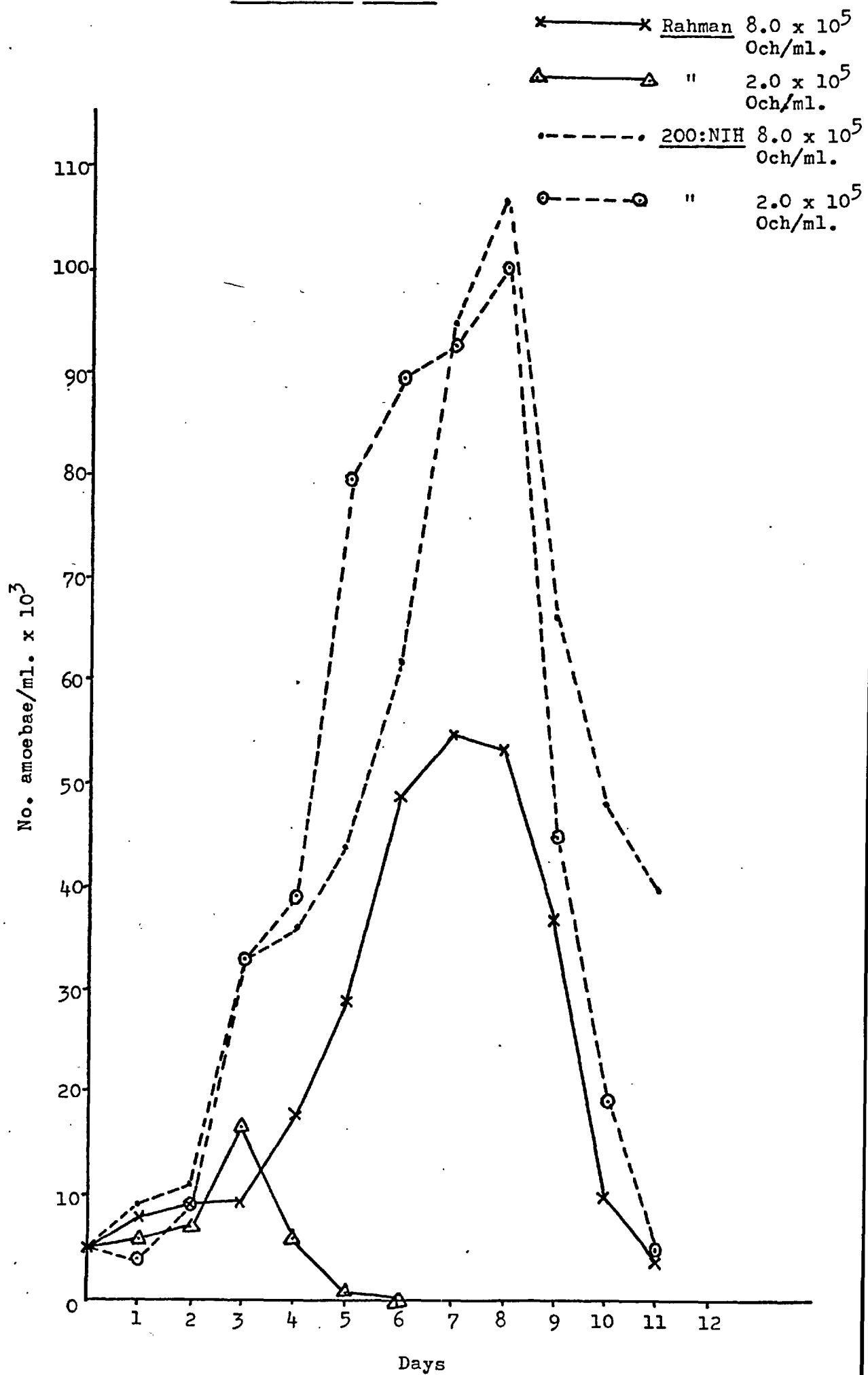


Figure 2. Monoxenic growth of *E. histolytica* with *Ochromonas danica*



in cultures associated with Crithidia sp. strain ReF-1, see figure 1, chapter 4a. Here peak growth was attained at day 5 for strain 200:NIH but a similar prolonged lag phase and marked culture longevity was observed. In consequence these cultures were remarkable for their longevity. There was a two to three day lag phase, rapid multiplication and rapid decline in numbers.

ii. Non-flagellate protozoa

This heading was chosen to include sporozoan and amoebic associates. The sporozoan associate was Eimeria tenella, the causative agent of coccidiosis in fowl. Both sporozoites and merozoites were used in attempts to support growth of E. histolytica and their preparation is described in chapter 2 pp. 31 . Although both stages of the life cycle used were engulfed by the amoebae no support was afforded by them. Cultures of amoebae with these organisms survived for 5-6 days which implied that some benefit was provided by the associates since this was two days longer than axenic survival.

The amoebic associate used was Hartmanella castellanii, strain R. This organism grew extensively in TP-S-1 medium at 37°C. It was observed within food vacuoles of E. histolytica. However sustained growth was not obtained with this amoebic associate. Cultures of E. histolytica survived for 5 days in its presence and thereby a slight degree of benefit must have been derived by the entamoebae.

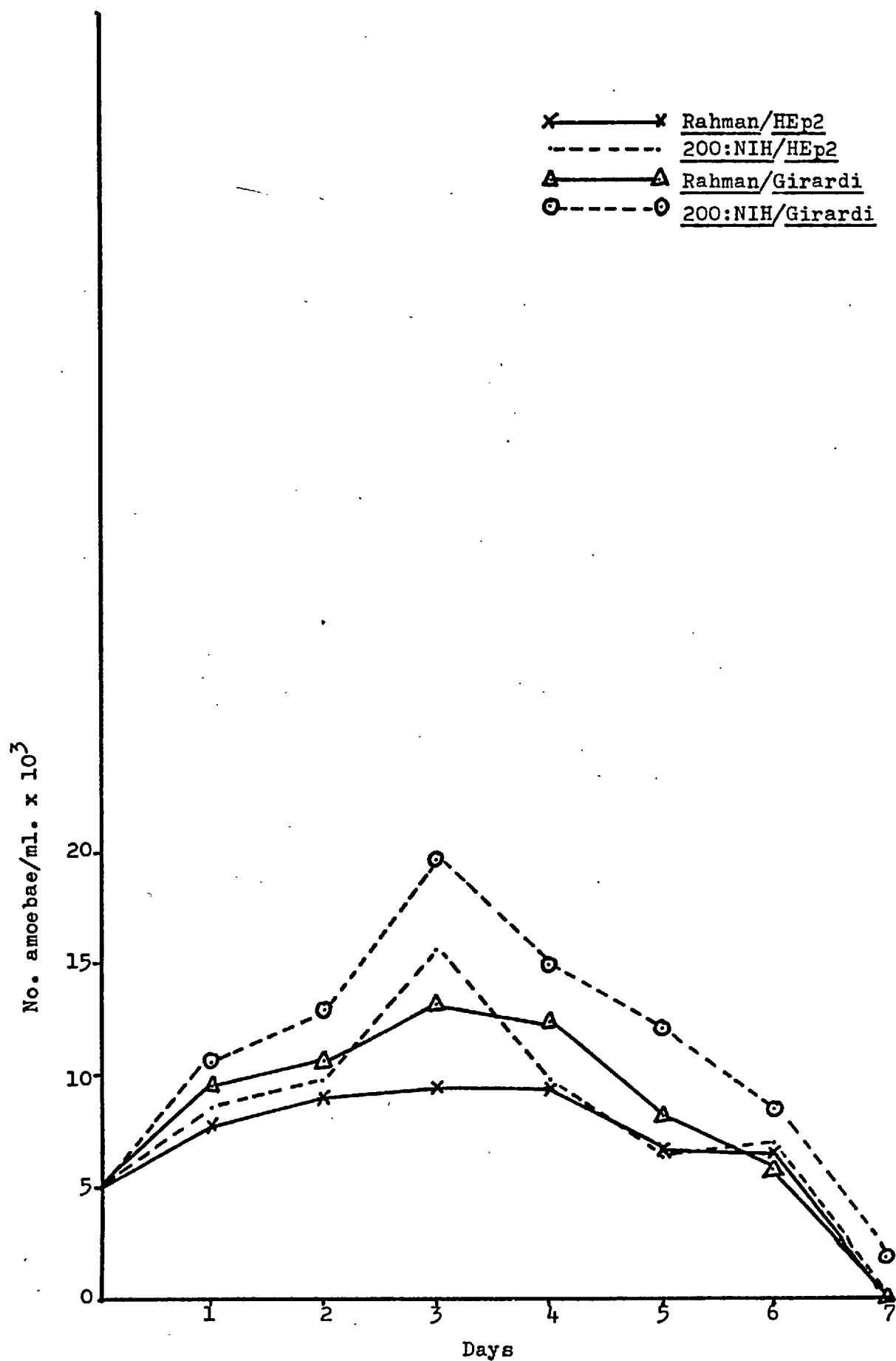
iii. Tissue culture cells

Two methods of cultivation were used with these associates. The first was to use a suspension of tissue culture cells separated

by trypsinisation. The second was the use of a fully grown tissue cell monolayer in situ by simple addition of the amoebae and TP-S-1 medium to a culture flask from which tissue culture medium had been discarded. Both methods and the strains of tissue culture cells used, are described in more detail in chapter 2 pp. Amoebic cultures associated with tissue culture cells were easily established by both methods of cultivation. In the second type of culture, amoebae were seen to flow over the tissue culture cells and engulf them avidly. Within twelve hours there were no tissue cells remaining. Probably growth using this system was better than that using the suspension system, but no accurate assessment of the initial concentration of associate could be made.

Growth curve experiments were conducted using the suspension method. The growth of two strains of E. histolytica with two strains of established tissue cell lines are described in figure 3. All cultures showed a reasonable degree of longevity but the yield was extremely poor. This appeared to be due to a very low level of multiplication rather than a prolonged lag phase. It was found that by adding tissue culture cells between subcultivation, a higher yield was obtained (2.8×10^4 amoebae/ml. in Rahman/Girardi cultures). Supplementing the numbers of initial associates was done routinely during maintenance. It is probable that E. histolytica would not have survived in association with tissue culture cells if this procedure had not been adopted. Although this technique was often tried with other apparently unsuccessful

Figure 3 Monoxenic growth of *E. histolytica* with tissue culture cells strains (HEp2 and Girardi)



associates, it was only worthwhile with tissue culture cells.

iv. Red blood cells

Red blood cells (RBC) from rabbit were used in these experiments. Their preparation and use in monoxenic cultivation is described in chapter 2 pp. 30.

The entamoebae were seen to engulf the RBC but no multiplication of the amoebae occurred. In fact survival in this situation was extremely short, being only three days. This was one day shorter than in axenic isolations, so the presence of the RBC was actually detrimental to the amoebae. As these results were contrary to the expected result, from the work of Shaffer & Iralu, and the in vivo situation in liver abscess and intestinal lesions, it was decided to follow up the work with further experiments. It was thought that by gradual association of the amoeba-trypanosome cultures with RBC using a far lower concentration than that used previously, over a period of several weeks, a degree of adaptation and tolerance might be developed by the amoebae to the toxicity caused by the RBC. Although a concentration of 1×10^2 RBC/ml. was found to be non-toxic to amoeba-trypanosome cultures, and apparently normal growth resulted for a period of ten weeks, it was neither possible to increase the concentration of RBC nor discard the trypanosomes without causing death of the amoebae. Therefore no tolerance to the RBC toxicity could be developed by this method.

One yeast, (found as a contaminant of an A-T isolate of Rahman) whose species was not known, was tried as a potential

associate. Growth continued for three weeks and thereafter declined until no amoebae were present at four weeks. Therefore no further attempts were made on yeasts.

Discussion

The aim of the work described above was to attempt to establish the diversity of suitable E. histolytica associates. Thereby some inference might be made to the specificity of the amoebic requirements in the monoxenic system. Since the amoebae have already been shown to require the presence of an associate that might be engulfed, a size limit was imposed on the range of associates. Taking this into consideration, effort was made to cover potential associates with as diverse a biology as possible. As the amoebae are heavily dependant on the associate it is logical to expect that whole organisms rather than part thereof would be more suitable, since they would be independant as regards their own metabolism.

Two large groups of organisms that fall within the size range, which were not included in the study, were bacteria and yeasts. Bacteria have been amply covered by other workers and apart from antibiotic-inhibited material, accurate quantitative assessment of the associate may not be made. The latter point also applies to yeasts. A detailed study of yeasts as potential associates was precluded as mentioned above.

Organisms whose metabolism might be considered to be very dissimilar to that of flagellates are algae. The failure of the smaller species to support the amoebic growth could be attributed to an unsuitable metabolism or possibly to the benefit derived being

less than the expenditure involved in engulfing the organisms. It is known that Euglena was in a dying condition when present in the amoeba monoxenic system. As such it is therefore not surprising that only poor amoebic growth occurred. The great success obtained with Ochromonas and the difficulty encountered due to the presence of too many associates is particularly interesting. Not only does this emphasise the importance of an actively metabolising organism but it also implies a similarity in the metabolism or perhaps the biochemical makeup of Ochromonas with protozoal flagellates, like T. cruzi and Crithidia sp. Hutner et al. suggested this with reference to the Crithidia factor and the similarity of folic acid requirements for Ochromonas malhamensis and Crithidia fasciculata (Hutner et al. 1957, Nathan et al. 1956). If E. histolytica requirements are highly specific, only one metabolic pathway and its products would be sufficient to cause this similarity. However the similarity cannot be continued too far since the maximum tolerated concentration of both T. cruzi epimastigotes and Crithidia sp. associates is considerably higher than that of Ochromonas (see chapter 4 pp 178).

It is not known why the non-flagellate protozoa did not support E. histolytica growth, but some suggestions may be made. Both sporozoites and merozoites of Eimeria tenella degenerated very rapidly, within hours, in the amoebic system and this fact may explain their lack of support. Both of these stages of the coccidian life cycle only exist in order to invade fresh host cells. They cannot continue their development extracellularly. Therefore as

they are incapable of metabolising as separate entities themselves, they are unlikely to be capable of supporting another organism. The same cannot be said of Hartmanella castellanii. This organism continued to grow in the amoebic system and was engulfed by the entamoebae. It might well have been competing with the entamoebae for nutrients. Unsuitable metabolism or toxic products are also feasible explanations of these results.

Growth of E. histolytica with tissue culture cells is significant in several aspects. Established tissue culture lines living in defined media may be regarded as the most undifferentiated or characterless living cells available in the laboratory. However the necessity of serum supplement to their medium complicates the situation somewhat. Undoubtedly established cell lines have lost many of the characteristics of the primary explant tissue and thus may be said to represent a "basic cell". Therefore by implication the E. histolytica requirements of its associate are not peculiar to flagellates or any other type of metabolism, but the picture is confused by availability and the effect of other substances present. The fact that additional supplies of tissue culture cells, between subculture, enhanced amoebic growth implies that the amoebic requirements were present in only limited quantities. It is probably fortuitous that better amoebic growth was encountered with Girardi cell line than with HEp2 line of tissue cells. This type of E. histolytica isolate might have significance for virulence studies, if it could be cultured in sufficient quantities.

Failure of RBC to support E. histolytica was undoubtedly due to their toxicity. Shaffer & Iralu (1961) encountered this toxic effect in their experiments supplementing CLG medium and Bacteroides associates with RBC. The monoxenic amoeba-trypanosome amoebae have already been shown to be less resilient to adverse conditions than polyxenic associated amoebae. Their inability to overcome the effects of the RBC, even during a period of adaptation might be due to the amoebae's increased sensitivity to suboptimal conditions. It was never possible to establish the capacity of RBC for amoebic support but the work of Shaffer and Iralu suggests that they would only be suitable as a supplement rather than associate.

CHAPTER 5 VARIATION OF THE SYSTEM : AMOEBAE
OTHER THAN E. HISTOLYTICA

Introduction

The work so far has only dealt with Entamoeba histolytica. This was because the most rational approach was considered to be to design and evolve a system providing optimal conditions for one organism first. Then the system can be tested on other related organisms with any adaptations that might appear necessary.

Of the most closely related organisms, other entamoebae, five species were used. E. hartmanni is particularly similar to E. histolytica morphologically but is considerably smaller, the trophozoite having a diameter of 5-11 μ as compared with 20-40 μ for E. histolytica, and the cyst having a diameter of 5-10 μ as compared with 12-15 μ for E. histolytica. It is considered that E. hartmanni is entirely confined to the gut lumen and therefore nonpathogenic to man although often found in cases of amoebic dysentery caused by E. histolytica.

Another human parasitic Entamoeba used was E. coli. The E. coli trophozoite is large, 15-40 μ in diameter, and, although fairly similar to E. histolytica, it differs by having a large eccentrically located karyosome. In living specimens E. coli is more sluggish in its movements than E. histolytica. Cysts are generally large, 10-30 μ in diameter, and when mature are easily distinguished from E. histolytica as they contain 8 nuclei instead of 4. The life cycle of E. coli contrasts with E. histolytica by the absence of nuclear division in the metacystic stages. Eight uninuclear amoebulae are produced by cytoplasmic divisions. Although the production of the tetranuclear cyst of E. histolytica is similar to the first stage of schizogony the analogy cannot be carried

further. During the production of amoebulae cytoplasmic division keeps pace with nuclear division, contrasting with the segmentation of cytoplasm after nuclear division in schizogony. E. coli presents an interesting anomaly in this respect.

The entamoebae, referred to above, are all parasitic in homeotherms. Parasites of poikilotherms that were tried in the culture system comprise E. invadens, and E. ranarum. Both of these closely resemble E. histolytica. Both have large trophozoites, E. invadens average 15.9 μ in diameter and E. ranarum 10-15 μ in diameter. Cysts are generally tetranuclear although those of E. ranarum are sometimes multinuclear, containing up to 16 nuclei, as often seen in other entamoebae. E. invadens is very actively motile. It is parasitic in various reptiles and is pathogenic to snakes infected experimentally, kept at 25° C causing lesions in stomach, duodenum, ileum, colon and liver. There is doubt as to whether this parasite is pathogenic in nature. E. ranarum, a parasite of various species of frog, is reported to have caused an amoebic abscess of the liver.

The only nonparasitic member of the genus, E. moshkovskii, was also tried in the A-T system. Again it is very similar to E. histolytica with trophozoite diameter of 11-13 μ and a tetranuclear cyst, diameter 7-17 μ . It has been isolated from sewage in most areas on most continents. It contrasts with E. histolytica in being extremely tolerant to a wide range of pH, temperature and tonicity. Neal (1953) was unable to produce any experimental infection in guinea-pigs, rats or frog tadpole with this organism.

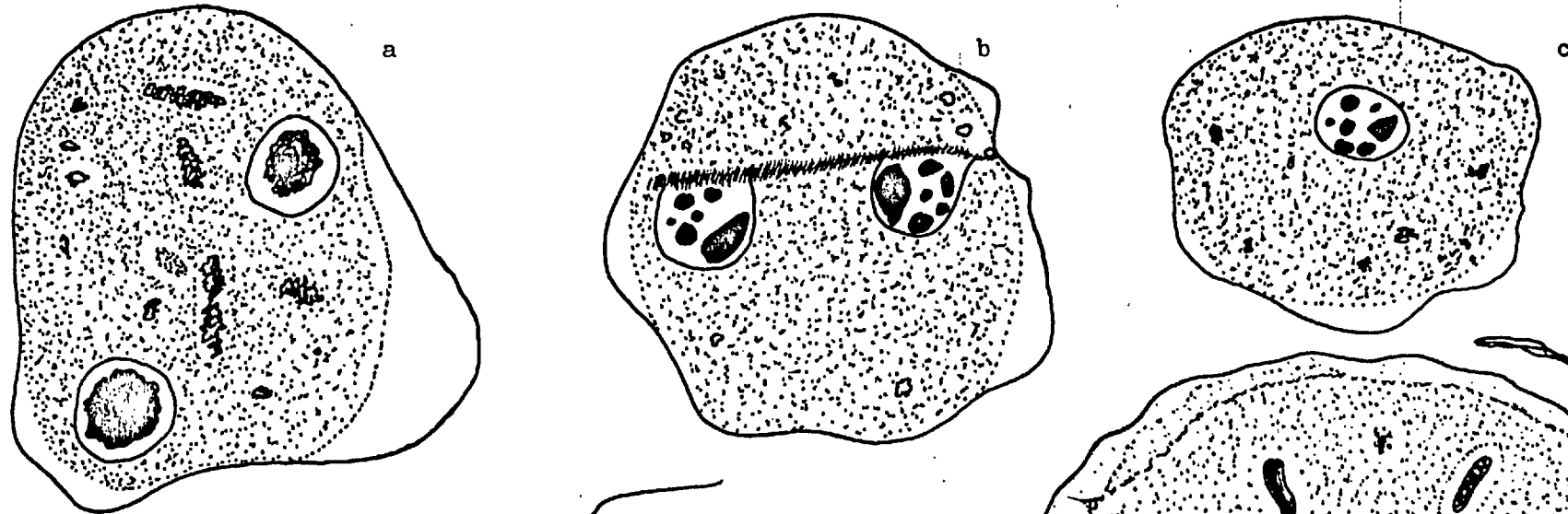
The genus Dientamoeba includes small amoebae which are very often binuclear. The trophozoite of D. fragilis is 5-12 μ in diameter. The nuclei have a vesicular structure, each containing 6

chromatin granules. During binary fission the binuclear amoeba gives rise to 2 uninuclear amoebae, having a large karyosome and peripheral chromatin granules lining the nuclear membrane. An incomplete mitotic division (arrested at telophase) produces the binuclear condition. This parasite lives in the lumen of the human colon and is considered to be non-pathogenic. Dobell, comparing this amoeba with Histomonas meleagridis, suggested that D. fragilis might be an aberrant flagellate similar to Histomonas, (see figure 1 for comparison).

According to the classification used by Kudo (1966), the family Endamoebidae belong to the order Amoebida, subclass Rhizopoda, class Sarcodina. Histomonas of the family Mastigamoebidae, belongs to the order Rhizomastigida, subclass Zoomastigida, class Mastigophora. One might therefore expect D. fragilis and H. meleagridis to be quite dissimilar. However they possess in common a circular shaped nucleus with large karyosome, the amoeboid shape and locomotion and of course food vacuoles with similar contents. Cysts have not been observed in either species. H. meleagridis, like D. fragilis is small, the trophozoite having a diameter of 8-12 μ . They contrast in the occurrence of some binuclear trophozoites in D. fragilis and the frequent possession of fine flagellae arising from a blepharoplast close to the nucleus by H. meleagridis. H. meleagridis is a parasite of gallinaceous birds, in particular young turkeys where it is responsible for the enterohepatic disease known as "blackhead". In accordance with the close similarity of these organisms, both were tried in the A-T system. Although Histomonas is an amoeb-flagellate it will be referred to as an amoeba in the following text.

All but one of the amoebae mentioned above may be cultured,

Dientamoeba fragilis (adapted from Hoare 1949) a & b binuclear forms c, uninuclear forms



Histomonas meleagridis
(adapted from Kudo 1966)

a, amoeboid form

b, large individual
from pheasant



Figure 1

without great difficulty, in conventional media in association with a mixed bacterial flora, in a similar manner to E. histolytica. However, workers agree that some difficulty is experienced in the maintenance of E. coli cultures. There is no obvious explanation for this. As mentioned earlier (chapter 1) monoxenic cultures of E. invadens, E. terrapinnae and E. moshkovskii have been isolated. Diamond prepared axenic cultures from the above monoxenic cultures and H. meleagridis was cultured in the absence of bacteria in association with slices of hamster liver by Lesser (1961). Reports of monoxenic or axenic cultures of the other entamoebae have not appeared in the literature.

This chapter is divided into three subsections. The first subsection reports the result of attempts to establish the other amoebae in monoxenic culture association. The second and third subsections pertain to the particular preferences of the amoeba species, both chemical and biological. As the comparison of the precise conditions for all the amoeba species used, was unlikely to yield outstanding differences, this method was not used. Instead, one species, E. invadens, was used extensively to confirm the suitability of the conditions evolved using E. histolytica. Obvious variations between species requirements such as temperature, and serum concentration in the case of E. moshkovskii were investigated in their own right.

a) Attempts at establishment of monoxenic cultures

Introduction

This subsection deals exclusively with attempts at monoxenisation of various amoeba species related to E. histolytica. Specific preferences in cultural conditions are described in subsections 5b and 5c.

Entamoeba species that are parasitic in poikilotherms and the free living species are naturally cultured at room temperature, 25°C. As a consequence of this, cultural techniques involved in maintenance of these species differ slightly from those used in the culture of species parasitic in homeotherms. Since the associates, used in the monoxenic systems described here, also grow at room temperature, it might be expected that reaction to monoxenization may be particularly interesting. In fact it could be argued that the system is not the same.

For convenience results with species of amoebae used were divided into two groups:-

i.) Entamoeba species parasitic in poikilotherms and free living species.

ii) Amoeba species parasitic in homeotherms.

Experimental results of monoxenic cultivation

Monoxenisation attempts for all species tried, started using the basic amoeba-trypanosome system, developed and described in chapter 3. If unsuccessful, further attempts were made using alternative associates as described in chapter 4. Successful attempts were not followed by repetition using other associates,

except in the case of E. invadens, as this was deemed unnecessary. The further study involving E. invadens will be described more thoroughly in subsections 5b and 5c.

i. Entamoeba species parasitic in poikilotherms and free living species

The two Entamoeba species parasitic in poikilotherms used in this group were E. invadens, strain BAH, and E. ranarum, strain R. Strain DC of the free living E. moshkovskii was also used.

Initiation of amoeba-trypanosome cultures and their maintenance was carried out as described in chapter 2, pp 48 using epimastigotes of Kessel strain of T. cruzi. Axenic isolations were also attempted at the same time.

The success of attempts at monoxenisation of all the various amoeba species tried, are described in table 1. As success, in terms of growth, was not obtained with some other species survival times also have been recorded. As already mentioned in chapter 2b, growth was considered to occur if a culture could be maintained for at least ten subcultures. All the strains included in group i were easily isolated in amoeba-trypanosome association and could be maintained for an apparently indefinite period of time. All three species are still being maintained with weekly subcultures after varying periods as follows:

<u>E. invadens</u>	1 year 7 months
<u>E. ranarum</u>	4 months
<u>E. moshkovskii</u>	4 months

Table 1 Results of attempts at monoxenisation of various amoebae

AMOEBA

	A S S O C I A T E													
	Axenic		<u>E. cruzi</u> epimastigote		<u>T. cruzi</u> amastigote		<u>Crithidia</u> sp.		<u>E. gracilis</u>		<u>O. danica</u>		<u>N. coccoides</u>	
	S	G	S	G	S	G	S	G	S	G	S	G	S	G
<u>E. invadens</u> Strain <u>BAH</u>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<u>E. ranarum</u> Strain <u>R</u>	+	+	+	+	NT		NT		NT		NT		NT	
<u>E. moshkovskii</u> Strain <u>DC</u>	+	+	+	+	NT		NT		NT		NT		NT	
<u>E. coli</u> Strain <u>Tooti</u>	NT		6	x	NT		NT		NT		NT		NT	
Strain <u>Said</u>	NT		6	x	NT		NT		NT		NT		NT	
<u>E. hartmanni</u> Strain Chattergee	4	x	8	x	10	x	7	x	3	x	1	x	5	x
<u>D. fragilis</u> Strain <u>Gokul</u>	5	x	8	x	12	x	8	x	4	x	2	x	5	x
<u>H. meleagridis</u> Strain <u>382III</u>	4	x	6	x	7	x	5	x	4	x	1	x	4	x
Strain <u>Ft</u>	5	x	7	x	8	x	6	x	4	x	1	x	5	x
<u>E. histolytica</u> Strain <u>Rahman</u>	4	x	+	+	+	+	+	+	+	+	+	+	4	x

+ = Success
x = Failure

S = length of survival days
G = growth

Growth occurring during the monoxenisation initiation phases of these species is described in figures 1, 2 and 3. These figures also include initiation of axenic isolations. Immediate success in establishing amoeba-trypanosome cultures was attained on the first attempt with all three species, using a normal size original inoculum of amoebae (10×10^3 amoebae/ml). Multiplication took place straight away and the growth curves presented for the first six days of monoxenisation initiation are very similar to normal growth of these amoebae in mixed bacterial cultures in HSre+S medium, see figures 4, 5 and 6. As seen in these figures peak growth was attained at the same time with E. ranarum and E. moshkovskii and was 1 day earlier in monoxenic initiation of E. invadens. After peak numbers of amoebae were seen, the numbers of amoebae all decreased for two days at the end of which the first subculture took place. The drop was not so marked as that seen with E. histolytica strains (see Chapter 3d pp 153). The monoxenic system was apparently established very rapidly as seen by the familiar alternating cycles of multiplication and decline.

It was particularly interesting to note the ease with which axenic cultures were established of these three strains. The growth during the first week in axenic culture was considerably better than that which followed. However the series of multiplication and decline cycles was set up in all cases. Although axenic cultures were established the type of growth seen even during initiation was very poor in comparison with the monoxenic cultures.

Figures 4, 5 and 6 portray the growth curves of various type cultures of each species. In all cases the monoxenic growth and growth with a mixed bacterial flora was quite similar both in type of multiplication and quantity. The axenic growth was outstandingly poor in terms of quantity. In the cases of E. ranarum and E. moshkovskii attainment of peak growth was delayed by one day in the monoxenic cultures. This must have been caused by the slight

Figure 1. Initiation of monoxenic culture of E. invadens in TP-S-1 with Kessel strain of T. cruzi

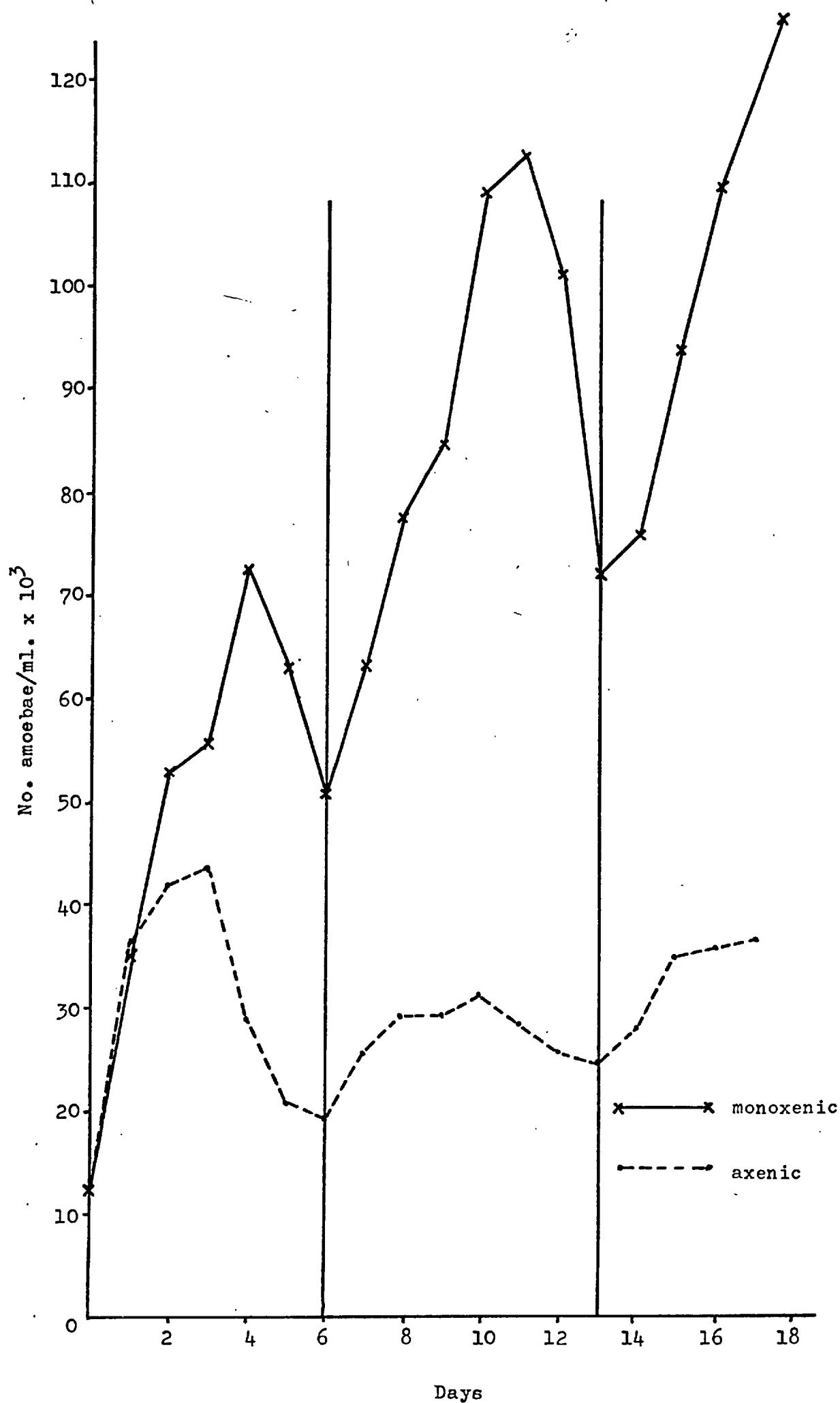


Figure 2 Initiation of monoxenic culture of E. ranarum in TP-S-1 with T. cruzi (strain Kessel)

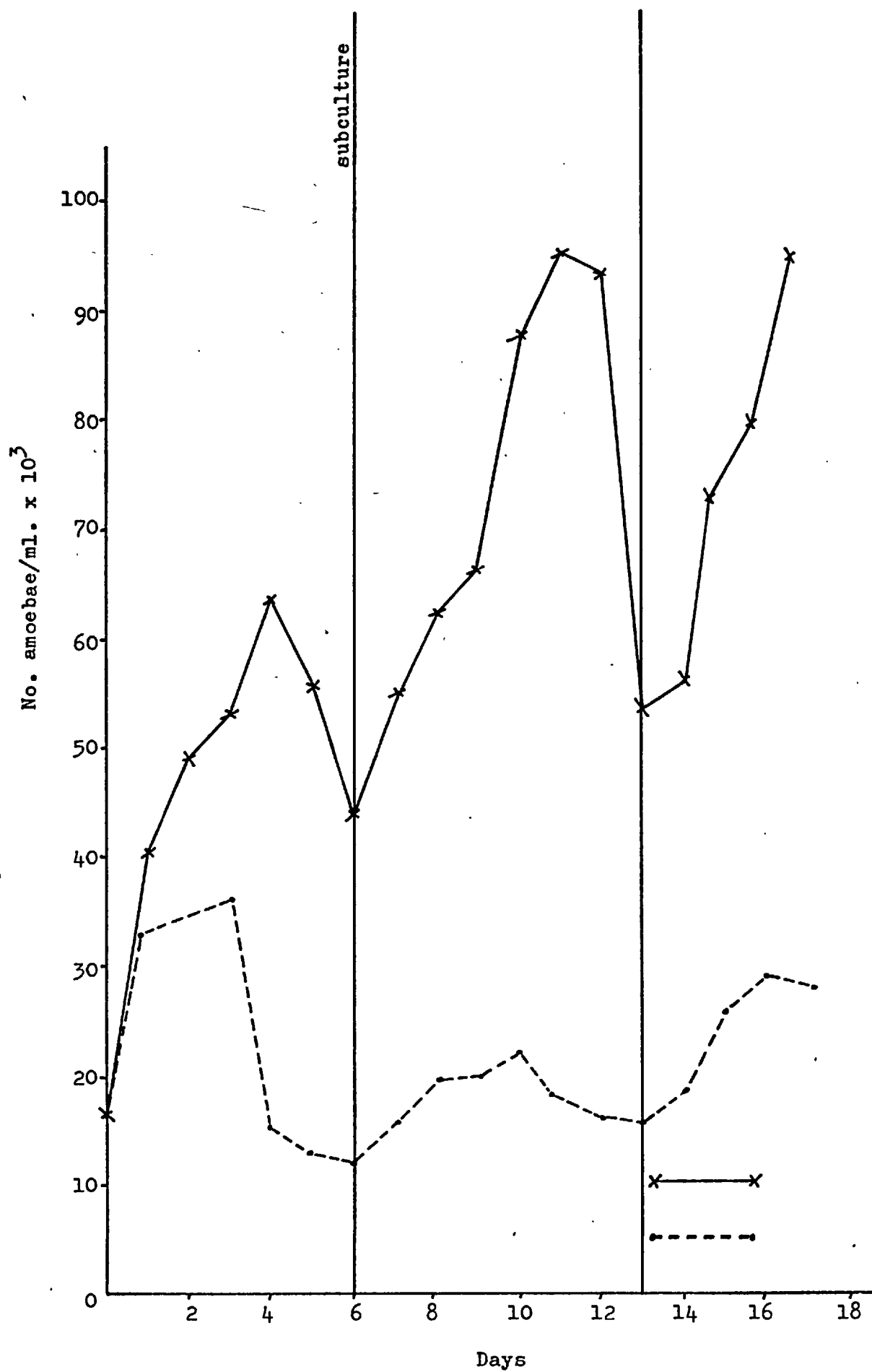


Figure 3 Initiation of monoxenic cultures of E. moshkovskii in TP-S-1 with T. cruzi (strain Kessel)

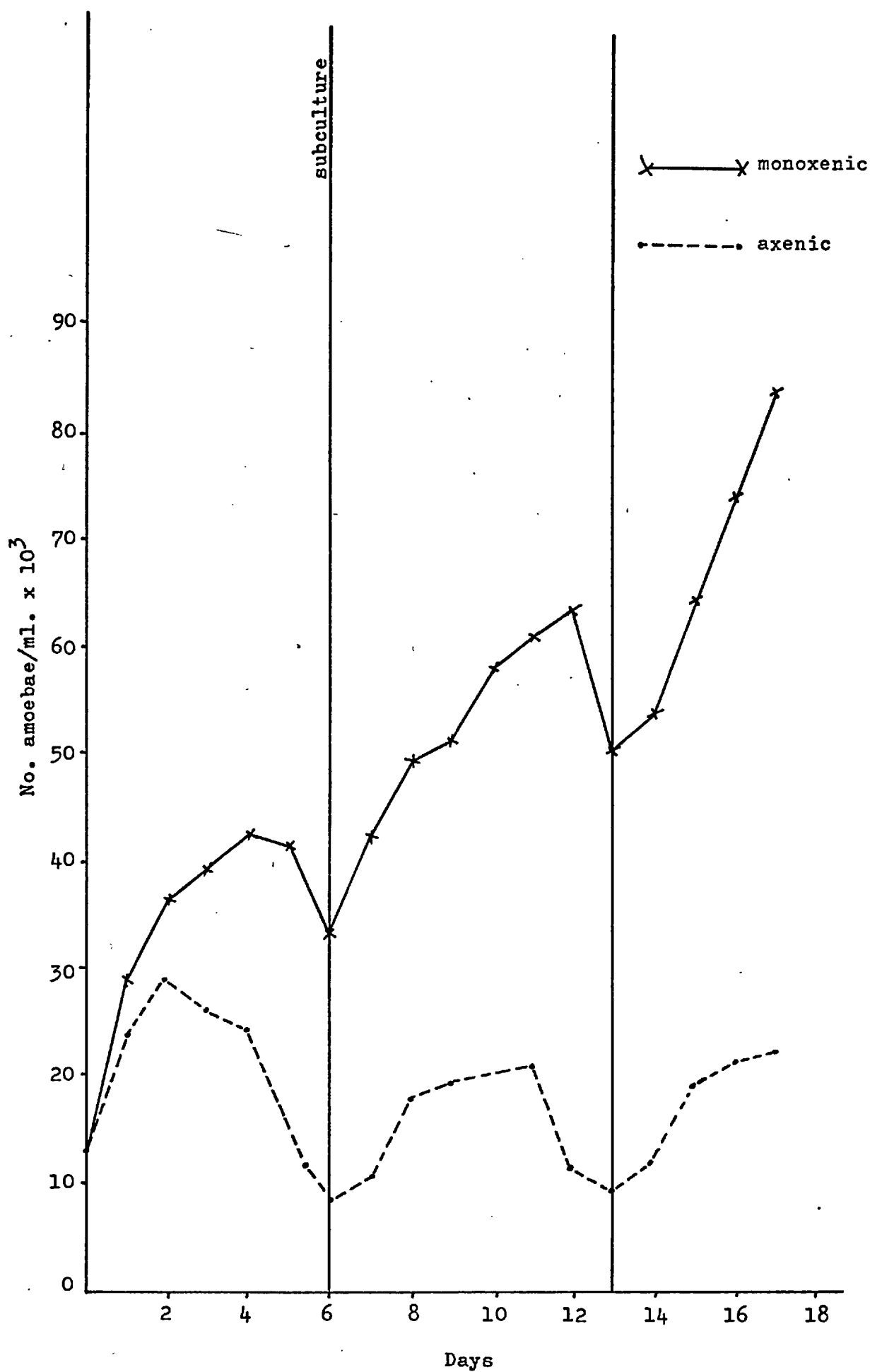


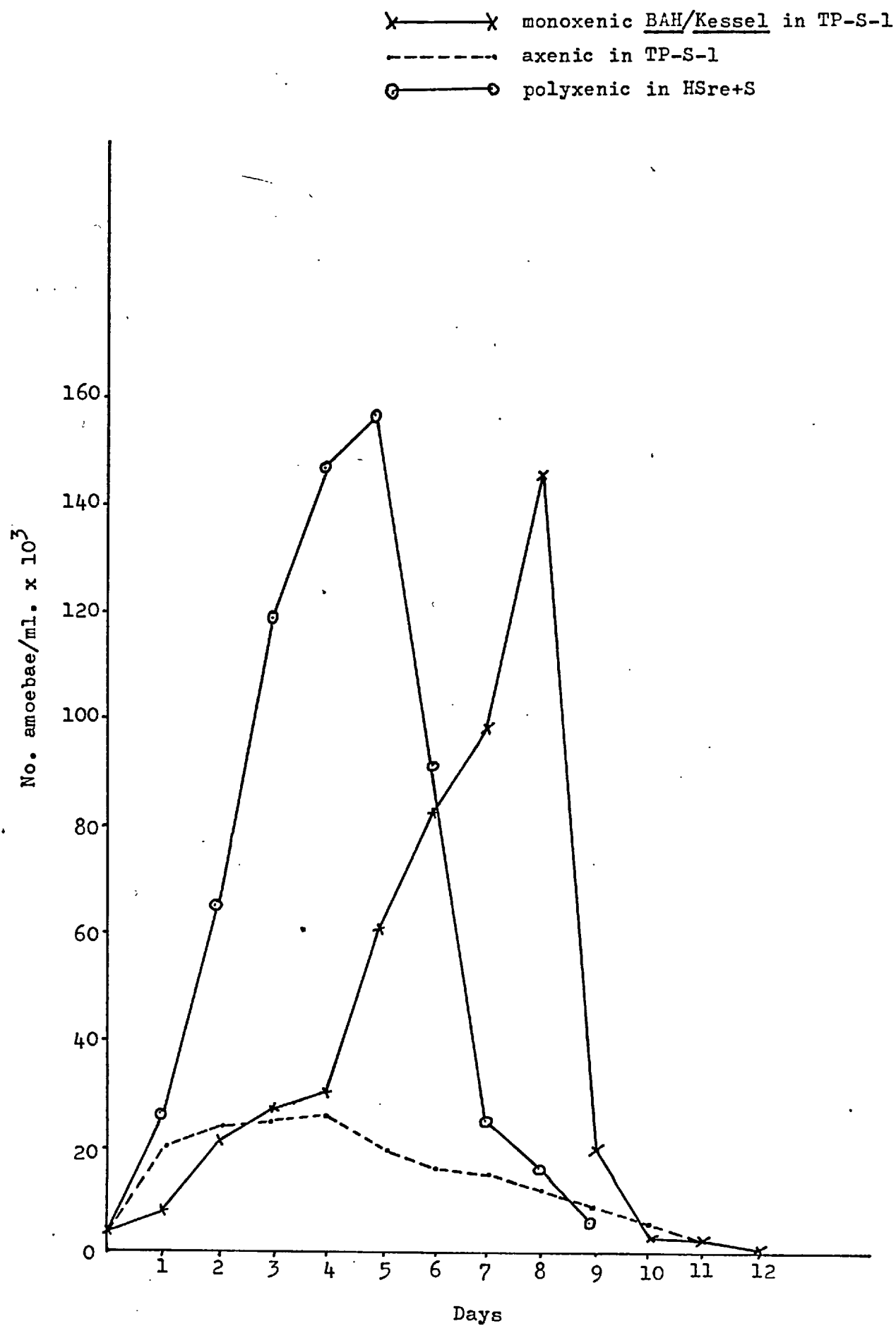
Figure 4 Growth of *E. invadens* (strain BAH)

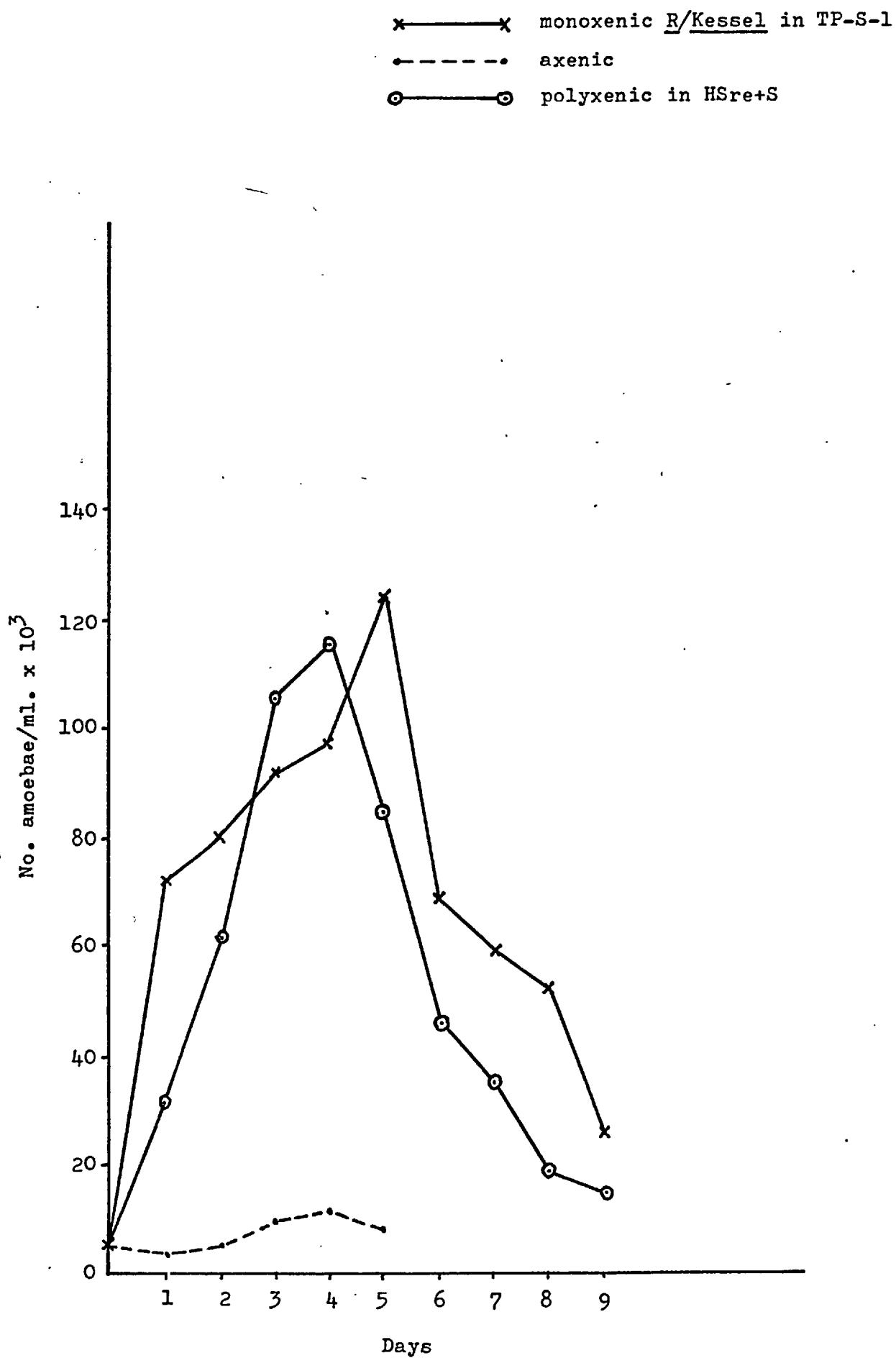
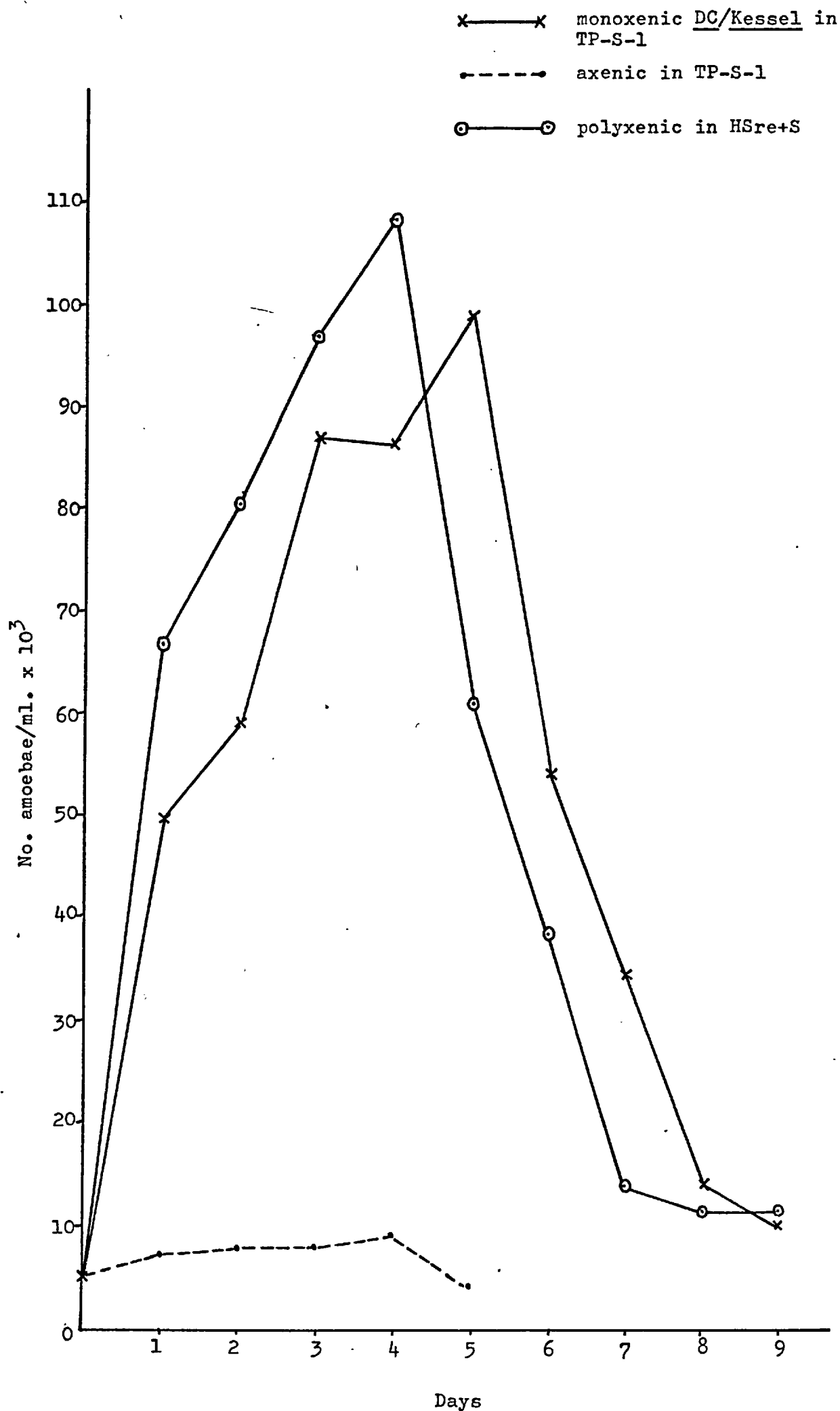
Figure 5 Growth of E. ranarum (strain R)

Figure 6 Growth of Entamoeba moshkovskii (strain DC)

lag phase seen between days 1 and 4. The lag phase experienced with monoxenic E. invadens extended from days 0 to 4. This contributed to the late attainment of peak growth on day 8, three days after that attained with mixed bacterial cultures. In all cases the rapid logarithmic growth ending with peak numbers was followed by an equally rapid drop in numbers.

Fold-increases found with these species ranged from 20-fold for E. moshkovskii to 30-fold for E. invadens. Differential counts, showed that less than 5% amoebae were dead of those present at peak growth.

ii. Amoeba species parasitic in homeotherms

E. coli is the only large amoeba dealt with in this group. The other amoeba species, E. hartmanni. D. fragilis and H. meleagridis, all have small trophozoites.

Initiation of monoxenisation was first carried out as described for the room temperature amoebae. As this resulted in failure, it was followed by attempts using other associates in the following order:-

T. cruzi amastigotes strain M1

Crithidia sp. strain ReF.1:PRR

Euglena gracilis

O. danica

N. coccoides

A. convolutus

H. castellanii strain R

tissue culture cells in suspension strain HEp2

red blood cells from rabbit

water soluble portion of T. cruzi cells strain Y

Preparation of cultures using the above associates were carried out as described in Chapter 2 pp. 48, Unfortunately the only attempts at

monoxenisation of E. coli were done using T. cruzi epimastigotes as associates. Further attempts could not be made as the stock cultures in HSre+S with mixed bacterial flora were lost and replacements were not found.

The results of attempts at monoxenisation of these species were unsuccessful as seen in Table 1. Since counts, using a haemocytometer slide, are inaccurate in assessment of very low concentrations, survival is only described in the term of time. In spite of surviving for 6 days (including 1 subculture) both Tooti and Said strains of E. coli failed to become monoxenic.

Length of survival of E. hartmanni in axenic conditions was only for 4 days. This length of time was doubled by the presence of epimastigotes of T. cruzi Kessel strain. Two more days survival in the presence of amastigotes of T. cruzi M1 strain was encouraging. However this was never improved by any of the other associates used.

The effect of epimastigotes on the other amoeba species, D. fragilis and H. meleagridis, prolonged the survival time to 6-8 days compared with the axenic situation of 4-5 days. Again the survival time was increased still further by the presence of amastigotes of T. cruzi. Although reasonable lengths of survival were obtained using tissue culture cells with these species it was only better than that obtained with amastigotes in the case of strain 382III of H. meleagridis. It is interesting to note that the two very small algal associates yielded longer survival time of amoebae than the larger algal associates. Ochromonas appeared to have an actively adverse effect on the amoebae. Association with Crithidia sp. gave survival times comparable with those obtained with epimastigotes of T. cruzi. H. castellanii and red blood cells gave no benefit to the amoebae as reflected by survival periods.

Discussion

The studies on these various amoeba species reflect not only species differences but also slight differences due to the specific characteristics of separate isolates. Thus the survival times for two strains of H. meleagridis are not identical for identical conditions. Therefore too much attention to detail in assessment of these results might be misleading. The general pattern should be examined rather than the minutiae. Another source of variation in reaction to the monoxenic system, is the bacterial flora which characterise their environment during prior cultivation.

The results were compared with those of the "axenic system" so that there was some form of "constant" by which to judge the effect of the associates. This "constant" represented the length of survival time during which "carry over" nutrients were depleted. Thus the positive or negative effect of the associate could be assessed.

Comparison of the growth curves during initiation of monoxenic cultures of E. invadens, E. ranarum and E. moshkovskii with those presented of E. histolytica in chapter 3d is particularly interesting. After the initial multiplication phase, depletion of carry over nutrients and lack of amoebic adaptation to the new environment is manifested by a drop in numbers in all cases. However this is far more marked in cultures maintained at 37°C than those kept at 25°C. The growth curves of the Laredo strain and other room temperature entamoebae species are very similar. Perhaps this apparently greater facility for adaptation, possessed by room temperature cultures, is a reflection of the slightly different characteristics of the amoeba-trypanosome system at 25°C.

It is also conceivable that a lower rate of metabolism at lower temperatures is better suited to adapt to the "shock" presented by the new system. While multiplication producing tolerant individuals takes place the stored nutrients are probably not completely finished. However at 37°C the cycle of growth and multiplication takes place so quickly that the carry over nutrients will be utilised more quickly and therefore diminish rapidly.

Another characteristic common to all the entamoebae successfully established in monoxenic culture is their large size. This is not startling in itself but might have considerable bearing on the failure of other amoeba species used.

The failure in attempted monoxenisation of E. coli cultures is surprising although it has often appeared to be more sensitive to environmental change. The survival time in standard amoeba-trypanosome conditions was quite long. E. coli is reported to be less fastidious in its diet than E. histolytica (Markell, E.K. & Kuritsubo, R., 1967). Cultivation using conventional media is generally successful and considered to be similar to that of E. histolytica. Since nonpathogenic entamoebae have been cultured at room temperature it is unlikely that nonpathogenicity would preclude monoxenisation, although some correlation of degree was seen within the E. histolytica species. The only conclusion is that probably success would be achieved if persistent attempts could be made.

Consideration of the failure met with, in monoxenisation of other amoebae species inevitably involves the amoeba size. A description has already been given in chapter 3 of how E. histolytica actually engulfs epimastigotes of T. cruzi. Other experiments also

described in the same section prove that the immediate presence of the whole live epimastigotes is essential for the support of amoebic growth. E. hartmanni, D. fragilis and H. meleagridis trophozoites are all only 5-10 μ in diameter. Considering that T. cruzi epimastigotes are 20 μ long these amoebae would be physically incapable of engulfing the flagellates, and it is therefore hardly surprising that no growth was obtained. However some benefit must be supplied by the presence of the flagellates as shown by the prolonged survival time.

It was in consideration of this problem that other suitable associates were sought and tested in the E. histolytica monoxenic system. Tissue culture cells (strain HEp2) in suspension were very slightly larger than red blood cells (7 μ in diameter). Perhaps some amoebae were able to engulf these associates or some metabolites of these cells could enter the amoeba by diffusion. Benefit only seems to have been derived from tissue culture cells as shown by the prolonged survival of Histomonas and Dientamoeba. The smallest whole living cell associates used were the algae, Nannochloris and Ankistrodesmus, whose respective diameters are 2 μ and 3-6 μ . Neither of these enhanced the growth of the amoebae but like red blood cells they did not support the growth of E. histolytica. The smallest associate tried was the water insoluble portion of lysed T. cruzi epimastigotes. This associate had been shown to enhance the growth of E. invadens but not E. histolytica. But since E. invadens is perhaps a special case, as exemplified by the ease of axenic cultivation, it is not too surprising that these associates and cellular fractions provided no benefit to these very small amoebae.

The only associates that were consistently beneficial were the amastigote cells of T. cruzi. When these developmental forms are seen within the parasitised host cell they measure 2-5 μ in diameter. However it must be remembered that the amastigotes used in this

system were produced extracellularly by special cultivation and may be abnormal compared with their natural counterparts. Their size was uniform and microscopical examination comparing them with other organisms of known size indicated that they were about 5 μ in diameter. A small amoeba might be able to engulf only one amastigote at a time and digest that one before further feeding. E. histolytica was often seen to contain many flagellates at one time. Probably insufficient nutrient material of the required kind could be obtained from single amastigote cells to promote growth and multiplication.

b) Particular species preferences for physical and chemical factors of the system

Introduction

In the study of cultural preferences of different amoebic species in a monoxenic system, naturally there is the prerequisite of an established isolate. Unfortunately, monoxenic cultures of a number of amoebae were not isolated and therefore the study of species preferences in the monoxenic system is only concerned with E. histolytica, E. invadens, E. ranarum and E. moshkovskii.

E. histolytica has already been dealt with in chapters 3 and 4.

Study of the literature (see chapter 1), indicated that the other amoebae are very similar to E. histolytica in their cultural requirements. It was pointless to repeat all the work involved in the design of the monoxenic system when excellent growth was obtained with other species in this system. However, attention to certain details might reveal specific preferences. For these studies, E. invadens only was used unless there were obvious indications otherwise.

Considerable studies were made of the effect of various associates in an attempt to find a very small but suitable associate. Non-biological aspects studied were concerned with the serum and temperature factors.

All these factors might be considered to show variation between amoebae species as a reflection of environments within different hosts, in the case of parasitic species, and the free-living habit, in the case of E. moshkovskii. Serum preference

might show the degree of adaptation to a particular host.

Temperature tolerance would certainly be expected to reflect the tolerance requirements of the natural environment. Free-living amoebae and parasites of poikilotherms would require greater tolerance than parasites of homeotherms.

Experimental Results

i Serum factor

E. invadens was grown in the presence of 10% serum from various animals as described in chapter 2 pp. 73 . Other factors in the system were constant between the groups.

Results of these experiments are portrayed in figures 1, 2 and 3. If these are compared with the results of similar experiments using E. histolytica (see figures 7,8,9, Chapter 3b), it may be seen that they are very similar. In most cases, growth of E. invadens was better than that of E. histolytica. The preference for various sera differed only slightly. E. invadens preferred calf serum to colostrum deprived calf serum and chicken serum to Rhesus monkey serum. In both cases rat serum allowed no growth.

The fold increase obtained with rabbit serum for E. invadens was twice as good as that obtained with either horse serum or calf serum. It is interesting to note that in this experiment peak growth was reached fairly late, days 14 and 15, in cultures containing the preferred animal sera. Peak growth of the poorer animal sera cultures was reached 3 to 5 days earlier and as much as 9 days earlier in the case of Rhesus monkey serum. On the whole the shape of the growth curves varied only in degree of multiplication during the logarithmic phase. The lag phases of cultures supplemented with bovine and horse serum were of longer duration

Figure 1 Growth of monoxenic *E. invadens* (BAH/Kessel) with sera from different animals

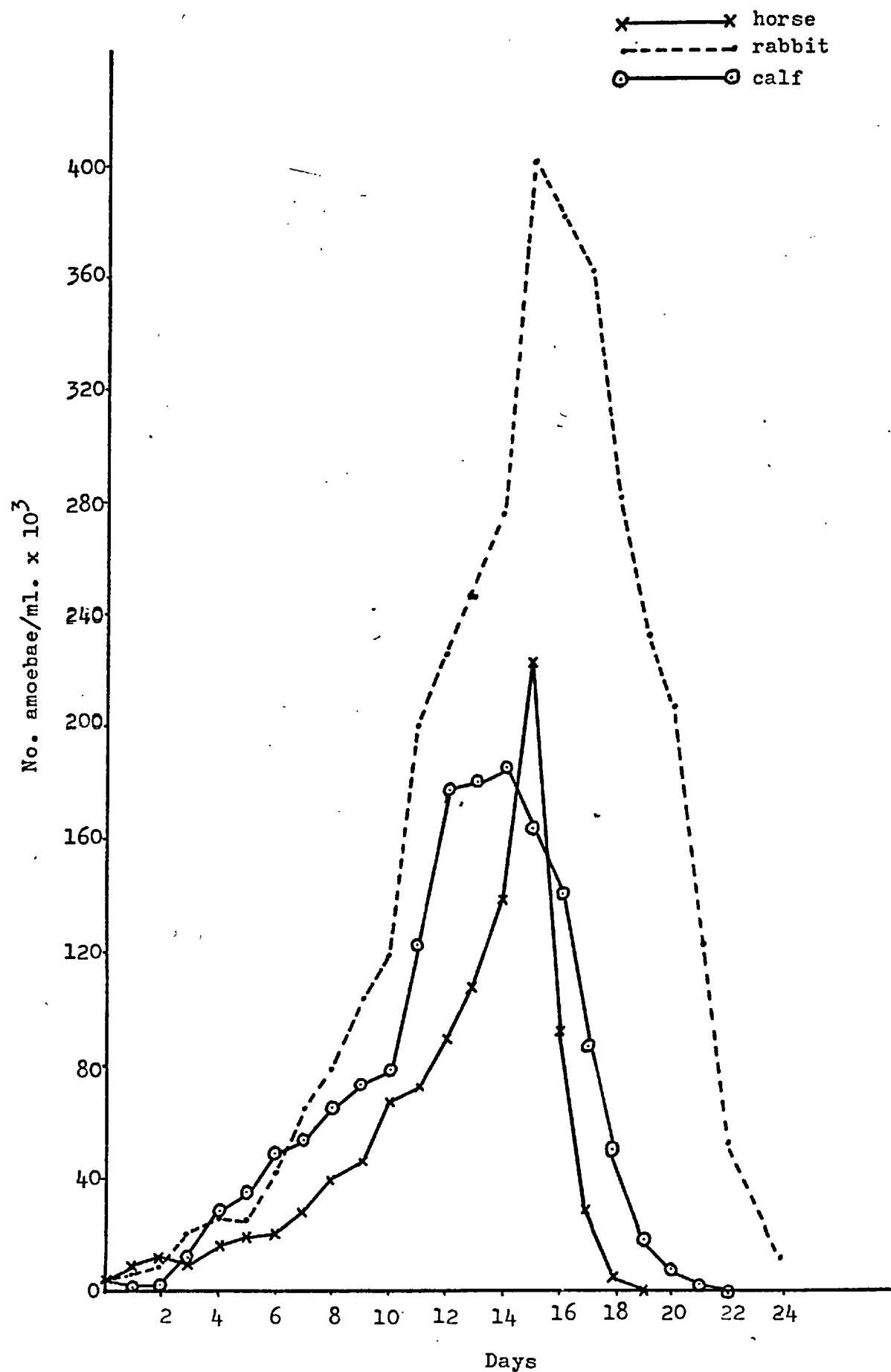


Figure 2 Growth of monoxenic E. invadens (BAH/Kessel) with sera from different animals

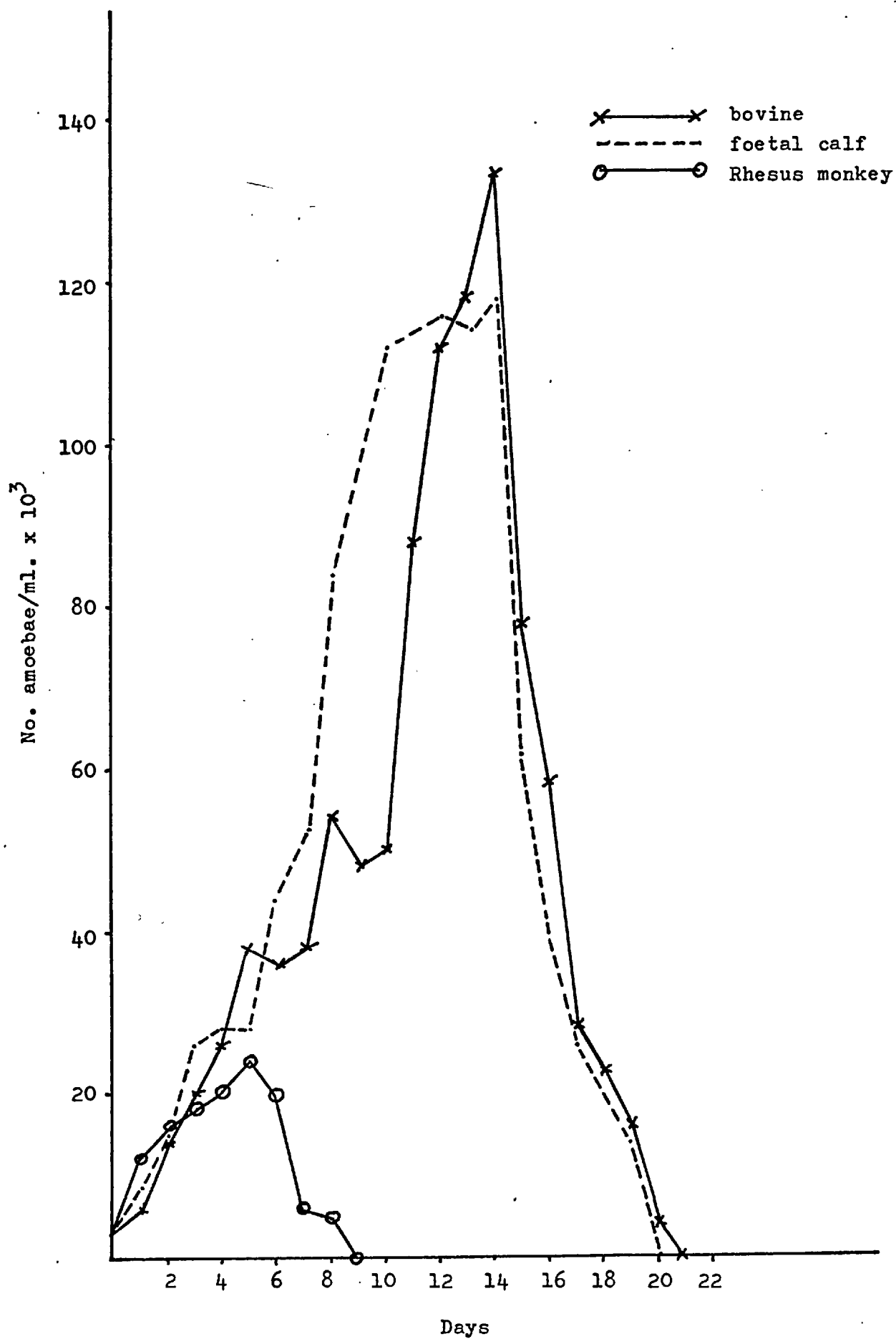
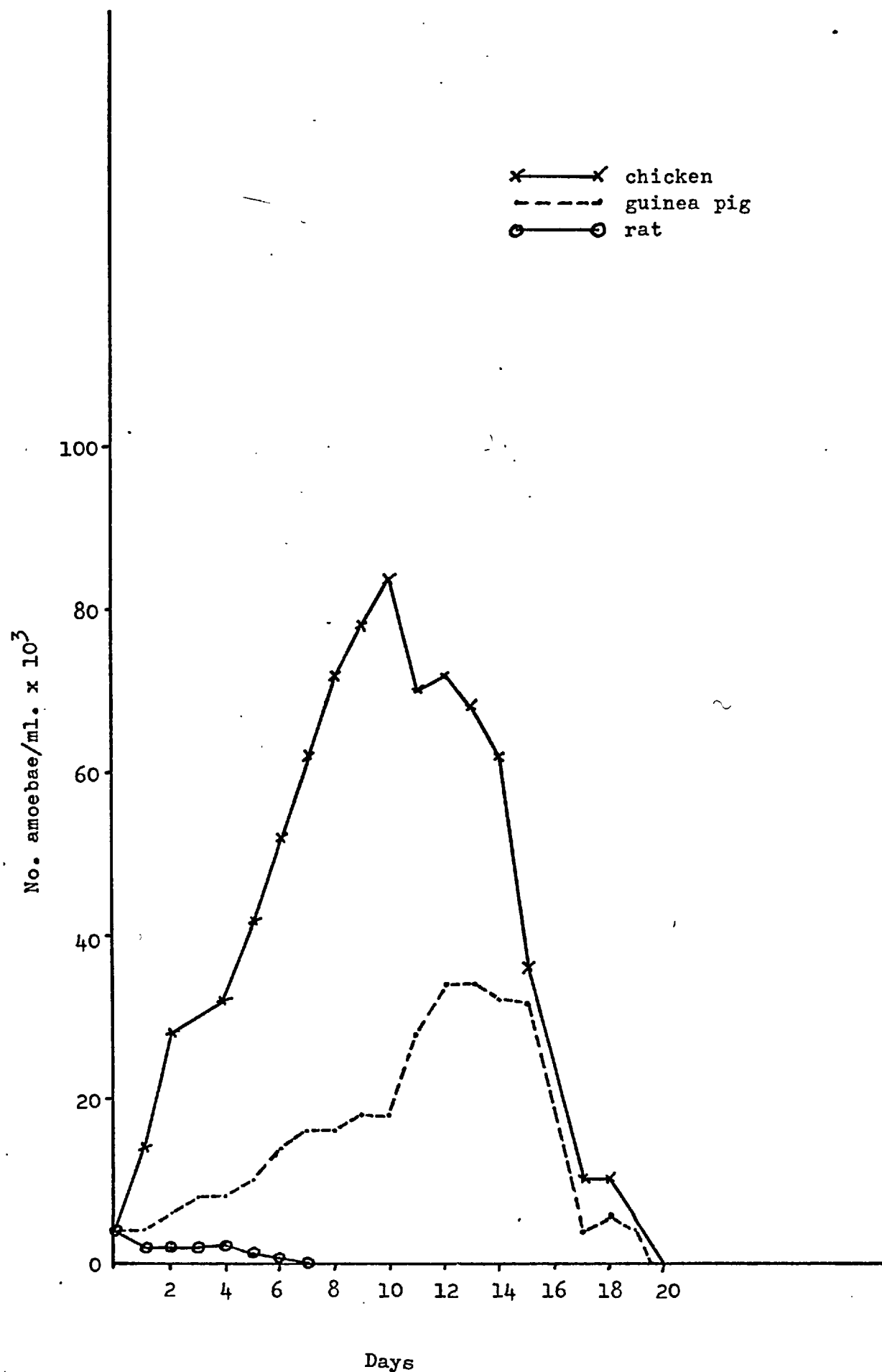


Figure 3 Growth of monoxenic *E. invadens* (BAH/Kessel) with sera from different animals



than the other cultures.

Another experiment was conducted to ascertain the optimum concentration of serum to supplement the medium in cases of E. invadens. E. invadens was grown in the presence of various concentrations of horse serum as described in chapter 2 pp. 75. The concentration of the other constituents was not adjusted accordingly as it was considered that these were already present in excess, but perhaps allowances should be made for this in the assessment of the effect of the highest concentrations of serum.

Growth produced under these conditions is described by peak yields of amoebae, recorded in table 1. (This table also includes results of similar experiments using E. moshkovskii, referred to later). Comparison of the results from the same experiment using E. histolytica as seen in table 1 chapter 3b shows that both species preferred 10% horse serum. Growth was supported with as little as 1.25% and as much as 40% serum. The generally low level of growth seen in this experiment compared with the previous one on sera from different animals was a reflection of the associated Y strain of T. cruzi instead of Kessel strain (see chapter 5c).

As Diamond and Bartgis (1970) found it necessary to use 1% horse serum in the axenic cultivation of E. moshkovskii, a similar experiment to the one described above for E. invadens was also completed using the monoxenic isolate, strain DC, of E. moshkovskii. The results of this are recorded in table 1. As was expected from the work of Diamond and Bartgis the picture of growth of this species in different concentrations of serum was different from the one seen with E. histolytica and E. invadens. Growth was extremely poor with the highest concentration of serum and appeared to worsen as the concentration was increased above the optimum of 1.25%. However a

Table 1. Peak yields of amoebae produced in medium containing
varying concentrations of horse serum

Initial concentration of amoebae : $5.0 \times 10^3/\text{ml}$.

% Concentration of Horse Serum	Peak yeild of amoebae x $10^3/\text{ml}$.	
	<u>E. invadens</u>	<u>E. moshkovskii</u>
40	20.5	NT
20	39.0	25.0
10	54.5	64.0
5	22.0	76.5
2.5	19.0	78.0
1.25	10.5	101.0
0.625	NT	86.5

reasonable degree of tolerance was shown in that growth was achieved with 20% serum and very good growth was achieved with all other serum concentrations tried. The lag phase was much reduced in cultures with 2.5% serum or less and multiplication continued steadily to reach peak numbers at day 7. Cultures containing a greater amount of serum showed a more marked lag phase which was followed by some degree of multiplication, but death seemed to supercede multiplication rather earlier in 20% serum cultures. As several varying concentrations were used, a range of results was obtained from optimum growth at 1.25% to poorer growth at 20%. It is interesting to see that reducing the concentration of serum below 1.25% did not enhance the yield of amoebæ compared with that of 1.25%. However very good growth was obtained with the concentration of 0.625% serum. Presumably growth would have declined as the serum concentration was reduced below this level, if tests had been made.

ii Temperature studies

Two types of experiment were carried out on temperature tolerance. The first one was to establish thermal death points and tolerance on subjection to a period of ten weeks subculture at varying temperatures. The second measured the growth of cultures set up from the above cultures after the tenth week. This one step treatment is rather more severe than might be expected to occur in nature, but the experiments were designed to give a realistic assessment of tolerance without involving too much adaptation.

All three room temperature entamoebæ were used in these experiments. Techniques used are described in chapter 3 pp 109. Unfortunately the strain of E. moshkovskii, that was most readily available, had lost the ability to grow at 37°C during a prolonged period in polyxenic cultures.

The results of the first experiment are described by means of a histogram, figure 4. E. invadens and E. ranarum showed similar tolerance, each surviving over a minimum range of 8°C, and dying at 37°C within a week. E. moshkovskii was outstanding in its temperature tolerance. It grew over a minimum range of 13°C and survival at 37°C was quite prolonged, suggesting that stepwise adaptation to growth at this temperature was possible. E. histolytica was included in the histogram for comparison. Like the other parasitic amoebae it was limited in its temperature tolerance, surviving over a range of only 4°C. Survival at temperatures outside this range was exceedingly short, the longest being one week at 28°C. It must be born in mind that E. histolytica was the only species whose minimum tolerated temperature was assessed. Apparatus for maintaining temperatures slightly below the ambient was not readily available. Therefore it is highly probable that the temperature tolerance ranges for the room temperature entamoebae was even wider than indicated by the above results.

The results of the second series of temperature tolerance experiments are described by figures 5, 6, 7 and 8. Again E. histolytica was included for comparison. In all four species peak growth at the optimum temperature was markedly higher compared with peak growth obtained at other temperatures. On the whole, peak growth at sub-optimal temperatures was reached earlier than that of optimal temperatures. This was particularly obvious in the growth curve of E. invadens (figure 5) at 28°C. Growth of this species at 20°C was exceedingly poor and it is surprising that it was maintained at this temperature for 10 weeks without difficulty.

The growth of E. ranarum cultures (figure 6) at different temperatures was very similar until day 5 when the optimal temperature began to show much increased multiplication compared with the cultures

Figure 4 Survival of monoxenic isolates subjected to different temperatures

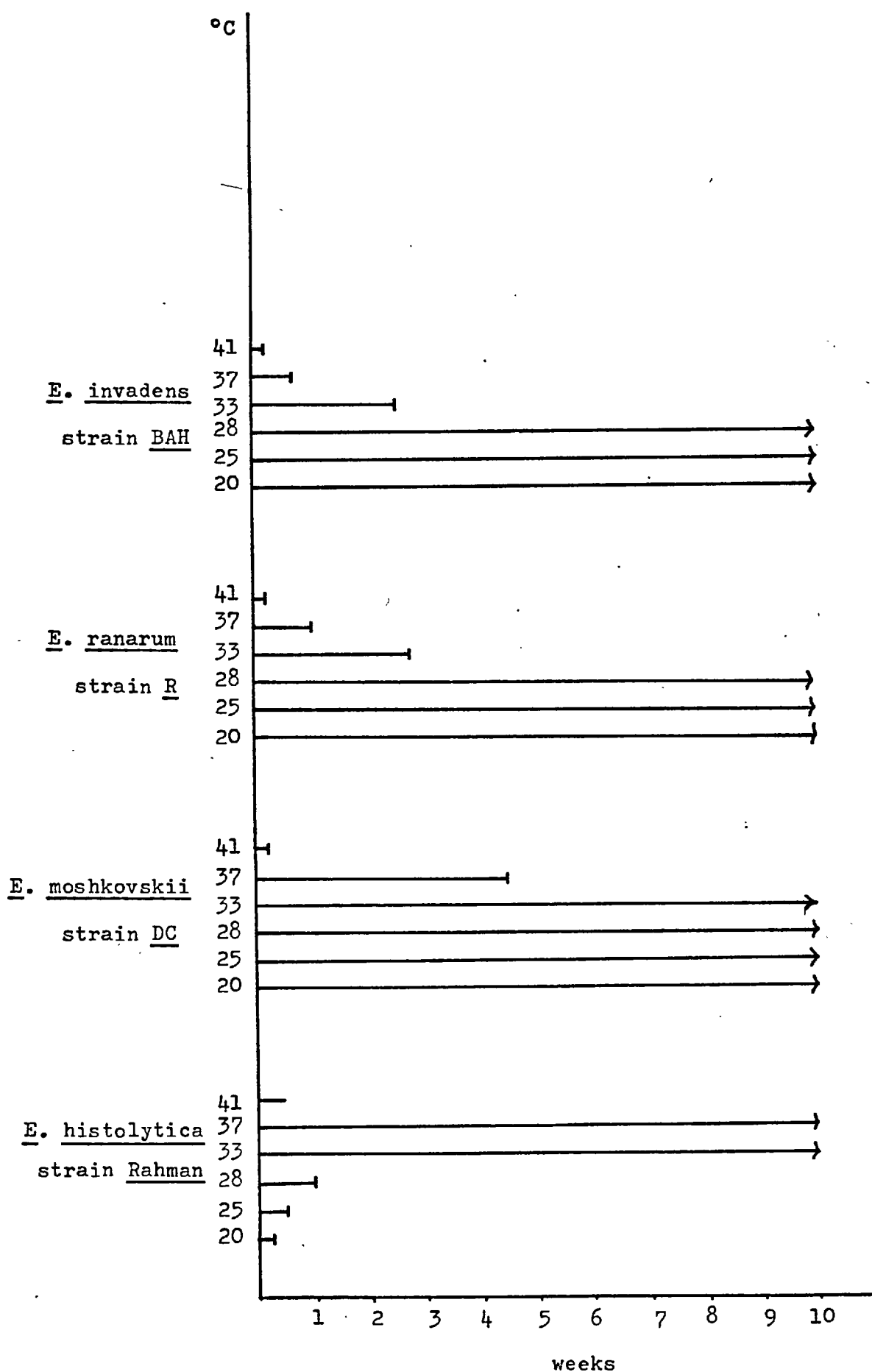


Figure 5 Growth of monoxenic E. invadens (BAH/Kessel) at varying temperatures

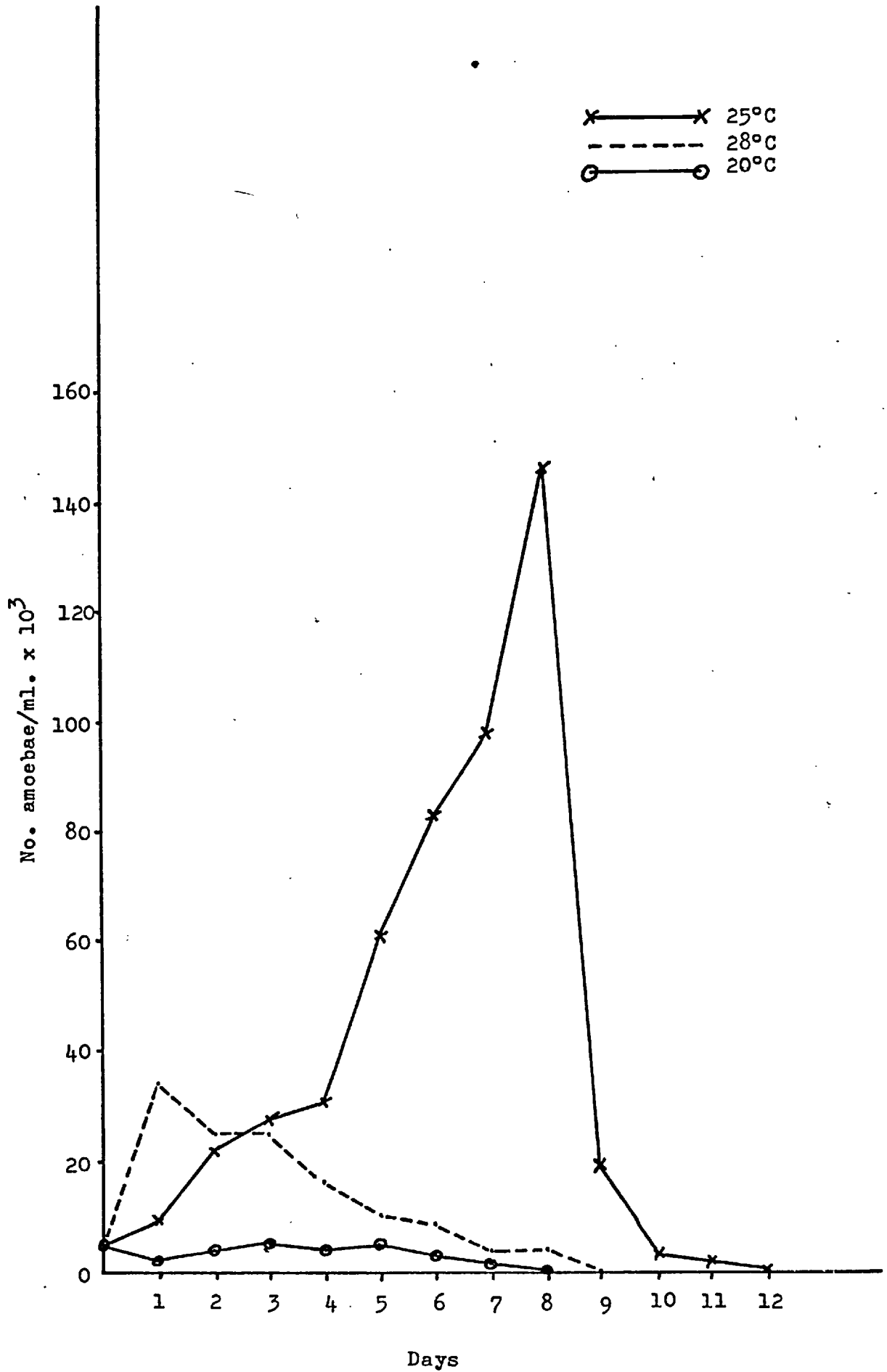


Figure 6 Growth of monoxenic E. ranarum (R/Kessel) at varying temperatures

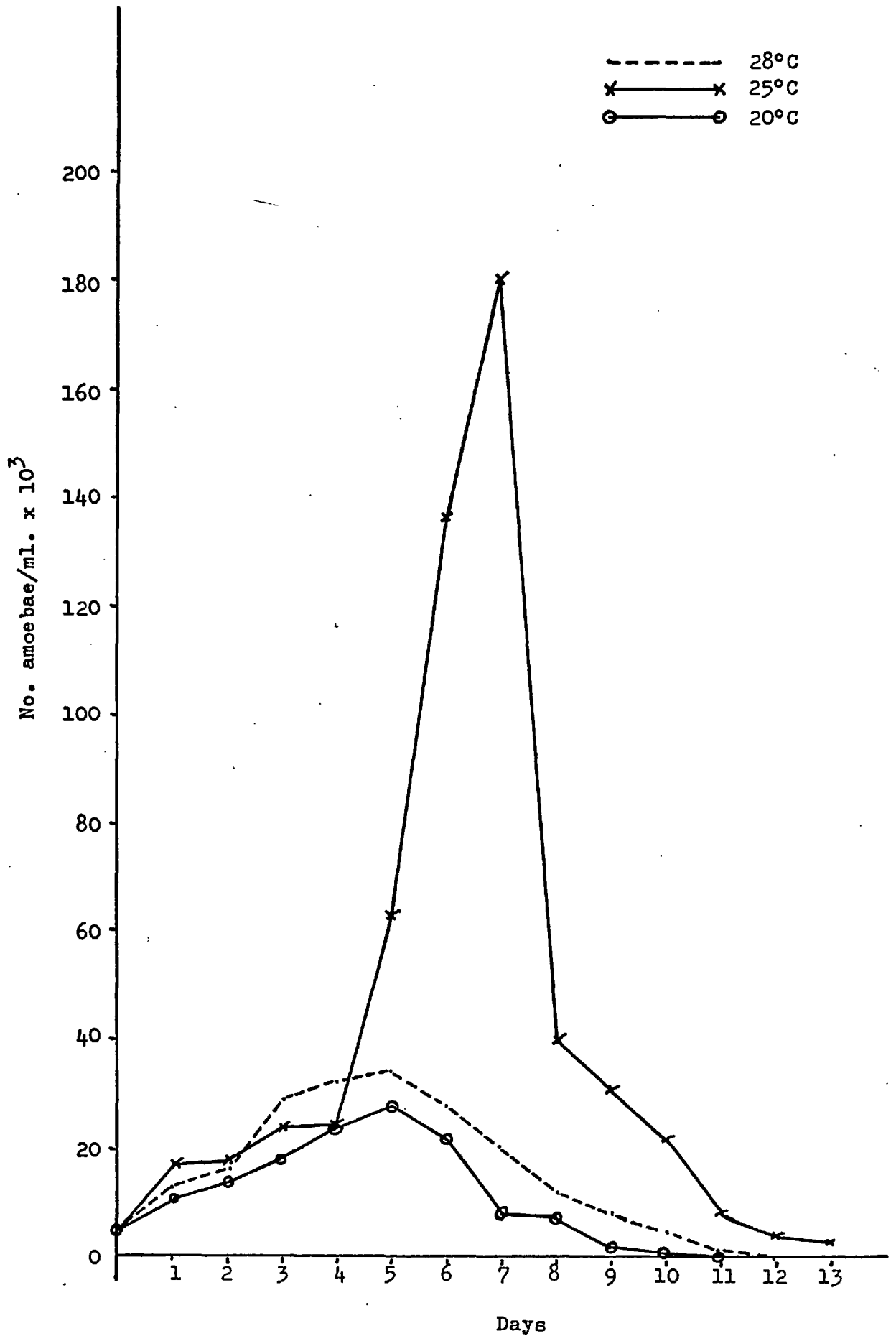


Figure 7 Growth of monoxenic *E. moshkovskii* (DC/Kessel) at varying temperatures

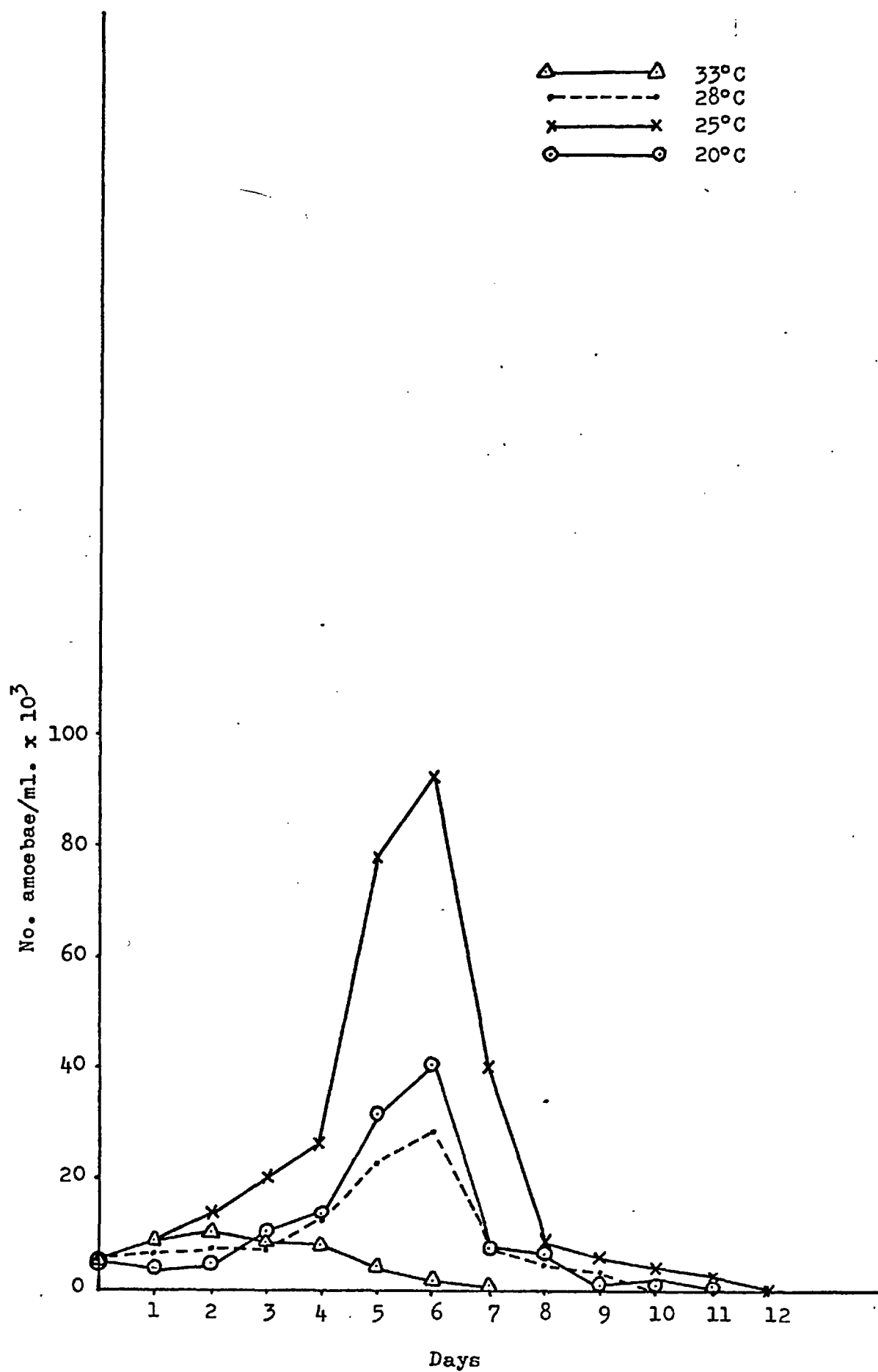
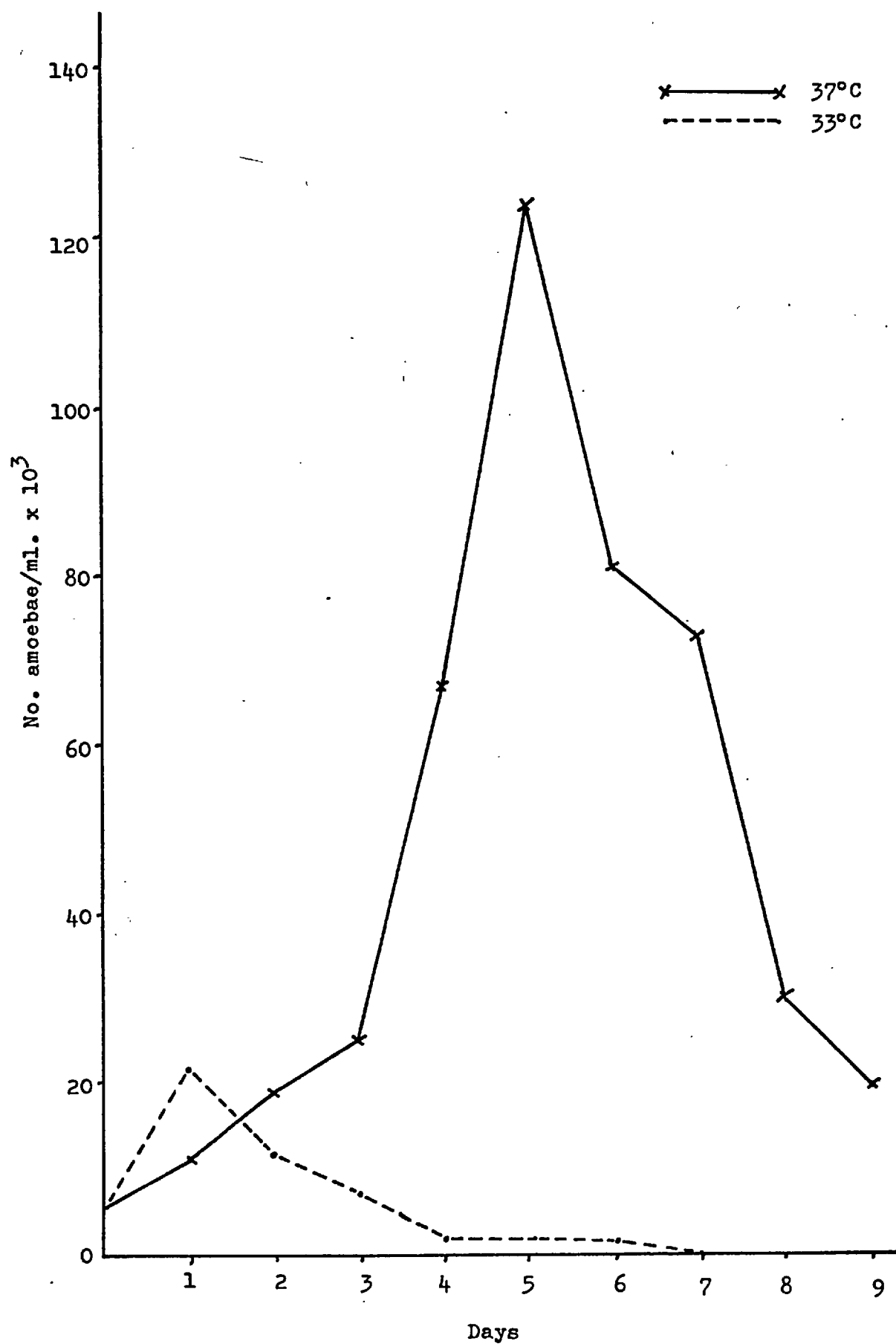


Figure 8 Growth of monoxenic *E. histolytica* (Rahman/Kessel) at varying temperatures



at the other temperatures which remained similar. Peak growth at 25°C was obtained two days later than that at 28°C and 20°C.

The growth curves for cultures at 28°C, 25°C and 20°C of E. moshkovskii (figure 7) are very similar and vary only in degree. Growth at the extremely high temperature of 33°C was poor and numbers dropped gradually after an early peak at 2 days. The growth curves of both this species and E. ranarum at 20°C indicate that growth would probably have been obtained at lower temperatures.

E. histolytica (figure 8) showed only limited growth at 33°C. A very early peak at day 1 was followed by a fairly rapid decline in numbers.

Discussion

Only two factors (serum and temperature) have been examined in this subsection but probably they are the most pertinent in consideration of species preferences of the particular species isolated in amoeba-trypanosome culture. The serum factor plays an active part in the characterisation of the system. Serum provides nutrients, including growth factors, amino acids and proteins. It also acts as a pH buffer and slightly increases the alkalinity of the medium. All conventional media used for polyxenic cultivation of E. histolytica have included serum and it is generally recognised as being necessary for sustained growth.

The results of the experiments testing sera from different animal sources indicate that preference for particular sera is not governed by host specificity. If this had been so, markedly different preferences would be expected from E. histolytica and E. invadens. However their orders of preference were extremely similar. If host specificity played any part at all, rat and guinea pig serum might be expected to support good growth of E. histolytica as they are susceptible to experimental infection with this parasite. However they did not support good growth. Rabbit serum supported excellent

growth and this is a susceptible host. This is an indication that host specificity plays no part in this problem. As the buffering capacity and pH of all mammalian serum is likely to be very similar, the nutritional aspects probably dictate the preference for particular type of serum. This is also born out by the similarity of experimental results from two Entamoeba species. One can only presume that rabbit serum contains more of a certain requirement than the other sera tested. This might be a growth factor but it is more than likely that it is an amino acid or protein. The other constituents of TP-S-1 medium are so diverse that most growth factors must surely be present in excess. If the amoeba needed some nutrients to be presynthesised to a certain degree, due to an inadequate enzyme system of their own, the availability of this nutrient would have a marked effect on their growth. As the amoebae species were both parasitic it is quite likely that they might have lost the ability to synthesise every amino acid and protein they require.

Another aspect that should be mentioned is the adverse effect of any antibodies that might be present in the sera. However, it is most improbable that the sera used contained antibody to any Entamoeba species.

The concentration of serum required for optimum support of entamoebae in monoxenic cultivation was studied using horse serum. Both E. histolytica and E. invadens preferred the relatively high concentration of 10%. This is probably the resultant of the beneficial and adverse characteristics of the serum. The poorer growth with higher concentrations of serum was reminiscent of the reaction of tissue culture and other cells to these conditions. Certain toxic substances when present in too high a concentration will override any beneficial nutritional factors. Naturally the

higher concentrations of serum will dilute the concentration of other medium constituents but these are present in such excess that this should not effect the results unduly. As both species tested were so similar in their preference for type of serum it is not surprising that both prefer the same concentration. These results indicate that to some extent nutrition of E. histolytica and E. invadens is similar in the amoeba-trypanosome system.

E. moshkovskii contrasted with the other two species of entamoebae tried, in its preference for a serum concentration of about one tenth of the E. histolytica/E. invadens optimum. This must surely be a reflection of the free living habit as opposed to the parasitic habit. E. moshkovskii would have to be more independent and therefore capable of completing most of its required synthesis itself, as there is no host to provide presynthesised material. This would account for E. moshkovskii requiring a small amount of serum. On account of this amoeba being accustomed to low concentrations of protein it is probable that it finds high concentrations toxic, as seen by the poorer growth in concentrations above 5% serum.

The temperature studies also show that the characteristics of the amoeba species, as defined by certain factors in monoxenic growth, are closely related to the requirements of their habitat and habit.

It was unfortunate that the lower level of temperature tolerance could not be established with the room temperature amoebae. However, from the evidence of the ten week experiment the degrees of temperature tolerance fall into three groups. They are first, E. histolytica, with a very narrow range; second, E. invadens and E. ranarum, with a wide range; and third E. moshkovskii with an extremely wide range.

Consideration of the type of temperature variation that the amoeba is likely to encounter in its habitat correlates with the

experimental results. The homeothermic host of E. histolytica maintains a very constant temperature that only fluctuates a degree or so except during illness. The poikilothermic hosts of E. invadens and E. ranarum have temperatures that are dictated by the environment. However, the habits of these animals will generally prevent them from reaching extreme temperatures. Thus the animal will seek out habitats with optimum local conditions. Sewage temperatures fluctuate with the weather and the season. The only buffering effects are the underground position of pipes, the metabolism of sewer dwelling animals and the temperature of industrial effluent, in particular.

The temperature tolerance shown by trophozoites of E. histolytica is quite adequate to cover conditions that would occur inside its host. Persual of parasitism in the same host would have led to selection of enzymes most suitable for metabolism within this range and gradual loss of less sensitive enzyme systems. Temperature tolerance as shown by the parasites of poikilotherms, E. invadens and E. ranarum, is probably adequate to cover conditions encountered. Sudden or rapid temperature changes would not impinge directly on the parasite as the host's body and habits would act as a buffer. The ambient of habitats where the respective poikilothermic hosts are found is rarely hotter than 28°C. However, it is often below 20°C. The experimental results certainly show that the parasite is able to tolerate temperatures up to the anticipated upper limit. The same may be said for the temperature tolerance of E. moshkovskii. Although local areas of sewage may exceed 33°C in temperature the major part is unlikely to, in temperate climates. Again, as with poikilothermic parasites the chances of the temperature falling below

20°C are great but the growth of E. moshkovskii and E. ranarum at 20°C suggested that growth could be sustained at lower temperatures.

Therefore the experimental results showed that growth of trophozoites of certain Entamoeba species can continue over a temperature range suitable for the type of environment. However, the Entamoeba species have another method of coping with adverse temperatures, encystment. This could not be assessed in the monoxenic system due to an inability to encyst in these restricted conditions. Encystment has a dual role in the life of the Entamoeba. It allows completion of the life cycle and also enables the organism to withstand adverse conditions. As cysts cannot remain viable indefinitely the tolerance range for the growth of the trophozoite must be adequate to cover average conditions for a greater part of the year. The above experiments have shown this to be true for E. histolytica and for the upper limit of the range for E. invadens, E. ranarum and E. moshkovskii.

Adaptation, also will play a part in the ability of the amoeba to cope with temperature fluctuations. As this has been studied in detail by other workers (Gordeeva, 1966; Siddiqui, 1963; Lachance, 1963; Cabrera, 1958) their work was not repeated here. Adaptation would be of particular use for room temperature entamoebae tolerating seasonal changes.

c) Particular species preferences for biological factors of the system

Introduction

As already mentioned the purpose of the work described in this chapter was two-fold. Firstly, it was to confirm that the monoxenic system, already designed for E. histolytica using trypanosomes and other associates, was equally suitable for the culture of other entamoebae. In so doing any slight variation in nutritional requirements of different species would become apparent. Secondly, it was an attempt to find a very small suitable associate that might be used in a monoxenic system with the amoeba species not already isolated in amoeba-trypanosome association. All the experiments reported here involved the use of E. invadens. They were often done in close conjunction with similar work on E. histolytica reported in chapters 3 and 4. E. invadens had certain advantages over E. histolytica, in particular the ease of axenic cultivation which enabled easier assessment of associates affording only poor support. Reasons for choosing the various associates have already been expounded (see chapter 4 pp. 172) so no further explanation will be given here.

For convenience the experimental results will be dealt with in two sections:-

i Trypanosoma cruzi associates

- a. Strains
- b. Stages in life cycle of trypanosome
- c. Physiological states of trypanosome

ii Other associates

- a. Flagellates
- b. Algal associates
- c. H. castellanii
- d. Tissue culture cells
- e. Red blood cells

Experimental results of monoxenic cultivation of E. invadens with various associates

i. T. cruzi associates

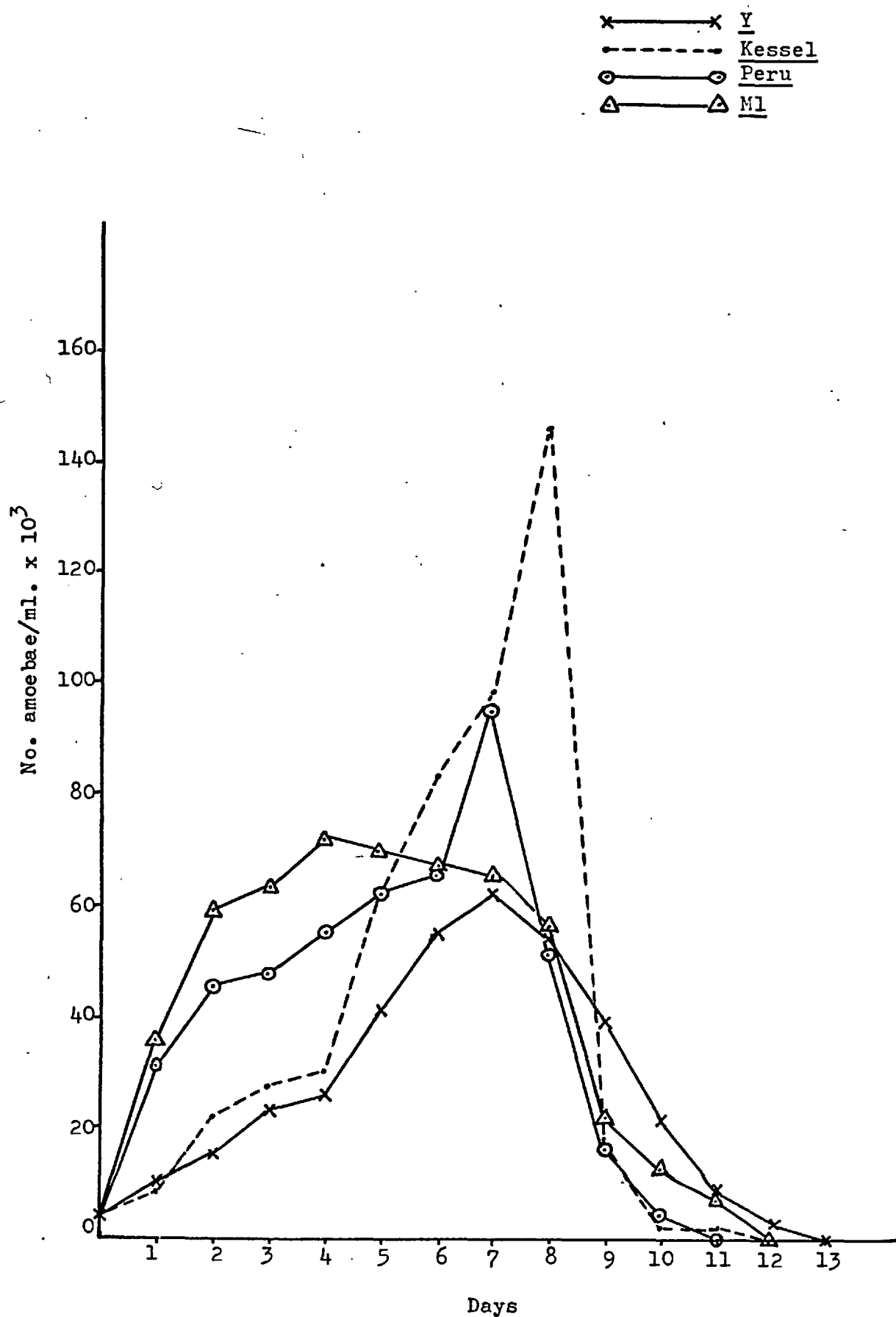
Extensive work was done using E. histolytica to establish the optimum initial concentration of T. cruzi epimastigotes. As these epimastigotes do not multiply in the amoeba culture medium at 37°C the initial concentration might be expected to have considerable bearing on the growth of E. histolytica. The system at 25°C for E. invadens is different because the epimastigotes continue to live and multiply. Therefore no attempts were made to establish the optimum initial concentration of epimastigotes for E. invadens.

a. T. cruzi strains

Details of the strains used and the methods employed in study of their support for E. invadens were similar to those used for E. histolytica and are described in chapter 2 pp.25 , together with details concerning other associates used in this subsection.

The growth curves of E. invadens associated with various strains of T. cruzi are plotted in figure 1. As with similar experiments using E. histolytica (see figure 4 chapter 3b), there

Figure 1 Growth of *E. invadens* (strain BAH) with epimastigotes of different strains of *T. cruzi* in TP-S-1



was considerable variation between support given by various T. cruzi isolates. This variation was possibly more marked in the growth of E. invadens. Variation was shown in three main aspects; the number of amoebae obtained at peak growth, the time when this was reached, and the length of initial lag phase. Kessel strain was outstanding in its support of amoebic growth, giving a yield one and a half times greater than any other strain. However this peak growth was attained very late, day 8, when some other cultures had already started to decline. Together with the characteristic of late peak growth there was a prolonged lag phase lasting four days. Both of these features characterised the Brazilian strain Y and Kessel strain. A slightly different growth curve was shown by E. invadens associated with the Peruvian isolate. The onset of the lag phase was delayed for 2 days and it lasted three days. Peak growth was again late, day 7, and was followed by a rapid decline in numbers. E. invadens associated with the Argentinian strain M1 was particularly unusual in the apparent lack of obvious lag phase. Peak growth was reached by fairly rapid multiplication at day 4 and the decline thereafter was only gradual, viable amoebae were still present at day 11.

b. Different stages in the life cycle of T. cruzi

Preparation of the different morphological forms of T. cruzi characterising various stages in its life cycle have been described in chapter 2 pp.28 & 30.

Where possible the same strain of T. cruzi was used for the preparation of different morphological forms. However the ease of metacyclic trypanosome preparation was considerably greater

using M1 strain and was therefore used. Results have been presented accordingly. The growth curves of cultures supported by various forms of Y strain, being given in figure 2 and those of M1 strain in figure 3. Cultures associated with epimastigotes showed the best growth in terms of yield and longevity. Although growth of cultures associated with trypanomastigotes appeared to be good during initial stages, due to the lack of pronounced lag phase, there was a rapid decline in numbers after peak growth obtained at day 5. The yield was only mediocre, but was however considerably better than that obtained in association with amastigotes. A five-fold increase attained by day 5 was reached by gradual multiplication and followed by rapid decline in numbers. This growth with amastigotes was no better than axenic cultures (see figure 4). The growth curves of cultures associated with both morphological forms of M1 strain tested were similar but varied in degree. Both cultures were characterised by the lack of pronounced lag phase, peak growth at day 4, followed by two phases of decline. The first decline phase being gradual and the second very rapid. Metacyclic trypanomastigotes offered poorer support, in terms of yield and longevity, than the epimastigotes.

c. Physiological states of trypanosomes

Firstly, growth was assessed in cultures associated with whole but dead epimastigotes of T. cruzi. Thereafter the growth of cultures associated with fractions of T. cruzi epimastigote cells was assessed. The results of the first set of experiments

Figure 2 Growth of *E. invadens* (strain BAH) with different morphological forms of *T. cruzi* (strain Y)

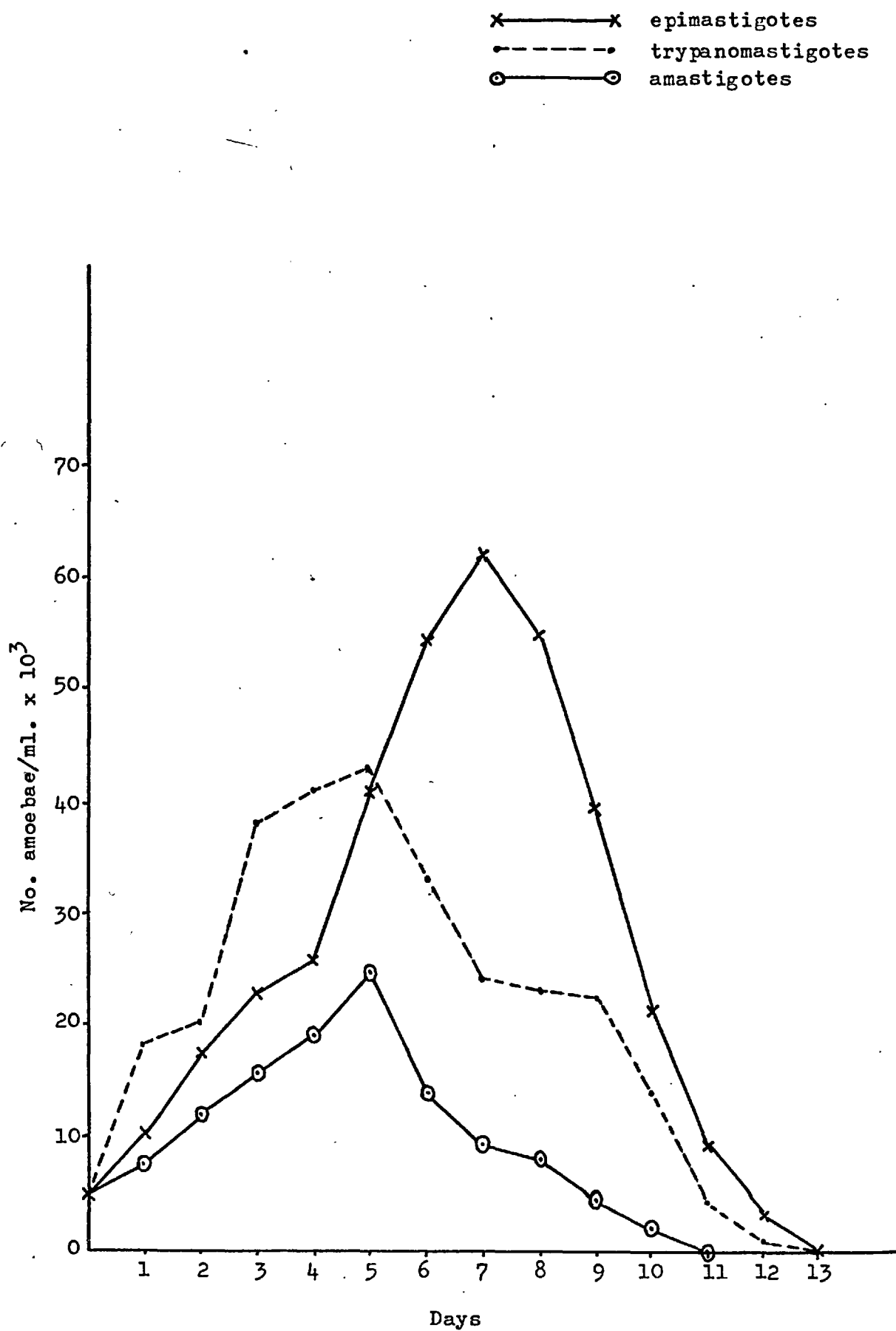


Figure 3 Growth of *E. invadens* (strain BAH) with different morphological forms of *T. cruzi* (strain M1)

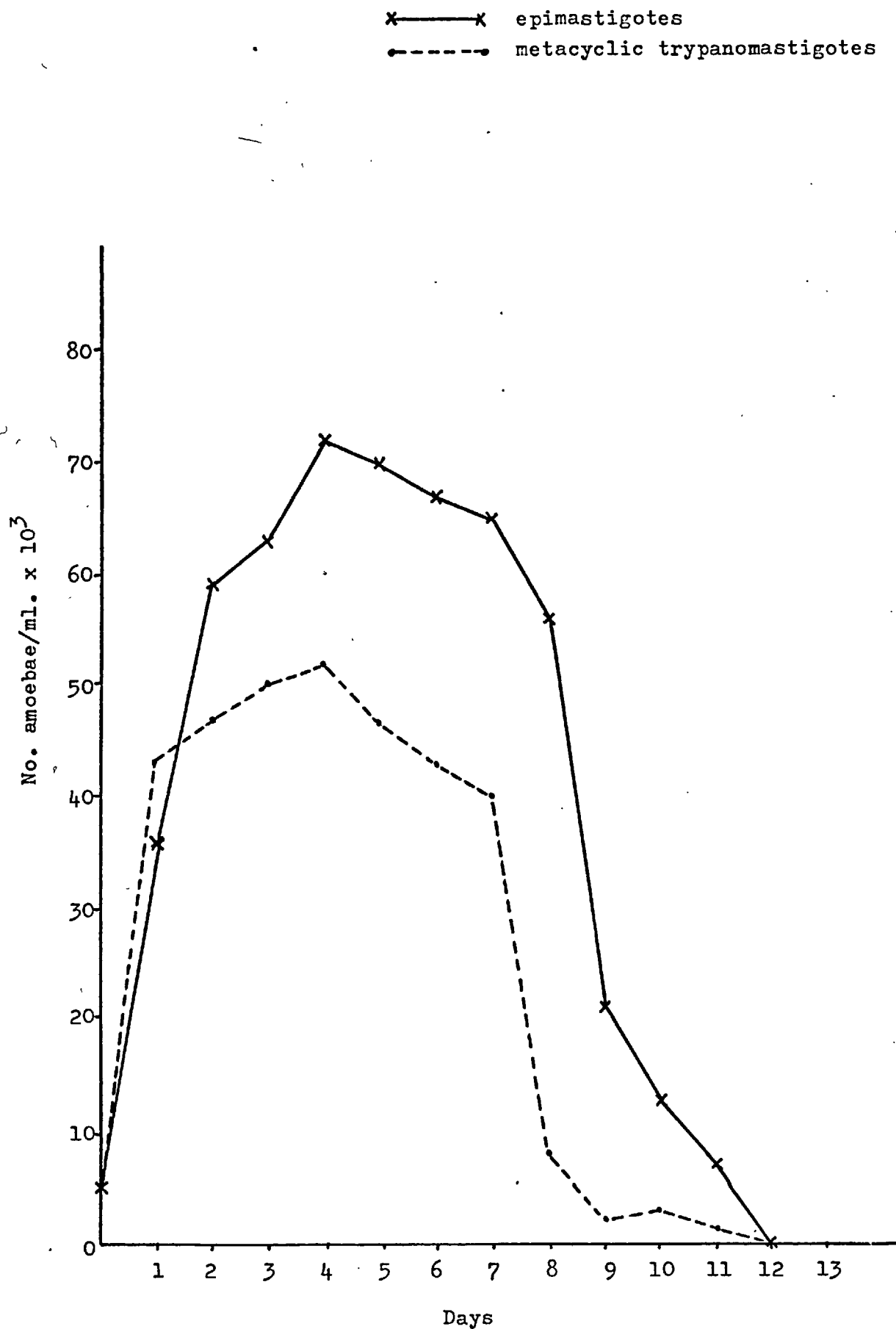
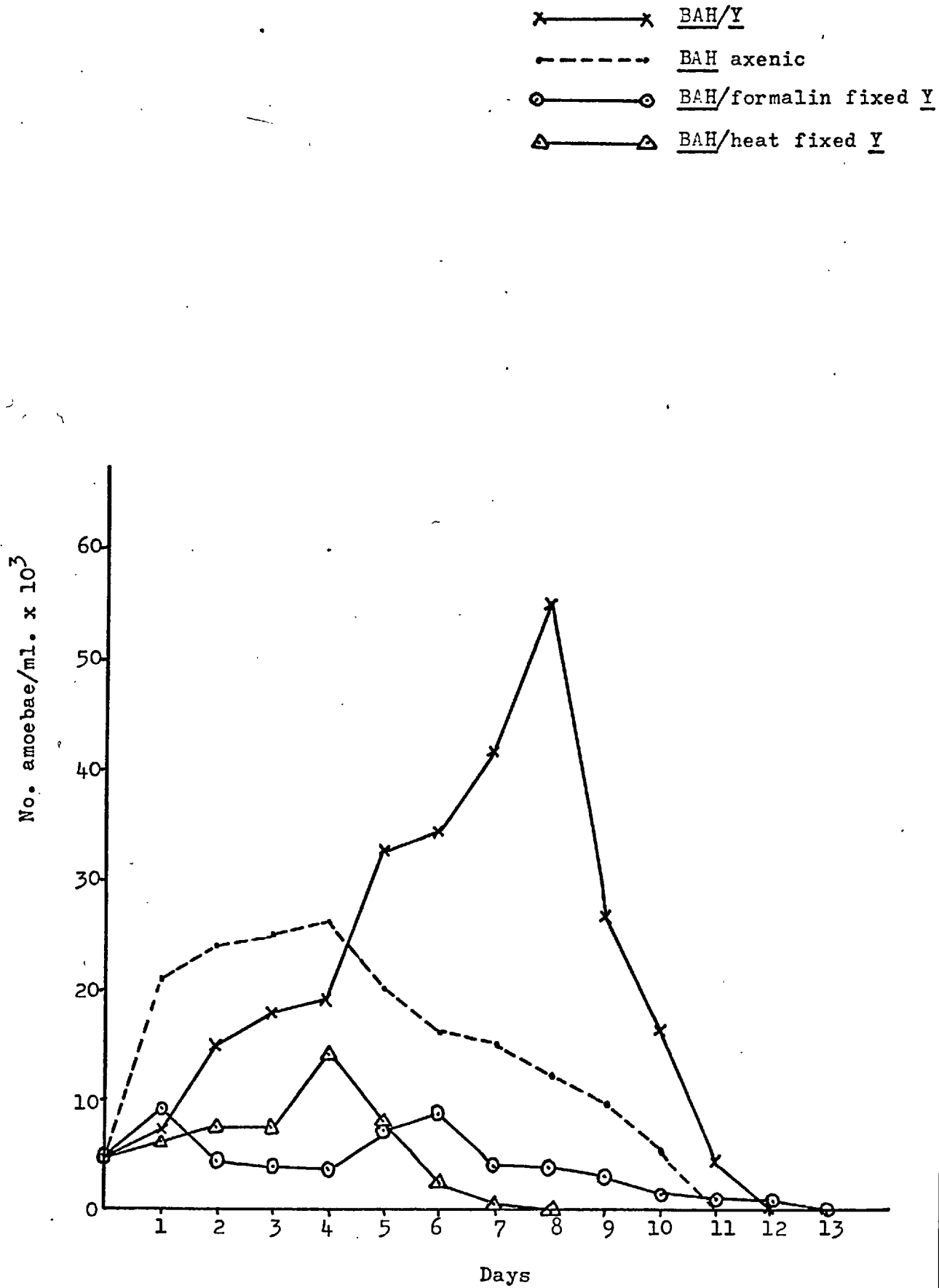


Figure 4 Growth of *E. invadens* (strain BAH) with formalin and heat fixed *T. cruzi* epimastigotes



are described by figure 4 together with growth curves of standard monoxenic (BAH/Y) and axenic isolates for comparison. Growth with dead epimastigotes killed either by heat or formalin fixation (0.01%) was very limited. The best yield was attained in association with heat fixed epimastigotes but this was not even a 3-fold increase. Growth with formalin fixed epimastigotes was most erratic. Both of these cultures, associated with dead epimastigotes, showed much poorer growth than the axenic culture which implied that the presence of the dead epimastigotes was actually detrimental to the amoebae.

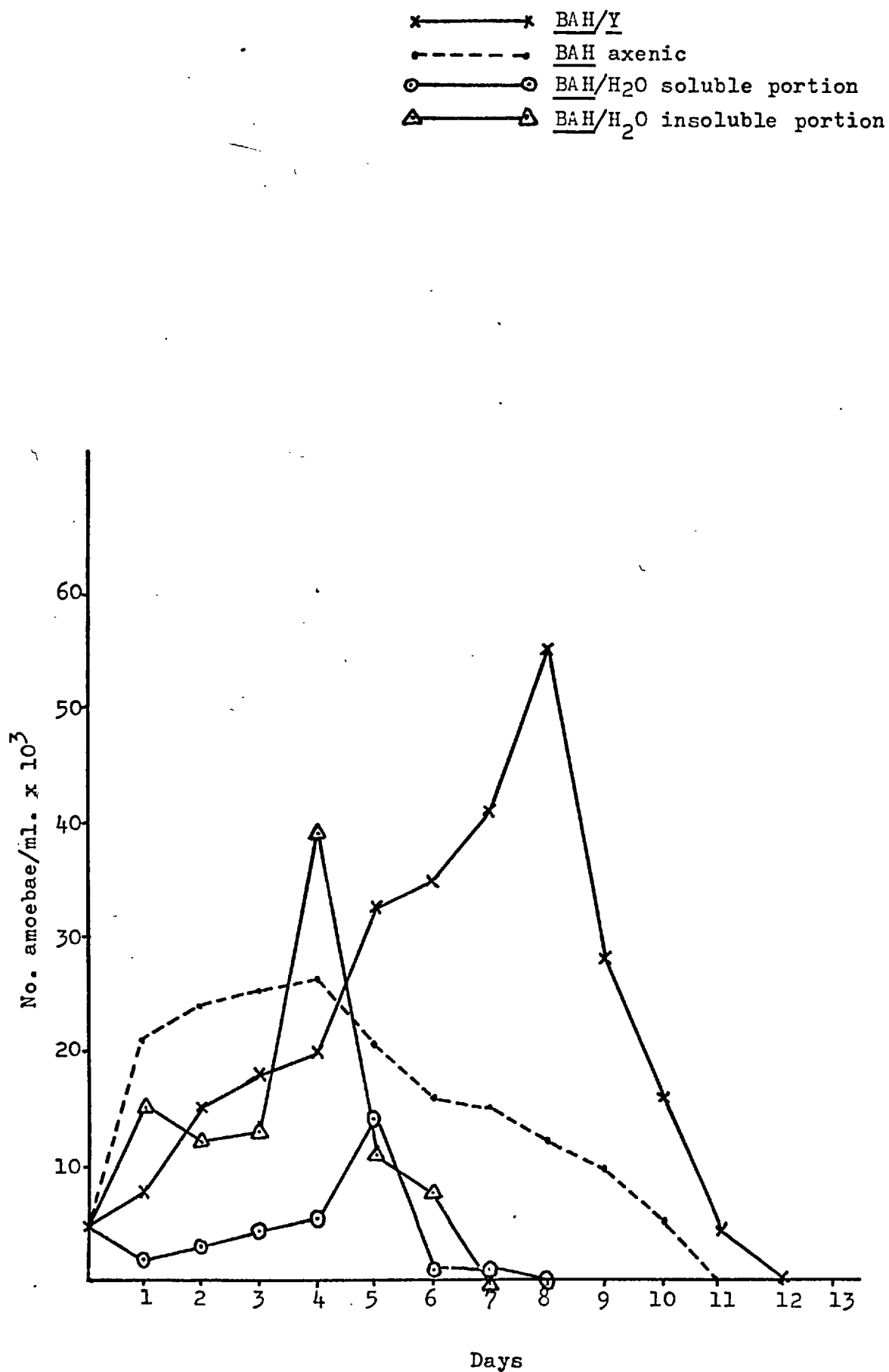
Growth of cultures associated with fractions of T. cruzi epimastigote cells is plotted in figure 5. Again growth curves of normal monoxenic and axenic cultures have been included for comparison. Growth with the water soluble portion of epimastigote was extremely poor and in fact worse than axenic growth. Nearly 3-fold multiplication took place but the lag phase and decline after peak growth were extensive. On the other hand growth with the water insoluble portion of epimastigotes indicated a definite advantage afforded by the associate. Maximum yield was one and a half times greater than that of axenic cultures and this was attained early. The lag phase was delayed by one day but was of short duration. However although growth was reasonable it was not as good as that afforded by association with the whole living epimastigote cells.

ii Other associates

a. Flagellates

Cultures associated with other flagellates besides T. cruzi

Figure 5 Growth of *E. invadens* (strain BAH) with water lysed trypanosomes



all incorporated the cultural stage; epimastigote for trypanosomes, and Crithidia, promastigote for leishmanias. Growth was obtained with each flagellate tried. Pan (1960) found support of amoebic growth was provided by a wide variety of flagellates.

Growth in association with various flagellates is described by figures 6 and 7. Excellent growth in terms of both yield and longevity was obtained in cultures associated with Crithidia sp. and L. tarentolae. The lag phases of both these cultures were too short to be defined by the measurements made. Peak growth with L. tarentolae was reached very early, day 3. The multiplication rate of Crithidia sp. associated cultures was not so great as L. tarentolae associated cultures and thus peak growth was attained two days later. Numbers of amoebae in both these cultures declined fairly steeply. The other two flagellate associates used in these experiments afforded adequate but not exceptional support for amoebic growth. Cultures associated with T. lewisi epimastigotes showed no pronounced lag phase but numbers declined after an early peak at day 4. Cultures associated with L. enrietti promastigotes had a very prolonged lag phase lasting seven days, before rapid multiplication to peak numbers a day later and an equally rapid decline thereafter.

Although it appears from the graphs that growth supported by Crithidia sp. and L. tarentolae was better than that supported by T. cruzi it should be noted that the Y strain of T. cruzi

Figure 6 Growth of E. invadens (strain BAH) with flagellate epimastigotes

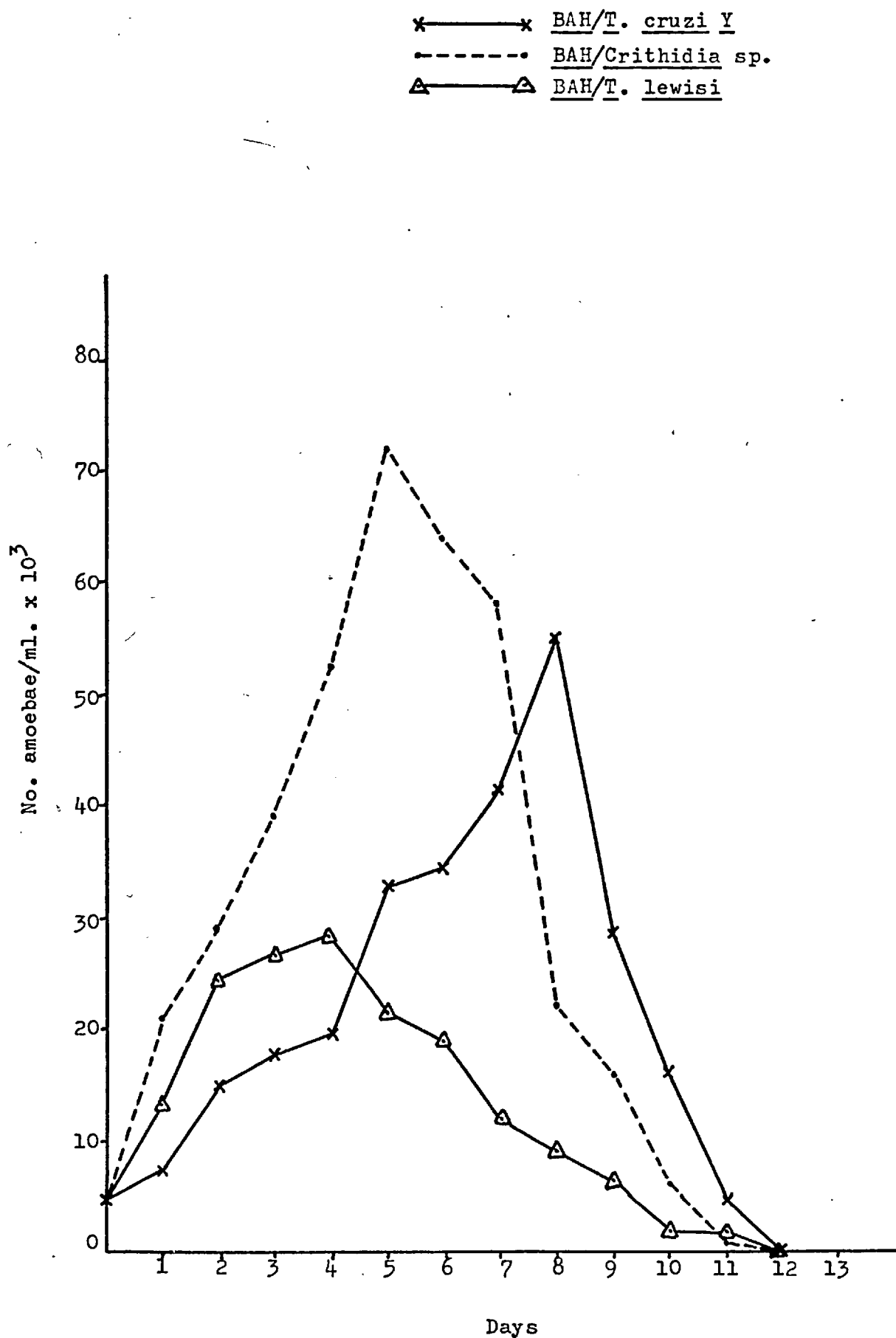
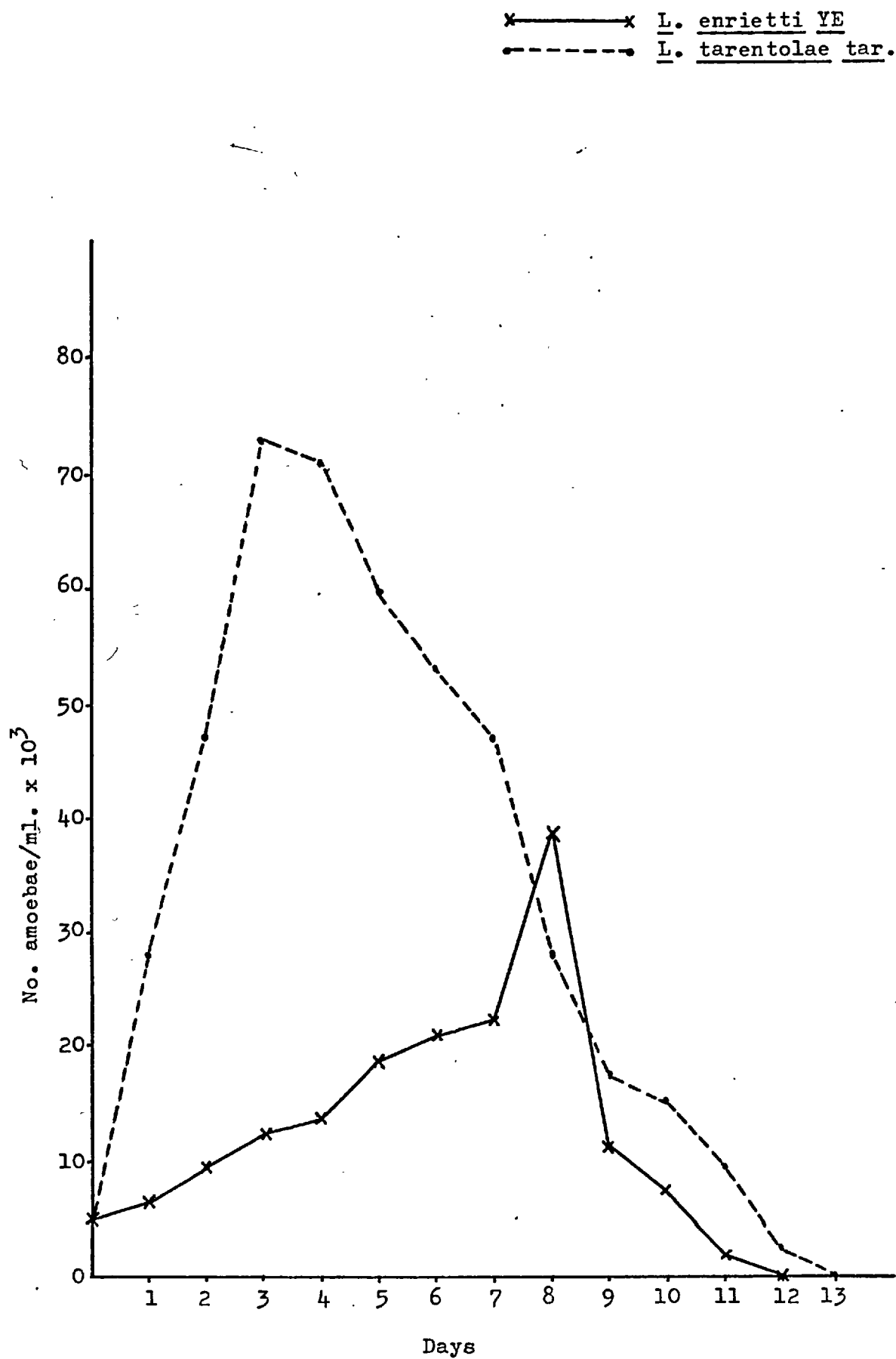


Figure 7 Growth of E. invadens (strain BAH) with flagellate promastigotes



was not outstanding in its amoebic support. Kessel strain of T. cruzi surpassed all other flagellates tried in this respect

b. Algae

Growth of cultures associated with various algae was disappointing. The growth curves are plotted in figure 8, together with the growth curve of a normal axenic culture of E. invadens. As may be seen in the figure none of the algal associates enhanced amoebic growth, in fact Euglena gracilis and N. coccoides appeared to be detrimental. Growth with O. danica was comparable with the axenic growth for the first two days but declined rapidly thereafter.

c. H. castellanii

R. strain of H. castellanii was used as an amoebic associate. Growth of this culture, as described by figure 9, was not satisfactory. Only five-fold multiplication of the original number of E. invadens took place and peak growth was reached very early at 2 days. A steady decline in numbers followed. This type of growth was again poorer than that shown by axenic cultures. Differential counts showed there to be at least 50% dead E. invadens present at day 2.

d. Tissue culture cells

Tissue cells of HEp2 strain, were used in suspension as amoebic associate. It was found during long term maintenance that fresh tissue cells added twice a week, instead of once a week, as with other associates, enhanced longevity. However so that some comparison might be made with the other associates

Figure 8 Growth of E. invadens (strain BAH) with algal associates

x—x axenic
- - - E. gracilis
o—o O. danica
Δ—Δ N. coccoides

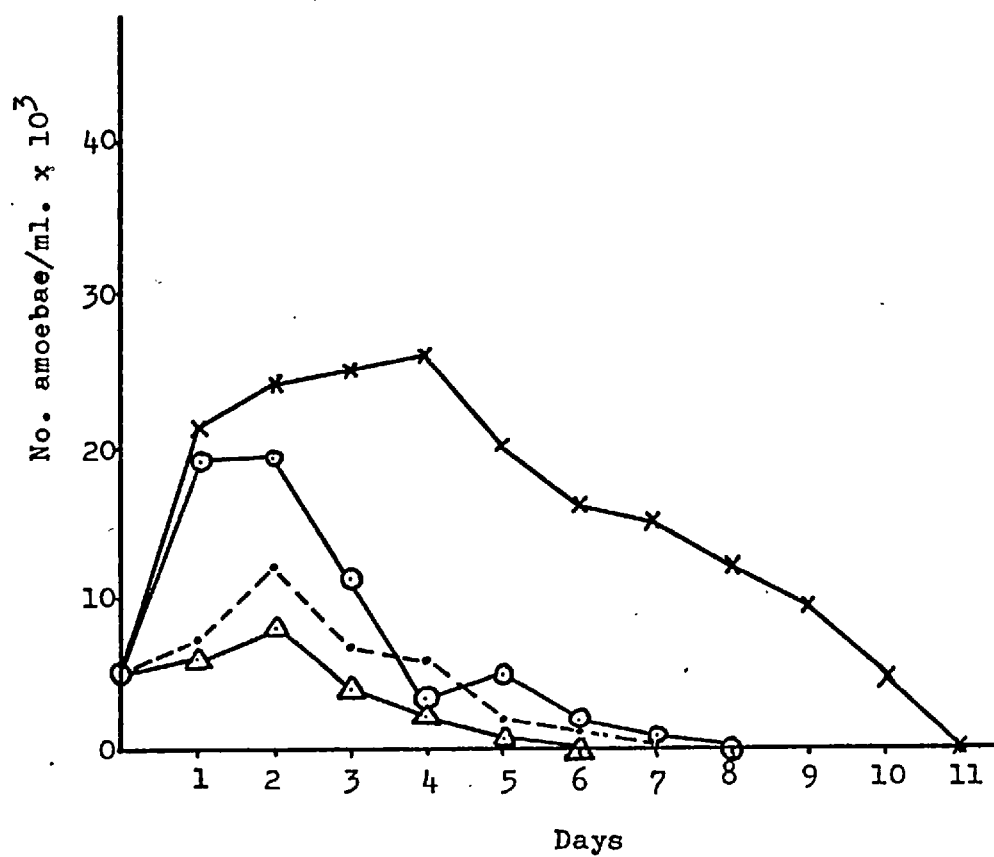
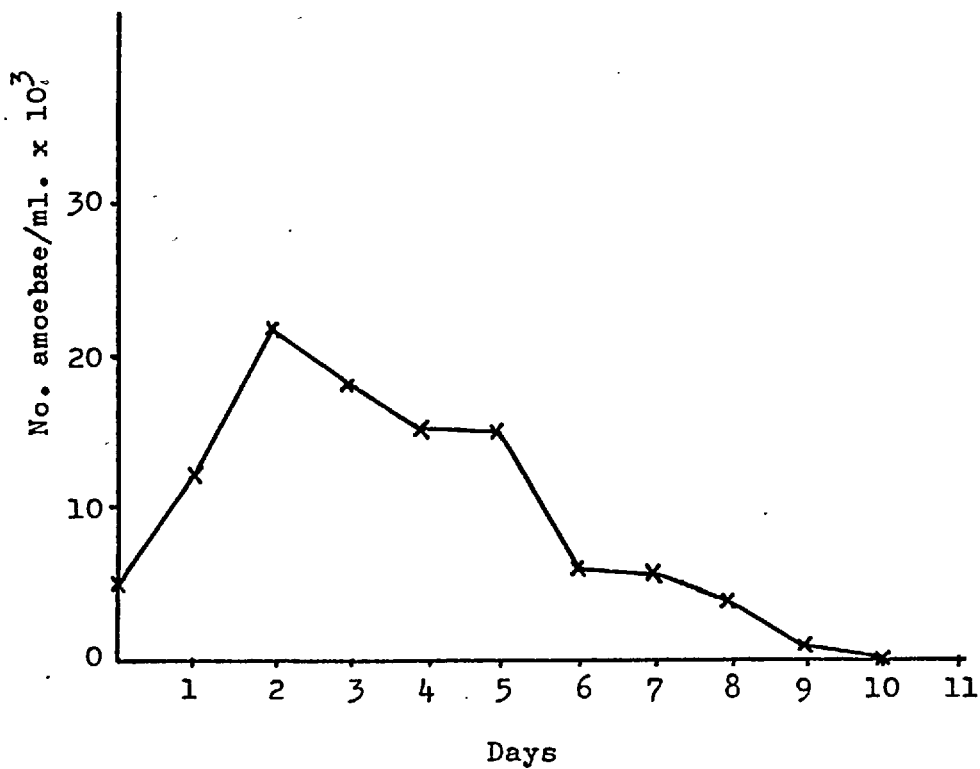


Figure 9 Growth of E. invadens (strain BAH) with
H. castellanii (strain R)



employed tissue culture cells were only added once at the initiation of the growth assessment experiment. The results, represented by figure 10, indicate that, although growth was satisfactory, it was not better than growth of axenic cultures. In fact in terms of longevity it was definitely poorer, showing far shorter survival of the greater numbers of amoebae expected after peak growth. A 2 day long lag phase delayed multiplication to a five-fold increase in numbers. Differential counts taken at this time, day 3, showed there to be 23% dead amoebae.

e. Red blood cells

Contrasting with E. histolytica (see chapter 4 pp.200) excellent growth of E. invadens associated with rabbit red blood cells was achieved. Cultures with two concentrations of this associate were set up and the results of their respective growth are shown in figure 11. Initial multiplication was good yielding a 6-8-fold increase in 2 days. There then ensued a lag phase which lasted for 2-3 days. Thereafter multiplication increased to a peak attained at day 7 for the higher concentration and day 8 for the lower concentration. A 15-20-fold increase was the average yield from these cultures and differential counts at peak growth revealed no more than 5% dead amoebae. Live healthy amoebae were often observed to contain vast numbers of red blood cells in their food vacuoles.

Discussion

The experimental results of this chapter have to be considered in the light of those reported for E. histolytica in

Figure 10 Growth of E. invadens (strain BAH) with tissue culture cells (strain HEp2)

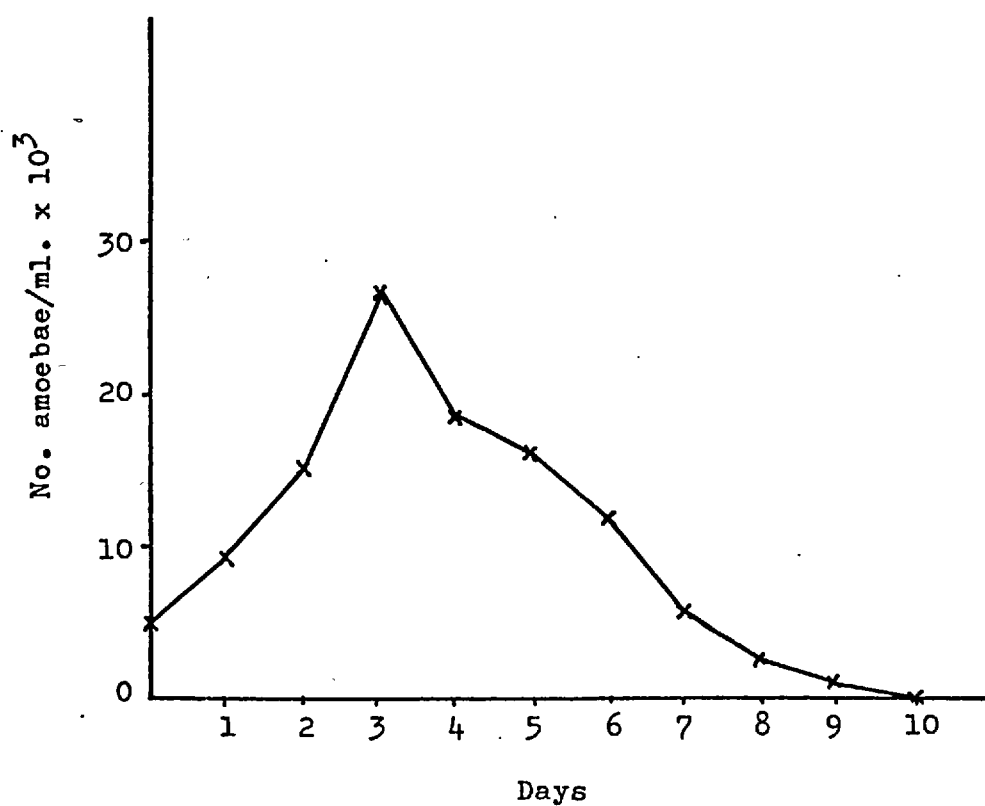
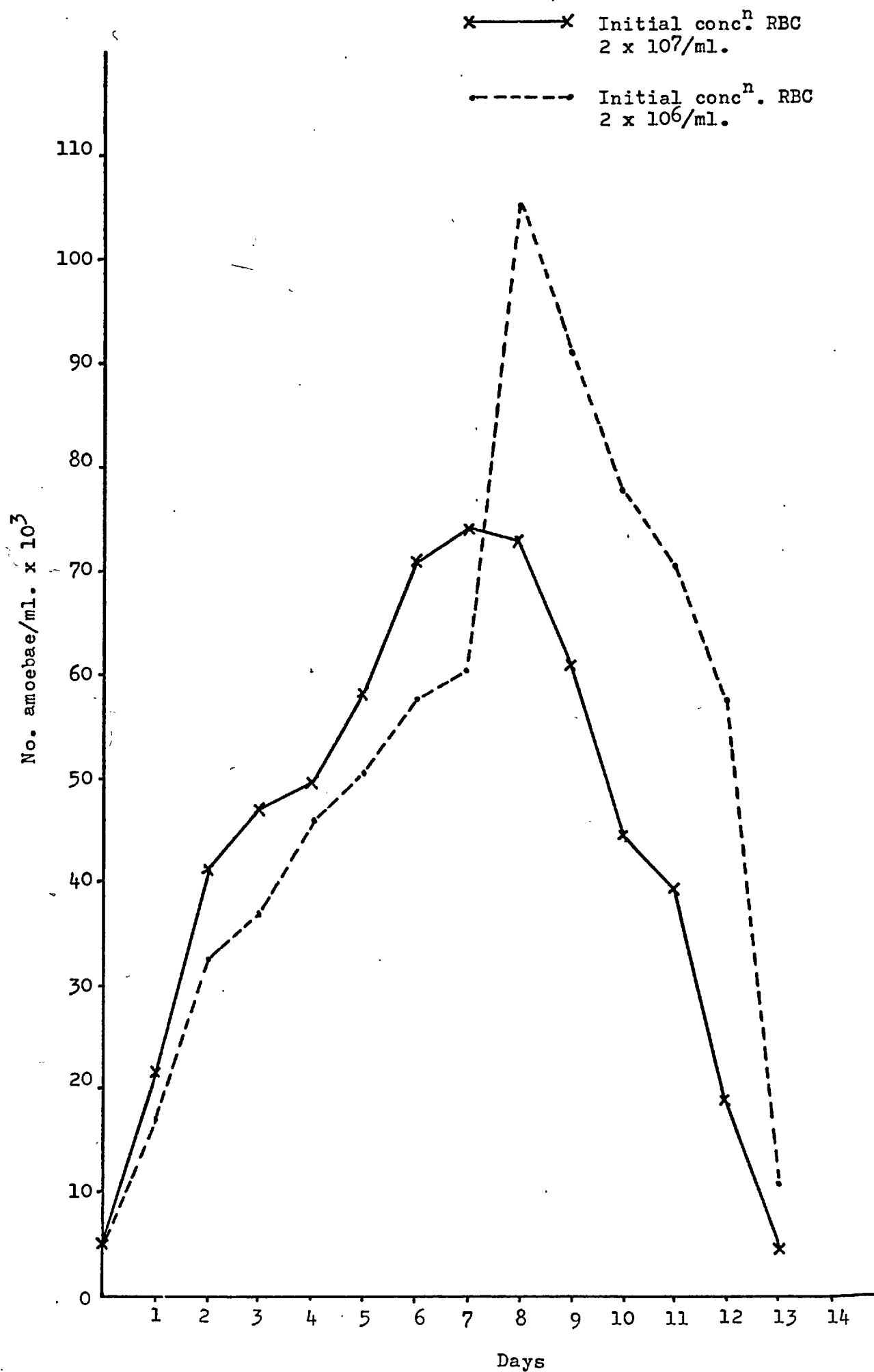


Figure 11 Growth of *E. invadens* (strain BAH) with rabbit red blood cells



chapter 3 and 4. Generally, growth of E. invadens was more readily obtained with unusual associates. However the facility of axenic growth of this species might tend to give misleading results unless careful attention is given to the growth curves. Axenic cultures of E. histolytica may be obtained by way of amoeba-trypanosome cultures but less easily than with E. invadens. The absence of the axenic facility in E. histolytica means a heavy reliance on the associate.

Differences between T. cruzi strains were shown by their amoebic support. The order of these differences was similar for both Entamoeba species, implying that both species were utilising the same factors in their metabolism. As associates were observed within amoebic food vacuoles, it may be assumed that certain benefit was derived by digestion of these cells. Benefit might also be derived by the environment containing associate metabolites. Further benefit would be derived through the inducement of phagocytosis, see pp 137 chapter 3. The various effects of different strains on growth of E. invadens were more marked than with E. histolytica. This could be a consequence of the living, multiplying associates at 25°C contrasted with their dying state at 37°C. Amoebic growth with both the Brazilian Y strain and Kessel strain of T. cruzi was similar and that afforded by Argentinian and Peruvian strains was also alike. Perhaps consideration of the geographical location of these strains is worthwhile. Peru, Argentina and Brazil are geographically different both in distance and altitude. The similarity between the Argentinian and Peru strains might be purely fortuitous. It is not surprising, therefore, that slight variations in the

T. cruzi epimastigote metabolism have been detected. Another possible source of metabolic variation between T. cruzi strains is the long cultivation of the Brazilian strains, contrasting with the recently isolated strains Argentinian M1 and Peru.

Results of growth with different morphological forms of T. cruzi are difficult to interpret when comparing growth of E. invadens and E. histolytica with amastigotes and trypanomastigotes. Growth of E. histolytica was excellent with amastigotes contrasting with poor growth of E. invadens. Experiments using trypanomastigotes gave converse results; good growth of E. invadens and very poor growth of E. histolytica. Further experiments were carried out to elucidate the problem. Various morphological forms of T. cruzi were kept in TP-S-1 at different temperatures and observed at intervals. Survival of epimastigotes and metacyclic trypanomastigotes at both 37°C and 25°C was similar for 3 - 4 days, thereafter degeneration occurred at 37°C. Amastigotes remained healthy and multiplied at 37°C for 7 - 10 days. However, at 25°C, they died and disappeared from the culture within 12 hours. Trypanomastigotes disappeared within 24 hours at 37°C but were still healthy after 7 days at 25°C. Pan (1968) conducted experiments on cultivation of T. cruzi at different temperatures in various media. Assessing the proportion of different morphological forms he found very similar results to those recorded above, using A-TPH medium (see chapter 2).

The conclusion from the Entamoeba growth with trypanomastigotes and amastigotes, is that both associates are suitable providing that the amoeba lives at a temperature which allows associate growth.

Growth of E. invadens with dead or acellular fractions of T. cruzi epimastigotes was slightly different from that of E. histolytica. Both entamoebae showed some growth with the water insoluble fraction. Growth with fixed epimastigotes varied depending on method of fixation. However since the growth was so poor little significance need be attached to these differences. However quickly the epimastigotes were killed by fixation, certain breakdown products would have been formed. The effect of these together with the absence of actively metabolising cells resulted in the poor amoebic growth.

Growth of E. invadens associated with the water insoluble cell fraction of epimastigotes was better than with fixed whole epimastigotes. Water lysis might cause less damage to cellular proteins than fixation. Even if both acellular fractions contained essential nutrients better growth would be expected with the particulate fraction due to the effect of non-specific phagocytosis. As the monoxenic amoebae have been shown to be particulate feeders, this mode of nutrition would be preferable to diffusion and pinocytosis. However, the difference between growth with the two fractions is so marked that some additional nutrient must be present in the water insoluble fraction.

The variation in growth of cultures supported by other flagellate species is no more marked than that of cultures supported by different strains of T. cruzi (see figure 1). Observations during the experiments help to explain slight species variations. Associates that continued to grow and multiply after association with the amoebae and medium, invariably gave rise

to good amoebic growth. Crithidia sp. was found to be very tolerant to different temperatures and media, and grow copiously in both E. invadens and E. histolytica cultures. Both amoebae species grew excellently in its association.

Since species belonging to the two genera, Trypanosoma and Leishmania, showed comparable variation in amoebic support, variation does not appear to be generic. However Pan (1960) using a larger number of species, found that better support was afforded by Trypanosoma species (see chapter 1). As Pan was using a less defined medium it is conceivable that there was more scope for metabolic variation of the associate.

Although some kind of support was afforded by three algal species, only Ochromonas is worth consideration. This was the only species used which, being facultatively photosynthetic, could survive in the dark. This accounts for better growth with Ochromonas. E. invadens was observed to ingest and grow in association with H. castellanii, which itself remained viable in the system, but the entamoebic growth was similar to that in axenic isolates. As discussed above (p 202) the reason for the failure produced with all these organisms is not known.

The tissue culture cells had no capacity for living in TP-S-1 medium, particularly at 25°C. Most of these cells were rapidly ingested by the amoebae, but occasionally dead tissue cells were found after 12 hours. Growth with one addition of tissue culture cells was only comparable to axenic growth but this could be enhanced by further additions. Negligible amounts

of nutrient in one addition might become significant on further additions.

It was remarkable that good support was afforded by rabbit red blood cells for E. invadens in spite of their lack of support for E. histolytica. The red blood cells degenerated within 24 hours exposed to the high tonicity and anaerobic environment of TP-S-1 medium. However the amoebae were seen to ingest the red blood cells avidly. There does not appear to be a feasible explanation for the contrasting results of the two Entamoeba species. In other culture systems, E. histolytica ingests red blood cells (Lowe & Maegraith, 1970) and also in vivo. The specific requirements of E. histolytica may well be the limiting factor in these circumstances.

CHAPTER 6 VIRULENCE OF E. HISTOLYTICA STRAINS IN MONOXENIC CULTIVATION

Introduction

No evaluation of a cultivation system of a parasite is complete without an assessment of the effect of the cultivation on the nature of the parasite. Probably one of the most important characteristics of a parasite is its pathogenicity to its host. When experimental animals can be used to measure pathogenicity, a standardised test can be used over a period of time and valid comparison made of results from such a test.

A review of the literature on virulence of E. histolytica grown in amoeba-trypanosome cultures was presented in chapter 1. This work was largely the studies of Phillips and coworkers (Phillips et al 1955, Phillips and Gorstein 1966). Wittner and Rosenbaum (1970) have also attempted to elucidate the type of virulence using axenic cultures of E. histolytica. Both workers conclude that bacteria associated with E. histolytica play an important part in the enhancement or induction of amoebic infection.

Chapter 6 is divided into two subsections. The first, 6a, describes a detailed study of virulence loss during prolonged cultivation of E. histolytica in amoeba-trypanosome cultures as developed earlier in this work. The second subsection, 6b, reports the results of experiments conducted in an attempt to elucidate the role of bacteria associated with the amoebae in virulence of E. histolytica.

a) Demonstration of virulence of amoeba-trypanosome cultures

Introduction

Two series of virulence tests were carried out using monoxenic amoeba-trypanosome cultures and the polyxenic stock culture from which they were derived. In the first series of experiments only one monoxenic isolate was used in order to assess its virulence over a period of maintenance in amoeba-trypanosome culture. The second series of experiments approached the problem in a slightly different manner. Several monoxenic isolates prepared from the polyxenic stock culture at varying time intervals were assessed on one occasion. In the second experimental series amoeba-crithidia cultivation was used. The second experimental series was to confirm the original findings using a different flagellate culture associate and to show that observations relevant to one isolate were relevant to several similar isolates of E. histolytica. It was also hoped that a more detailed account of virulence loss would be obtained than had been shown in the results of the first experiments.

Experimental results of virulence studies on amoeba-trypanosome isolates

The methods used for initiation and maintenance of the monoxenic isolates of E. histolytica have been described in chapter 2 pp 46-51. The virulence assessment test chosen for these experiments was caecal inoculation of weanling rats. In addition the second series of experiments was repeated using intrahepatic inoculation of weanling hamsters (for methods see pp 81). The methods involved in this procedure

and the subsequent assessment of infection have also been described in chapter 2 pp 82.

The results of two long term experiments have been described in tables 1 and 2. Three terms of virulence assessment were made to give a complete picture of virulence degree. Control rats inoculated with a suspension of T. cruzi epimastigotes in TP-S-1 never developed caecal abnormalities. Although the precise terms of these two experiments are not directly comparable, the pattern of virulence loss shown by the two strains is similar. Biswas strain had become non-invasive after a period of a very few months of amoeba-trypanosome association and no specific lesions developed, the average caecal score being less than 2. This invasiveness was only slightly improved by the use of an excessively high amoeba inoculum. The virulence history of Rahman strain in amoeba-trypanosome culture was of a similar calibre to that of Biswas; after only eight weeks it gave a caecal score of 2.2 and invaded 7/10 rats. Strictly speaking this is still an invasive strain. As monoxenic cultivation of the strains was maintained, so invasiveness decreased steadily. Greater loss of virulence of the Rahman strain occurred than of the Biswas strain. By two years of monoxenic cultivation every virulence assessment was lower for Rahman strain than Biswas strain. Even after a period of nearly four years monoxenic maintenance, Biswas strain amoebae invaded 2/10 rats and viable amoebae were found in 7/10 rats. The Rahman strain long term experiment was not continued for

Table 1 Virulence of amoeba-trypanosome culutres of Biswas strain

No. of subcultures	Period of maintenance in weeks	No. amoeba x 10 ⁴ inoculated per rat	Average caecal score of infected rats	No. rats invaded	No. rats infected
Polyxenic	controls	5	2.8	10/10	10/10
45	14	5	1.9	8/10	10/10
45	14	20	2.1	8/10	10/10
Polyxenic	controls	5	2.9	10/10	10/10
111	54	5	1.6	6/10	10/10
111	54	20	1.7	6/10	10/10
Polyxenic	controls	5	3.0	10/10	10/10
223	95	5	1.4	4/10	10/10
223	95	81.6	1.44	4/9	8/9
Polyxenic	controls	5	3.2	10/10	10/10
445	206	5	0.9	2/10	7/10
445	206	50	0.9	3/10	6/10

Table 2 Virulence of amoeba-trypanosome cultures of Rahman strain

No. of subculture	Period of maintenance in weeks	No. amoebae x 10 ⁴ inoculated per rat	Average caecal score of infected rats	No. rats invaded	No. rats infected
Polyxenic	controls	5	3.1	9/10	10/10
24	8	5	2.2	7/10	10/10
24	8	50	1.8	5/10	9/10
Polyxenic	controls	5	3.4	21/22	22/22
130	63	5	1.25	3/22	16/22
Polyxenic	controls	5	3.2	9/10	10/10
210	101	5	1.1	2/10	9/10
Polyxenic	controls	5	3.1	10/10	10/10
268	134	5	0.4	1/10	3/10

as long as that of Biswas strain, since the virulence of the monoxenic isolate of 134 weeks standing was almost lower than could be reliably assessed.

A second type of experiment, utilising a number of amoeba-crithidia isolates obtained from the same polyxenic stock culture, was carried out. Virulence assessments were made at one time in a similar manner to that of the first experiments. Results have been presented in table 3.

Although the number of rats infected with Rahman strain isolated for only one week in monoxenic culture was the same as with the polyxenic stock and only one fewer rat was invaded by the monoxenic isolate, the caecal score was greatly reduced. Monoxenic isolates of two and three weeks' culture maintenance produced comparable caecal scores to their one week counterpart, but the proportion of rats whose caeca were invaded by the amoeba was lower. Monoxenic isolates which had been maintained for four or more weeks could be termed non-invasive on grounds of the low average caecal score. The caecal score of rats inoculated with amoebae from monoxenic cultures of several months' standing, was considerably lower than that of rats inoculated with up to one month culture amoebae. The number of rats, inoculated with amoebae from older isolates, whose caeca contained lesions, was about half that produced by more recently isolated amoebae. The very old isolate of over two years' continuous cultivation was also tested at the same time as the amoebae from cultures especially isolated for this experiment. The virulence

Table 3 Virulence of various amoeba-crithidia culture
isolates of Rahman strain

No. of subculture	Period of maintenance in weeks	No. amoebae $\times 10^4$ inoculated per rat	Average caecal score of rats infected	No. rats invaded	No. rats infected
Polyxenic	control	5	3.1	10/10	10/10
2	1	5	2.1	9/10	10/10
4	2	5	2.2	6/10	10/10
6	3	5	2.0	6/10	10/10
8	4	5	1.8	6/10	9/10
24	12	5	1.3	5/10	10/10
26	13	5	1.22	3/10	9/10
28	14	5	1.2	3/10	10/10
50	24	5	1.1	2/10	9/10
268	134	5	1.1	1/10	3/10

assessments for amoebae of this culture have been included in table 2 as part of the first series of experiments. Comparison with its newer counterparts in monoxenic isolation in this experiment, showed that its virulence was lower than newer isolates in every respect, in particular in the number of rats in which amoebae could be found. One point that should be emphasised during consideration of the results of this last experiment, is that none of the monoxenic isolates of less than ten weeks' standing were confirmed for bacterial sterility. As it was necessary to supplement cultures with antibiotics until six subcultures were completed, antibiotic inhibited bacteria were probably present in the newer isolates to a greater or lesser extent.

This latter experiment was also carried out using intra-hepatic inoculation of weanling hamsters as virulence test. The results are described in table 4. Compared with the rat caecum virulence test, this proved to be far less sensitive. No significant amoebic abscesses resulted from inoculation with amoebae grown in association with crithidia species. However, the very recent isolates were able to produce slight abscesses and to survive in the hamster livers.

Table 4 Virulence of various amoeba-trypanosome culture isolates of Rahman strain to hamster livers

Inoculum: 1×10^5 amoebae/hamster

No. of subculture	Period of maintenance in weeks	No. hamsters with abscesses	No. hamsters infected	Average abscess weight
Polyxenic	control	9/10	10/10	0.67gm.
4	2	7/10	7/10	<.1gm.
8	4	1/10	2/10	<.1gm.
26	13	0/10	0/10	0
28	14	0/10	0/10	0
50	24	0/10	0/10	0
268	134	0/9	0/9	0

Discussion

The two series of experiments reported here have shown the phenomenon of virulence loss to rat caeca occurring in strains maintained in amoeba-trypanosome and amoeba-crithidia association. Phillips (1962) had reached a similar conclusion using inoculation of guinea pig caeca. Virulence loss of Biswas strain was less marked as it was still slightly invasive after four years of monoxenic cultivation. No other strain has been reported to have any virulence whatever after such a long period in monoxenic cultivation. The apparent degree of virulence could not have been due to the physical trauma of the inoculation or any other factor apart from amoebic activity. The results of almost normal caeca after inoculation of Rahman strain amoebae of two years' monoxenic standing, are similar to observations of other workers (Phillips et al 1958, Wittner, Rosenbaum & Einstein 1970).

During any kind of cultivation the organism is being subjected to very standardised conditions. Often, probably invariably, these conditions are suboptimal compared with those within the natural host. As a result of these two factors a strain of organism will change over a period of time. This change occurs by variation and selection by the cultural conditions. Individuals with a slight advantage gradually make up the predominant part of the population. However, when these individuals are put into a strange environment they may be incapable of dealing with the new environment. Neal and Vincent (1955) suggested that the

existence of a factor in E. histolytica encouraged tissue invasion. Cultures whose virulence had been lost by long-term polyxenic cultivation were passaged through hamster livers and virulence was reinstated. Possibly this factor was lost more easily in amoeba-trypanosome and amoeba-critidia cultures.

The experimental results of this subsection have only shown in detail that virulence is lost under circumstances of monoxenic cultivation. No real reason for this occurrence can be concluded from the results. It could be selection of a part of the population in preference to the rest or it could be the absence of bacteria. However, one noteworthy result was that virulence loss occurred gradually and apparently immediately after initiation of monoxenic cultures. Selection of individuals from the population is unlikely to have occurred as rapidly as bacterial eradication. Phillips and Gorstein (1966) found association of amoebae with different species of bacteria altered E. histolytica virulence.

b) Attempts to enhance virulence

Introduction

The results reported in the last subsection demonstrated the loss of virulence by E. histolytica isolated and maintained monoxenically. In the experiments described in this subsection, it was hoped to elucidate the cause for the initial rapidity of virulence loss.

As already mentioned in chapter 1, several authors have emphasised the role of concomitant bacteria in E. histolytica virulence (pp 22). Wittner and Rosenbaum (1970), using axenic amoebae cultures, showed that virulence could be restored by live bacteria. Experiments of amoebic reassociation with bacteria must of necessity be assessed using intrahepatic inoculation, since the presence of bacteria in the lumen of the gut would confuse any conclusions that might be made.

Experimental results of attempts at restoration of virulence

For these experiments the long established strain of Rahman/ReF.1:PRR was used as this showed the least degree of virulence to rat caeca and none to hamster liver (see tables 2, 3, and 4, chapter 6a). The groups of bacteria used for reassociation together with full details of the methods involved, have been described in chapter 2 pp 83. One group of bacteria received an antibiotic treatment. Intrahepatic inoculation was carried out as described previously (chapter 2 pp 81).

Results of these experiments have been described in table 1. Excepting the group associated with antibiotic treated bacteria, amoebic virulence was restored to some degree.

Table 1 Effect of Reassociation of E. histolytica (Rahman)
with bacteria on virulence to hamster livers

Associated bacteria	Length of association in hours	No. hamsters with abscesses	No. hamsters infected	Average abscess weight
Polyxenic	control	9/10	9/10	0.56gm
<u>E. coli</u> strain <u>B</u>	18 ¹ / ₂	3/10	3/10	<0.1gm
<u>E. coli</u> strain <u>A'</u>	18 ¹ / ₂	8/10	7/10	<0.1gm
<u>E. coli</u> strain <u>A'</u>	12	3/10	3/10	<0.1gm
<u>E. coli</u> strain <u>A'</u>	4	0/10	2/10	0
Unknown mixed bacterial flora from the <u>Rahman</u> stock	18 ¹ / ₂	8/10	8/10	<0.1gm
Unknown mixed bacterial flora from <u>Rahman</u> stock inhibited by antibiotics	18 ¹ / ₂	0/10	0/10	0

Lesions were generally so small that it was difficult to be certain that they were of an amoebic character rather than bacterial. Bacteria were present in all the livers although amoebae had been washed prior to inoculation. Both microscopical examination and cultivation confirmed the survival of E. histolytica as described in the table. The two strains of E. coli varied in their virulence enhancement. The unknown mixed bacterial flora gave best results but even these stood no comparison with the polyxenic stock culture used as controls. No abscesses, nor amoebae, nor bacteria were found in the livers inoculated with amoebae associated with antibiotic inhibited bacteria.

Using E. coli strain A the length of time of association with E. histolytica was of importance. Four hours' association did no more than allow survival of E. histolytica in two cases.

Discussion

Wittner and Rosenbaum (1970) criticised the studies of Phillips and Gorstein (1966) on the grounds that bacterial pathogenicity itself apparently modified their results. Wittner and Rosenbaum used nonpathogenic bacteria but nowhere in their experimental findings do they confirm this lack of pathogenicity or infectivity. Neither the size of amoebic abscesses nor the severity of liver invasion was reported in their paper. However, autopsy was performed at 14 days contrasting with the 4 days used in the experiments reported here. Michel and Westphal (1970a) confirmed that extra-intestinal infection of hamsters with bacteria free

E. histolytica produced no abscesses. They later found (1970b) that axenised amoebae were larger and showed a marked increase in enzyme activities compared with their bacteria associated counterparts.

Using the measurement, number of hamsters with abscesses, the results reported here and those of Wittner and Rosenbaum are similar. This measurement shows that E. histolytica virulence has been enhanced. It does not provide equivocal proof that the reason for this improved virulence is solely the characteristics of the E. histolytica. These results could be caused by the provision of a suitable environment for E. histolytica by bacterial induced necrosis. The amoebae themselves could then invade and multiply in the necrotic tissue.

Even though the hamsters used in the experiments, reported here, were immunised against the bacteria likely to contaminate the amoebic inoculum, they still developed abscesses containing bacteria. The abscesses might have been caused by the amoebae and the bacteria simply survived within them, or the process might have occurred in reverse. As the abscesses were so small compared with the controls it would appear that any virulence factor conferred on the amoebae by the bacterial association pretreatment was small. Alternatively the significance of the pretreatment lies in the provision of a suitable environment for amoebic survival. The time of bacterial association was of particular significance in increasing amoebic infectivity and invasiveness. This

indicates that sufficient time is required for ingestion or absorption of substances from the bacteria in order to enhance virulence. It seems likely that both provision of suitable environment and conference of a virulence factor apply.

The result that no benefit was derived from association with antibiotic inhibited bacteria, in a way parallels the rapid drop in virulence that occurred in very recently monoxenically isolated cultures. The bacteria could not assist amoebic virulence by producing infection in the liver and apparently no transference of a "virulence factor" took place.

CHAPTER 7 GENERAL CONCLUSIONS

Many cultivation systems are available for E. histolytica. Some of these have been used extensively to study the nutritional requirements of the parasite. The disadvantages of the polyxenic cultures are obvious; it being almost impossible to distinguish amoebic from bacterial activity. Of the monoxenic systems, the use of inhibited bacteria as associate has received considerable attention and has been correspondingly rewarding. Some detailed study on the use of flagellates as sole associate for E. histolytica was made by other workers, but a true quantitative evaluation was not made. Some qualitative aspects also, were not studied. Therefore the use of this system has been restricted because of the variation that might occur due to insufficient knowledge of the system.

The monoxenic, flagellate associate, system represents the intermediary stage in preparation of axenic cultures. It might therefore appear that no detailed interest in this stage is necessary as an axenic culture is theoretically ideal. Unfortunately axenic cultivation of E. histolytica has certain disadvantages, the greatest one being practical. Few workers have found axenic cultures simple to prepare and maintain. Another disadvantage is the fact that the amoeba appears to have lost some of its essential characteristics. The total dependence on pinocytosis, absorption, diffusion and adsorption contrasts greatly with particulate feeding. Although particulate feeding might only represent one part of the organism's method for obtaining nutrients it obviously plays an integral part. It is this particular change in habit and probably metabolism also, that requires the period of adaptation experienced during axenisation.

As the mode of feeding in amoeba-trypanosome culture is particulate the amoebae are closer counterparts to the parasites in vivo than axenic amoebae. Another disadvantage of axenic cultivation is the virulence loss. This is also characteristic, although not so extreme in amoeba-trypanosome cultures.

One advantage of the amoeba-trypanosome system is the insensitivity of T. cruzi to compounds. Therefore the system is good for chemotherapy.

The physical and chemical requirements of the amoebae not only represent the in vitro requirements of cells, such as pH salt content and glucose, but also specific amoebic requirements, such as high serum and protein content. Very probably the media used in this study provide an excess of most constituents but it would be tedious to find the minimal requirements.

The biological components of the amoeba-trypanosome cultures are dynamic components. Not only do they contribute to the system but also they take their share of nutrient. It has been demonstrated that physical contact between the amoebae and the flagellates is necessary for amoebic support thereby confirming the amoeba's dependance upon particulate feeding in this system. The flagellates provide the amoebae with nutrient and also have a toxic effect, as was shown by the effect of too high a concentration. Therefore an optimum range of epimastigotes to one amoeba should be used. For reproducibility the same initial concentration of epimastigotes must be used throughout. Although support for amoebic growth varied from strain to strain of T. cruzi epimastigotes, the degree of support was of a similar order. The

same can be said for the different stages of the T. cruzi life cycle tried in the experiments. The support afforded for amoebic growth is not limited to T. cruzi or even to flagellates.

Ochromonas is noteworthy because of the phylogenic correlation between these algae and zooflagellates.

The physiological state of the flagellate is of particular importance. The living flagellate affords best support although a degree of support is provided by dead or part organisms.

An interesting result was the variation with which various isolates of E. histolytica could be isolated in amoeba-trypanosome culture. The general rule that the degree of virulence correlated with the degree of culturability does not hold true for Laredo strain. This could help to justify a separate species for this and similar strains although this is not sufficient evidence by itself.

The degree of culturability associated with invasiveness rule, only applies to E. histolytica. Other entamoebae which are nonpathogenic such as E. ranarum and E. moshkovskii grew excellently in amoeba-trypanosome culture. The only entamoebae that did not grow in this system probably were too small to engulf the associates, with the exception of E. coli.

The problem of the invasive characteristic and its loss during prolonged cultivation is complicated and far from explained. The absence of encystation and bacteria might contribute to the explanation. Certainly in the case of axenic amoebae the loss of particulate feeding must be significant. The work has shown that the presence of bacteria is significant but no further explanation can be given.

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