Studies on the Cation Requirements of Entamoeba Histolytica.

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ABSTRACT

Much interest has been devoted to investigations of the physiology and biochemistry of the pathogenic, parasitic protozoan, *Entamoeba histolytica* as a means of providing a biochemical basis for the chemotherapy of amebiasis. In order for growth and reproduction to occur the ameba must be furnished with all of the substances essential in the synthesis and maintenance of its protoplasm, a source of energy, and suitable environmental conditions. These comprise the minimum growth requirements and are most important in studying the physiological aspects of *E. histolytica*. Like all other organisms *E. histolytica* requires certain inorganic salts for growth; however, the specific nature of these inorganic requirements and their metabolic roles have not been evaluated. This work constitutes a beginning of such a study. It was first necessary to develop a medium which allowed for relatively complete control of the inorganic constituents of the culture fluids. This was successfully accomplished by substituting amino acids for the natural peptones in the base medium, thus providing a particularly suitable medium for this type of investigation. In order to obtain some preliminary information
about the amebae's cation requirements experiments were carried out in which the calcium, magnesium, iron and zinc were either excluded as far as possible from the media or added singly and in various combinations. The need for these cations was assessed by their effect on amebae growth. It became apparent in the initial investigations that zinc was dispensable as an addendum to the medium. Added calcium, magnesium and iron were found necessary for maximal growth of amebae. Evidence that iron is essential for amebae growth was conclusively demonstrated.

More precise knowledge concerning the active form of the iron requirement was obtained by means of iron chelating agents. The ferric iron chelators, CHEL DP and Versene Fe-3 Specific, were found to have a slight or no inhibitory effect on amebae growth. On the other hand, the ferrous iron chelators, o-phenanthroline and chiniofon, had a pronounced amebicidal effect which could in turn be reversed by adding an excess of iron. The optimal iron concentration for amebae multiplication was found by directly varying its concentration. In an effort to determine if iron was strictly indispensable attempts were made to replace the iron in the medium with other cations. Of the nine cations tested none could be successfully substituted for iron in the medium. An investigation of the possible manner in which the iron is utilized in the amebae cultures was made by supplying
the iron in the form of iron-containing organic substances such as hemin, cytochrome c and peroxidase. The evidence obtained indicated that the iron supplied in this form was not more effective than the added iron salt.

Biochemical characteristics of *E. histolytica* have not yet been defined in pure culture. Some studies have been reported for amebae grown with an associated organism; however, these are necessarily more complicated and difficult to interpret. The present finding of a relatively great iron requirement was examined to ascertain whether the effect of the added cation was directly upon the amebae, the associate organism, or both. *Bacteroides symbiosus*, the bacterial associate employed in this study, retains some glucose utilization activity although its reproduction in the amebae cultures is inhibited by penicillin. Work was directed towards an investigation of this metabolic activity with particular interest focussed on the influence of iron. A comparison was made of the glucose utilization by the bacteria alone and parallel amebae-containing cultures. These studies indicated that iron plays an important role in glucose metabolism of penicillin-inhibited cells of *B. symbiosus*. This finding leaves unanswered the question of whether the added iron acts upon the amebae directly or indirectly through its effect upon bacterial metabolism.
I INTRODUCTION

A. Historical review

It is believed that to Lösch (1875) belongs the credit for the discovery of the pathogenic species of amebae now known as Entamoeba histolytica. While examining the stools of a patient suffering from a type of relapsing dysentery in St. Petersburg, Russia, Lösch found motile amebae, many of them containing red blood corpuscles. He successfully produced a typical amebic dysentery in a dog employing stools containing the motile amebae. Despite the evidence furnished by this successful experiment Losch did not regard the amebae, which he named Amoeba coli, as the cause of the dysentery. Rather, he suggested that these parasites might delay or prevent healing of the intestinal ulcerations which were present.

Following the discovery of Lösch there appeared in the literature reports of cases of dysentery in Egypt, some of them complicated with abscesses of the liver, in which amebae were demonstrated and believed to be identical with those described by Losch (Kartulis, 1886, 1887). Osler (1890) was the first to demonstrate amebae in a case of dysentery in the United States and his observations were followed by those of Stengel (1890), and of Dock (1891)
and Musser and Willard (1893). Harris (1901) was the first investigator to produce an abscess of the liver in an experimental animal after the production of dysentery by the rectal injection of *E. histolytica*.

Councilman and LaFleur (1891) at Johns Hopkins Hospital published a monograph on amebic dysentery in which they concluded that this disease is a clinical entity and that it is characterized by definite pathologic lesions produced by the amebae. They believed, furthermore, that both pathogenic and nonpathogenic species of amebae inhabited the intestine of man. A clear description of *Entamoeba histolytica* was given by Quincke and Roos (1893) who first described cysts of this parasite. However, they failed to study closely the morphology of the motile and encysted forms. These investigators clearly differentiated the pathogenic species from the common nonpathogenic species now known as *Entamoeba coli*. The first accurate description of the cysts of *E. histolytica* was given by Huber (1903).

Many authorities still refused to accept the amebae of Lösch as the etiological factor in dysentery or liver abscess due to the confusion caused by the presence of amebae in the feces of healthy individuals and those suffering from many diseases. It was not until Schaudinn (1903) published his researches on amebic dysentery that it became generally accepted that a pathogenic and a nonpathogenic species of amebae were parasitic in the
human intestine. This observation of Schaudinn was confirmed in whole or in part within the next few years by several investigators (Craig, 1905; Walker and Sellards, 1913). Schaudinn gave good descriptions of the trophozoites of the two species, but gave an erroneous account of the method of reproduction of the pathogenic species and entirely missed observing the cysts of this parasite. He placed the two amebae of man in the genus Entamoeba established by Casagrandi and Barbagallo in 1895 and gave the name Entamoeba histolytica to the pathogenic species and Entamoeba coli to the nonpathogenic species. The recognition of the true character of amebiasis as distinguished from the phase known as amebic dysentery came gradually. Craig (1921) stimulated much interest in the subject and today it is recognized that amebiasis is responsible for many clinical symptoms far different from those of acute or chronic amebic dysentery.

Several early workers made unsuccessful attempts to cultivate E. histolytica (Penfold et al., 1916; Yoshida, 1919). Although Cutler (1913) reported a method for culturing E. histolytica his results were not confirmed. Hence, the credit for the first successful cultivation is usually given to Boeck and Drbohlav (1925) who employed slants of inspissated whole egg overlaid with serum-glucose-Locke solution to maintain polybacterial cultures. After the publication of Boeck and Drbohlav several modifi-
cations became known. Dobell and Laidlaw (1926) conducted an extensive study on *E. histolytica* and other amebae from the human, using essentially the same medium but substituting particulate rice starch for glucose. Cleveland and Collier (1930) used liver infusion agar slants overlaid with serum and saline. Nelson (1947) introduced slants of agar containing alcoholic extracts of egg or mammalian tissues and covered with saline. Balamuth (1946) developed a monophasic medium consisting of buffered aqueous egg yolk infusion to which liver extract was sometimes added. Many other modifications were made in subsequent studies but the general pattern remained unchanged.

The media thus far developed for *E. histolytica* all contain highly complex materials such as egg yolk infusion, agar, serum, rice powder, organ extracts, and others. In developing culture media many workers made no attempts to elucidate any specific nutritional requirements or to ascertain the minimal amounts of the several ingredients, since the media were designed solely for the purpose of obtaining abundant or continuing growth of the trophozoites. Such undefined media were usually further complicated by the presence of living associate organisms which might consist of either a mixed bacterial flora (Boeck and Drbohlav, 1925; Cleveland and Collier, 1930); a single bacterial species (Chinn *et al.*, 1942); another protozoan, *Trypanosoma cruzi*
(Phillips, 1950); or living tissue (Lamy, 1948; Shaffer and Sienkiewicz, 1952).

Much effort is now being devoted to the search for simpler media which, while supporting good amebae growth, require fewer manipulations and would allow a considerable degree of control over the constituents of the culture fluids. The Shaffer-Frye (S-F) technique for the cultivation of *E. histolytica* involves preconditioning a thioglycollate-agar-containing medium by growth of a streptobacillus. Penicillin and horse serum are added to the cultures at the time of inoculation and a petrolatum seal used for protecting the medium from air. A modified Shaffer-Frye (MS-F) technique has been developed which offers advantages in ease of manipulation and adaptability to experimentation (Reeves, Meleney and Frye, 1957). In the modified procedure a sterile base medium and a small volume of bacterial culture or washed bacterial cells are substituted for the centrifuged, bacteria-containing supernatant fluid of the original S-F procedure. The troublesome vaseline seal is avoided by the use of screw-cap culture tubes. Recently a three-amino acid base medium which gave excellent results for the cultivation of *E. histolytica* with penicillin-inhibited cells of *Bacteroides symbiosus* has been described (Reeves, Latour and Frye, 1960). This medium allowed for the first time, relatively complete control of the inorganic constituents of the medium. It
contained the amino acids arginine, cysteine, and glutamic acid; salts of sodium, calcium, magnesium, iron and zinc; glucose; phosphate buffer; horse serum; and pantothenate and pyridoxine factors.

Inconsistent results have been reported in the study of various effects of physical and chemical factors in culture media for *E. histolytica*. The optimum temperature for the growth of *E. histolytica* is between 37 and 38 °C. (Boeck and Drbohlav, 1925; Dobell and Laidlaw, 1926). It has been shown to tolerate wide variations in pH in different culture media (Shaffer, 1952; Deschiens, 1929) even though a nearly neutral reaction has usually been reported to be optimal (Chang, 1948). In most cases, the pH appears to be a function of the interaction between the bacterial flora and substrate.

Information currently available indicates that anaerobic conditions are necessary for optimum growth and proliferation of *E. histolytica* (Snyder and Meleney, 1942; Shaffer, Ryden and Frye, 1948). Several investigators have found a close relationship between the maintainence of a low oxidation-reduction potential in the culture medium and the growth of *E. histolytica* (Hopkins and Warner, 1946; Chang, 1946). Jacobs (1950) has expressed some doubt about this factor reporting that *E. histolytica* can grow and reproduce within a wide range of oxidation-reduction potential. It has been established experimentally that reducing agents like cysteine favor amebae growth (Snyder
and Meleney, 1943), while oxygen is definitely deleterious (Chang, 1946).

Several studies have been made which indicate that *E. histolytica* can tolerate considerable changes in total osmotic pressure (Watson, 1945; Balamuth and Brent, 1951).

In view of the recent trend toward investigating the physiology and metabolism of a disease-producing parasite as a means of providing a rational therapy, more interest is being devoted to the physiology and biochemistry of *Entamoeba histolytica*. In studying the physiological aspects of any organism it is important to know its nutritional requirements. The most serious impediment to a successful solution of the problem of the nutrition of *E. histolytica* is that it has not been cultivated free of an associate, whether it be viable or inactivated. Some workers have attempted to isolate the ameba from its associate and have then studied its biochemical activities. Others have elected to study amebae-bacteria complexes, using a method of differences to subtract the bacterial contribution from the total processes. Caution should be exercised in interpreting results obtained by the latter of these two methods for it assumes that the separate contributions of the associates are directly additive.

With our present knowledge of the nutritional status of *E. histolytica* it is difficult to define exactly the role of various components of the culture media. However, some carbohydrate source seems indispensable in cultures
of amebae. The requirement is satisfied by rice starch, rice powder, other milled cereal grains, or in some cases by substitution of glucose or maltose. Hopkins and Warner (1946) observed the amebae feeding upon rice grains, bacteria, fat globules and yeast and were able to show the accumulation of glycogen reserves in the amebae.

Reeves, Meleney and Frye (1957) reported that amebae multiplied very well in a modified Shaffer-Frye medium if maltose was substituted for glucose, but not if glucosamine, galactose, fructose, mannose, sucrose or sodium gluconate was used as a substitute. Hallman et al. (1954) were unable to detect any direct utilization of glucose in antibiotic-inhibited cultures of E. histolytica containing Aerobacter aerogenes. Entner and Anderson (1954) searched for products of utilization of glucose and/or maltose by washed resting cell suspensions of E. histolytica using amebae-streptobacillus and amebae-trypanosome cultures. Succinate and lactate were identified among the fermentation products, but the bulk of the slight activity recorded was contributed by endogenous metabolism. These authors concluded that under the experimental conditions nonproliferating amebae showed feeble metabolic activity, a result which appears inconsistent with most recent work.

Loran, Kerner and Anderson (1956) reported that E. histolytica contains relatively large amounts of glucosamine and is dependent upon the bacterial associate for its
supply of this amino sugar. They further stated that this ameba does not utilize glucose as such but only the products of glucose metabolism of the monobacterial associate.

It has been reported, on the contrary, that *E. histolytica* isolated under certain conditions exhibited a reasonably fast rate of metabolism of glucose, which was readily distinguished from that of associated bacteria (Kun and Bradin, 1953; Kun, Bradin and Dechary, 1956). Suspensions of amebae formed CO$_2$ and H$_2$S under anaerobic conditions from sugars in the presence of cysteine or cystine. Evidence was obtained which indicated that glucose was phosphorylated and fructose 1-6 disphosphate was fermented to pyruvate which was then decarboxylated. It was shown that during the fermentation of glucose or fructose 1-6 disphosphate in the presence of broken cells, triose phosphate was oxidized by cystine. The oxidation of the resulting thiol occurred by way of a "labile S compound" which was enzymatically desulfurated and the S reduced to H$_2$S. On the basis of reactions to numerous substrates and metabolic inhibitors, Kun and coworkers have formulated a tentative pathway of biological oxidation in *E. histolytica*. Becker and Geiman (1955) experimented with labeled substrates to determine whether carbohydrates are incorporated into amebae. Glucose-U-C$^{14}$ was added to antibiotic-inhibited polybacterial cultures under anaerobic conditions and evidence of utilization was sought in the form of radioactive products. These authors recovered from cultures distinct
levels of C\textsuperscript{14} in the form of CO\textsubscript{2} or glycogen. No C\textsuperscript{14} was recovered as CO\textsubscript{2} or glycogen from any of the bacterial controls. Hilker and White (1959) made a study of the carbohydrate metabolism of \textit{E. histolytica} using cell-free preparations and presented evidence which indicated that the Entner-Doudoroff pathway is probably a major pathway of glucose metabolism of this organism.

Snyder and Meleney (1943) presented evidence to indicate that cholesterol is a specific growth requirement. Following this Rees, Bozicevich, Reardon and Daft (1944) found that cholesterol and eight vitamins of the B complex stimulated the growth of amebae in media containing egg white. Cholesterol alone or the vitamins alone had no effect, but in combination the stimulation was pronounced. Griffin and McCarten (1949) reported that cholesterol in adequate amounts could be used as a substitute for serum in culture media for \textit{E. histolytica} and that oleic acid in optimum concentrations reinforced the action of cholesterol.

There is little or no evidence of the essentiality of peptones or amino acids for \textit{E. histolytica}. The principal is well established that for a study of the nutritional requirements of a protozoan for amino acids the culture fluids must be rigorously free of protein (Kidder and Dewey, 1945). Such a study will have to be postponed until the amebae can be maintained in pure culture, for the accompanying bacteria are ingested by
the amebae and may thus provide nutrients for the amebae.

The vitamin requirements for the cultivation of *E. histolytica* have usually been satisfied by the addition of yeast extract or other vitamin-rich materials, or by a mixture of various synthetic vitamins. There are a few reports in the literature which make reference to a specific vitamin requirement. Reeves, Meleney and Frye (1959) reported a pyridoxine requirement for *E. histolytica* cultivated in an MS-F culture medium with penicillin-inhibited cells of *Bacteroides symbiosus*. Upon transferring the amebae from the MS-F medium to one containing an acid hydrolysate of vitamin-free casein a requirement for pantothenate was recognized (Reeves, Meleney and Isbell, 1959). Pantetheine or coenzyme A successfully replaced pantothenate in the new medium and these substances were effective at a considerably lower concentration than calcium pantothenate. The B vitamin requirement for amebae growth in a three-amino acid base medium was met by the addition of pantothenate and pyridoxine factors (Reeves, Latour and Frye, 1960).

There are several concepts postulated as to the possible role of the associated organism in cultures with *E. histolytica* (Nakamura, 1953). To study the physiology and metabolism of the amebae many attempts have been made to eliminate the bacteria from the amebae. Cleveland and Sanders (1930) were the first to obtain amebae without bacteria. They obtained the amebae from bacteria-free
hepatic abscesses, but the amebae did not survive without addition of bacteria when some of the material containing amebae was inoculated into various culture media. Rees et al. (1941) successfully isolated cysts by means of micromanipulation and washing but cultures could not be established unless bacteria were added.

Several workers have attempted to inactivate bacterial associates. Jacobs (1947) cultivated the amebae in the presence of heat-killed *Escherichia coli* (56-65 °C for 2 hours) for several months, following elimination of the original bacterial associate *Clostridium perfringens* by means of penicillin. Amebae growth was scanty and Jacobs did not establish whether the metabolic activity of the treated bacterial fraction was inhibited or just partially inactivated. Jacobs proposed the use of the penicillin technique as a method of investigating the effects of growth substances on *E. histolytica* in the absence of bacterial effects. Shaffer, Ryden and Frye (1949) have used this general approach extensively. With the aid of antibiotics Shaffer and Frye (1948) maintained continuous cultures of amebae without any appreciable growth of bacteria. A combination of several methods was used by Miller and Firlotte (1948); centrifugation and levitation were followed by passage of amebae by micromanipulation, through a series of dilutions containing penicillin and streptomycin. Reeves, Schweinfurth and Frye (1960) found that the growth and mul-
tiplication of B. symbiosus cells could be completely suppressed by Co⁶⁰ irradiation without loss of the ability of these cells to stimulate and support the growth and multiplication of E. histolytica.

Phillips and Rees (1950) found that the amebae grew with T. cruzi even when the trypanosomes were exposed to heat for 10 minutes at 48° C, a treatment which rendered them non-viable for subculture. Nakamura and Anderson (1951) also found that the amebae may be cultivated in a medium containing trypanosomes exposed to 48° C for 10 minutes but not in a medium containing trypanosomes exposed to 50° C for 10 minutes. Manometric studies indicated that heat-treated T. cruzi at 50° C no longer take up oxygen whereas 48° C merely reduces the respiratory rate. In contrast to the effect upon the trypanosomes, these investigators found that amebae survived treatment at 50° C for 10 minutes. They concluded that trypanosomes capable of motility and development are not required since the amebae grew in spite of the fact that T. cruzi after heat-treatment at 48° C for 10 minutes was non-motile and failed to grow under the cultural conditions employed. Even though motile organisms are not present, some heat labile respiratory enzyme system contributed by T. cruzi seems to be definitely necessary since at a temperature inhibitory to trypanosome respiration, the culture of the amebae failed.

Recently Diamond (1961) reported that he successfully
maintained *E. histolytica* in axenic culture employing a diphasic medium containing a cell-free extract of chick embryo. He claims that this is the first published report presenting unequivocal evidence of the indefinite subcultivation of *E. histolytica* in an environment free of metabolizing bacteria, fungi, protozoa or metazoan cells.

Another approach to the biochemistry of *E. histolytica* is the determination of the enzyme systems the amebae possess. Several investigators have sought to discover whether *E. histolytica* elaborates hyaluronidase as part of its tissue-invading mechanism. Bradin (1953) assayed supernatants from cultures of five different strains of *E. histolytica* maintained exclusively in cultures over long periods of time and found no detectable amounts of hyaluronidase. However, the four strains infective for the hamster did produce hyaluronidase as evidenced by studies with organisms removed from experimentally induced hamster liver abscesses. Bacterial controls proved negative in both instances. Hyaluronidase production persisted only through several serial transfers eventually disappearing as in the stock cultures. Bradin noted that his data supported the view of a changing physiological state under prolonged *in vitro* cultivation. DeLamater *et al*. (1954) were unable to demonstrate intra- or extracellular hyaluronidase production by four strains of *E. histolytica* either from stock cultures or from cultures recently isolated from experimental liver abscesses.
Jarumilinta and Maegraith (1960) repeatedly demonstrated hyaluronidase activity by three different methods in three stock cultures of *E. histolytica*. The authors state that the difference between their findings and those of Bradin and DeLamater and associates are probably due to differences in strains and techniques. They also pointed out that Seitz filtration of the extracts removed hyaluronidase activity which may explain why DeLamater *et al.* were unable to demonstrate hyaluronidase production.

By means of histochemical techniques Carrera and Changus (1943) demonstrated the presence of intracellular acid phosphatase in stock cultures of *E. histolytica*. High levels of acid phosphatase were found in the colon wall of experimentally infected guinea pigs, but only in the specific areas of amebic invasion (Carrera, 1950). Balamuth (1950) employed a modified Gomori histochemical technique for demonstrating the presence of acid phosphatase and applied it to fixed smears of four different strains of human amebae, and also of the non-pathogenic *E. terrapinae*. Strongly positive reactions for acid phosphatase were given by all three species of *Entamoeba* tested but not by *Dientamoeba fragilis* or *Endolimax nana*. Blumenthal *et al.* (1955) showed that *E. histolytica* contained two separate enzymes having phosphomonoesterase activity and reported the pH optima and unit activities of these two enzymes.

The presence of proteolytic enzymes which hydrolyzed
casein, dissolved formalin-denatured gelatin and reduced the viscosity of gelatin solutions has been demonstrated in extracts of *E. histolytica* (Neal, 1956). The bacterium which accompanied the amebae in culture, when tested under the same conditions showed no proteolytic activity. Harinasuta and Maegraith (1958) showed proteolytic enzyme activity of amebae by the use of photographic gelatin film. Washed suspensions of accompanying bacteria did not digest gelatin nor did the medium in which amebae had been suspended for two hours. Other workers (Nakamura and Edwards, 1959) were also able to show that casein is hydrolyzed by *E. histolytica* but not by the associated bacteria. For assay large drops of the material to be tested were placed on 1% agar plates containing 0.2% casein and these were incubated at 20-25 °C for 12 hours, after which the plates were flooded with a protein precipitating agent. Casein hydrolysis was indicated by the presence of clear areas in the opaque casein agar plates.

Growing cultures of *E. histolytica* have been found to elaborate a starch-splitting substance (Hallman and DeLamater, 1953). Hilker and associates (1957) studied the amylase activity of three strains of *E. histolytica* and two amebae from reptilian sources. Chromatographic studies showed the presence of amylase and maltase but not sucrase or lactase in lyophylized amebal preparations.

Several reviews on the chemotherapy of human amebiasis have appeared in the literature (Anderson and
Hansen, 1950; Findlay, 1950; Anderson, Bostick and Johnstone, 1953). Balamuth and Thompson (1955) reviewed the procedures now used to determine the effects of drugs on amebae in vitro and in vivo and presented a summary of the known amebicides.

There is little precise information on the mode of action of amebicides apart from studies of direct effects upon the amebae versus indirect effects resulting from inhibition of the culture associates. The mode of action of emetine was studied by Stewart (1949). Observations on the growth rate and cytology of treated cultures suggested that the amebistatic effect may come from interference with an essential cytoplasmic reaction before cell division. Cytoplasmic damage by emetine also was noted by Anderson and Hansen (1950); thioarsenate in contrast caused marked nuclear degeneration.

The mode of action of halogenated 8-hydroxyquinolines has thus far not been elucidated. All in common clinical use contain iodine and it is frequently stated in textbooks but without documentation (Fieser and Fieser, 1950) that the activity of such drugs as chiniofon and diiodohydroxyquinolines depends upon their iodine content. This seems unlikely since many non-iodinated 8-hydroxyquinolines have antiamebic action both in vitro and in vivo. In addition, massive doses of elemental iodine or of sodium iodide were ineffective in canine amebiasis (Balamuth and Thompson, 1955). Following the administra-
tion of radioactive chiniofon to seven subjects Albright et al. (1947) observed that absorption of the drug does occur in small amounts averaging 12.9% of the dose given. They stated that blood levels of clinical significance are not attainable with the dose used and chiniofon is partly broken down after absorption as a progressively greater proportion of iodine is split off from the compound.

A method has been described for measuring manometrically the acid and CO₂ production of *E. histolytica* and the effects of several drugs on its metabolic activity (Nakamura et al., 1953). Some evidence is presented of the synergistic action of drugs against the amebae when treated *in vitro*. These studies have been applied as a new technique in the evaluation of potential amebicidal agents and may be of value in studying the action of these drugs.

B. The cation problem

Little is known about the actual inorganic requirements of the amebae and their role in the living cell. The writer is aware of only a few published reports which make allusion to this subject. The results of Kun, Bradin and Dechary (1955) who studied the effect of certain chelating agents on the rate of CO₂ and H₂S evolution of *E. histolytica* suggests that a metalloprotein may be important in the metabolism of the amebae. Hansen and Anderson (1948) observed that the addition of iron as heme improved the nutrient properties of their medium.
These authors made no mention of the amount of heme employed and the accompanying porphorin fraction was not evaluated. Iralu and Shaffer (1961) studied the effect of red blood cell derivatives on the propagation of *E. histolytica* and found hemoglobin and hemin to be highly toxic to three amebae strains at levels of 8 \( \mu \text{g/ml} \) and 75 \( \mu \text{g/ml} \), respectively. They suggested that the heme moiety is responsible for the toxicity of both hemoglobin and red blood cells to the amebae. Low concentrations of hemoglobin and hemin, on the other hand, showed a stimulatory effect on the multiplication of *E. histolytica*. Hematoporphorin HCl showed only stimulatory effects which these authors state is in accord with the observations of Dubos (1954) indicating that iron should be present in the porphorin to exhibit toxicity. Nelson's results (1951) indicated that small amounts of agar were necessary for amebae growth; however, highly purified agar was incapable of supporting growth unless magnesium in the form of sulfate, chloride, or acetate was added. However, none of these reports can be interpreted as establishing a definite iron or magnesium requirement.

In order to approach the cultivation of *E. histolytica* in a defined or synthetic medium it would be necessary to take into account the cation requirements. The most common culture systems containing various natural crude products as well as actively multiplying bacterial cells, seem far too complex to allow any conclusions to
be drawn in regard to cation requirements. The modified Shaffer-Frye system substitutes penicillin-inhibited bacterial cells for actively multiplying associated organisms, but does not provide control over inorganic components of the media. The principal component of the S-F and MS-F systems is a peptone derived from casein which brings into the medium measurable concentrations of calcium, magnesium, iron and zinc. In such a system there appear to be two means by which cations might be studied (1) selective removal of cations, or (2) sequestering the cations with specific chelating agents.

Studies along these two lines were begun but did not yield very promising results. It was thought that a new base medium in which pure amino acids were substituted for the natural peptones would allow considerably better control over the inorganic constituents of the culture fluids. The amino acid base medium, containing cysteine, arginine, and glutamic acid was developed which was especially suitable for this type of study (Reeves, Latour and Frye, 1960). The need for certain trace elements is difficult to demonstrate, even in a defined medium, due to the fact that they may be effective in great dilution and may be present as a trace impurity in the organic ingredients, distilled water and salts used in preparing the media. It is also possible that antagonistic relationships exist between individual elements emphasizing the importance of maintaining the elements in the proper
ratio.

The medium most used in this investigation contained three amino acids (cysteine, arginine, glutamic acid). In preliminary cation studies two other amino acid media were employed, one containing six and the other fifteen amino acids. Sodium chloride was added to the amebae culture media to maintain isotonicity; hence, no information on the question of a sodium requirement was obtained. The major functions of the sodium ion in the animal body appear to be in connection with osmotic pressure regulation and acid–base balance, although other possible functions such as a catalytic effect on enzyme activity cannot be excluded. Its function in bacterial nutrition is unknown. Potassium was supplied in the form of phosphate salt which also served for buffering, and its requirement was not investigated. Phosphate plays an important role in energy transfer and utilization of carbohydrates and is, no doubt, important in amebae metabolism although its requirement was not explored in detail.

Experiments were designed to study the effect of calcium, magnesium, zinc and iron on amebae growth. These cations were either excluded so far as possible from the media, or added individually and in various combinations. During the initial phases of this work it was found that added zinc was not essential to amebae
growth; hence, in subsequent work it was not included as a constituent of the amino acid media. Calcium and magnesium were found to be necessary for maximal growth, but the quantitative requirements for these cations were not established. The need for magnesium in metabolic processes results primarily from its activation of many enzymatic reactions, particularly those concerned in carbohydrate metabolism. The complete function of calcium in bacterial nutrition is not clear, but it appears to play a part in decreasing the permeability of cell membranes and in the irritability of cells in general.

Iron was studied by use of chelating agents as well as by directly varying the iron concentration of the medium. An iron requirement for amebae growth could be readily and dramatically demonstrated, so it was decided to focus most attention on this aspect. A thorough investigation of the iron requirement proved to offer a comprehensive problem. Attempts were made to replace the iron by other cations or by various iron-containing organic compounds. Further work was devoted to a study of the effect of iron on glucose utilization by the associated organism B. symbiosus under penicillin inhibition as well as of the amebae-bacteria complex.
II METHODS

A. Cultivation of Entamoeba histolytica

The DKB strain of Entamoeba histolytica employed in this work was obtained from Dr. Quentin M. Geiman in 1955. As received it was growing in Cleveland and Collier medium with a mixed bacterial flora. It was subsequently transferred to modified Shaffer-Frye (MS-F) medium and then to a three-amino acid medium containing cysteine, arginine and glutamic acid (Reeves, Latour and Frye, 1960). It was maintained in this last medium during this work in association with the single organism, Bacteroides symbiosus¹ (streptobacillus), the latter always being under penicillin inhibition in the amebae culture fluids.

Composition and preparation of amebae culture media. The amino acid-containing media employed in this investigation allowed a considerable degree of control over the inorganic constituents of the culture fluids. The medium most used contained only the amino acids cysteine, arginine and glutamic acid. In preliminary experiments two other amino acid-based media were used, one containing six and the other fifteen amino acids. The L-forms of the amino

¹American Type Culture Collection Number 12329
acids were used. The last two media were prepared by incorporating into the three-amino acid medium additional selected amino acids in approximately the concentrations found in a 2 percent casein hydrolysate. The composition of these media is given in Table 1. Demineralized water was used exclusively in all phases of this work.

In developing the media the cations of magnesium, iron and zinc were maintained at $0.5 \times 10^{-3}$, $4 \times 10^{-5}$ and $2 \times 10^{-6}$ M, respectively, by the addition of salts of these cations. These are the approximate molar concentrations found in the MS-F medium, a Trypticase-containing medium. The concentration of calcium ion was reduced from $1.5 \times 10^{-3}$ M as found for the MS-F medium to $0.25 \times 10^{-3}$ M to avoid a troublesome precipitation of calcium phosphate. During the initial phases of this work it was found that the added zinc salt was not essential to amebae growth; hence, in subsequent work it was not included as a constituent of the amino acid media.

In other phases of this investigation a base medium prepared from an acid hydrolysate of vitamin-free casein was employed which has been described by Reeves, Meleney and Isbell (1959). The composition of this medium is given in Table 2.

All base media were autoclaved in 12 ml amounts in

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2 Trypticase is a pancreatic casein hydrolysate supplied by the Baltimore Biological Laboratory, Baltimore, Maryland.
Table 1

The Composition of Amino Acid-Based Media Used in the Cultivation of *E. histolytica*

<table>
<thead>
<tr>
<th>Amino acids in base media</th>
<th>Moles/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Three-amino acid medium</strong></td>
<td></td>
</tr>
<tr>
<td>L-Cysteine·HCl</td>
<td>L-Cysteine</td>
</tr>
<tr>
<td>L-Na glutamate</td>
<td>L-Na glutamate</td>
</tr>
<tr>
<td>L-Arginine·HCl</td>
<td>L-Arginine·HCl</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>L-Histidine</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>L-Leucine</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>L-Aspartic Acid</td>
</tr>
<tr>
<td></td>
<td>L-Serine</td>
</tr>
<tr>
<td></td>
<td>L-Tyrosine</td>
</tr>
<tr>
<td></td>
<td>L-Valine</td>
</tr>
<tr>
<td></td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td></td>
<td>L-Methionine</td>
</tr>
<tr>
<td></td>
<td>L-Alanine</td>
</tr>
<tr>
<td></td>
<td>L-Lysine</td>
</tr>
<tr>
<td></td>
<td>L-Threonine</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
</tr>
<tr>
<td><strong>Six-amino acid medium</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fifteen-amino acid medium</strong></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

Other components present in the base medium and their concentrations in moles per liter were: D-glucose, 0.055; calcium chloride, 0.00025; magnesium chloride, 0.0005; ferric chloride, 0.00004; sodium chloride, 0.0428; potassium phosphate (K₂HPO₄), 0.0004; and sodium hydroxide to pH 7.0 ± 0.1.
<table>
<thead>
<tr>
<th>Component</th>
<th>Grams per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein acid hydrolysate</td>
<td>20.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.7</td>
</tr>
<tr>
<td>Thiomalic acid (mercaptosuccinic acid)</td>
<td>1.5</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.788</td>
</tr>
<tr>
<td>Pyridoxal hydrochloride</td>
<td>0.005</td>
</tr>
<tr>
<td>Sodium hydroxide to pH 7.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>
16 x 125 mm screw-cap tubes at 120 C for 10 minutes, the caps being loosened while in the autoclave and tightened when the medium had cooled. The sealed tubes were stored in the refrigerator for as long as a week before use in amebae cultures. At the time of amebae inoculation the media were completed by the addition of 0.25 ml sterile horse serum, 5,000 units of penicillin G potassium, 13 μg each of coenzyme A and of pyridoxal phosphate sterilized by Millipore filtration and 0.2 ml of a suspension of \textit{B. symbiosus} cells containing 0.15 to 0.21 mg of bacterial nitrogen.

Inoculation, incubation and counting of amebae. The usual inoculum contained 10,000 amebae suspended in a small volume of fluid from the preceding culture. Total culture volumes after inoculation ranged from 13 to 15 ml. The cultures were incubated for 2 days in a slanting position after which microscopic examination was made directly on the culture vessel. The appearance and degree of compactness of the amebae along the side and bottom of the culture tube gave a good indication of the amebae harvest which could be estimated and graded from 0 to 4 plus. Those tubes with a 2 plus or less were generally counted in an especially designed counting chamber consisting of a small cylinder attached to a glass slide. The amebae settled to the bottom in about 10 minutes and could then be counted. Those tubes which received the higher gradings were counted using a spinal fluid counting chamber.
In obtaining an aliquot for counting, the trophozoites were carefully suspended in the culture fluids with a capillary pipette operated with a small rubber bulb. Aseptic techniques were employed in all procedures dealing with the handling of cultures. All cultures were examined for microbial contamination by direct observation and by plating\(^3\) on 4% rabbit blood agar plates. The reported results were drawn entirely from cultures which proved not to be contaminated.

B. Cultivation, harvesting and storage of *Bacteroides symbiosus*

The technique for the preparation, harvesting and storage of large lots of bacterial cells has been described by Reeves, Meleney and Frye (1957). The medium for the cultivation of the bacterial cells consists of an enzymatic casein digest, glucose, thiomalate, salts and yeast extract dissolved in demineralized water. Its composition is given in Table 3. The bacterial medium has the same composition as the medium base employed in the MS-F technique for amebae cultivation except for the added yeast extract. The thiomalic acid was dissolved in water and approximately neutralized with sodium hydroxide before adding the organic ingredients. A 10 X concentrated solution of

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\(^3\)The streptobacillus fails to grow aerobically on ordinary blood-agar plates.
Table 3

The Composition of Yeast-Enriched MS-F Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Grams per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase</td>
<td>20.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$·3H$_2$O</td>
<td>2.0</td>
</tr>
<tr>
<td>Thiomalic acid</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium hydroxide to pH 7.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>
the thiomalate could be stored in the refrigerator for several weeks.

The procedure for preparing large batches of streptobacillus cells is as follows: A flask containing 800 ml of freshly prepared medium was given a 2 to 5 percent inoculation with a 24 hour culture which had been transferred daily for several days. The large culture was incubated at 37 °C for 16 to 18 hours (never more than 24 hours). The cells were harvested by centrifugation in sterile centrifuge bottles and transferred to 50 ml centrifuge tubes. They were then washed two times by centrifugation with normal saline containing 0.02 M sodium thiomalate and stored under 0.1 M sodium thiomalate in the refrigerator. A 10 fold concentration of the cells was achieved in this manner. Subculturing and plating were done on each large batch of cells to make certain that the preparation had not become contaminated during handling. For use in amebae cultures the washed bacterial cells were suspended in the thiomalate solution and a volume of cells corresponding to 2 ml of original culture fluid was transferred to each amebae culture tube.

C. Estimation of calcium, magnesium and iron content of the media

**Determination of iron in cultural components.** In order to ascertain the quantity of background iron present in various components of the culture fluids, analyses were done employing a modification of the
colorimetric iron determination described by Drabkin (1941). Those steps dealing with pH adjustment, reduction of iron and formation of the color complex of ferrous-ö-phenanthroline were carried out using half the recommended quantity of reagents and half the total volume. No detectable amounts of iron impurities were found in the amino acids, or in solutions of penicillin G potassium, coenzyme A and pyridoxal phosphate (Table 4). Accurately measured quantities of horse serum, bacterial cells, thiomalic acid and acid-hydrolyzed casein were ashed in a platinum crucible and each residue dissolved in a small volume of hydrochloric acid (1 part conc. HCl to 2 parts water). The solution was quantitatively transferred to a 5 or 10 ml volumetric flask and the volume adjusted with water. When aliquots of the ashed samples were analyzed for iron it was found that the acid-hydrolyzed casein introduced 7.4 μg iron per culture and that the iron content of the various lots of horse serum varied somewhat and averaged 6.9 μg/ml. The serum and bacterial cells brought in an amount of iron equal to 3.4 μg per culture (4 x 10⁻⁶ M). Although the amount of background iron could be markedly lowered by dialysis of the horse serum against solutions containing iron chelators this decrease in the background iron content was of no consequence to amebae growth in three-amino acid medium as will be reported subsequently.

Determination of calcium and magnesium in horse serum
<table>
<thead>
<tr>
<th>Table 4</th>
<th>Iron Content of Various Components of Amoeba Culture Fluids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iron in ( \mu g/ ml ) or gm</td>
</tr>
<tr>
<td>Horse Serum</td>
<td>7.4 ( \mu g/ml ) (6.4)</td>
</tr>
<tr>
<td>Bacterial cells in M/10 thiomalate</td>
<td>8.1 ( \mu g/ml ) 1.6</td>
</tr>
<tr>
<td>2 x recrystallized thiomalic acid</td>
<td>11 ( \mu g/gm ) 0.03</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>31 ( \mu g/gm ) 7.4</td>
</tr>
<tr>
<td>A-3 base medium (No added ( \text{FeCl}_3 ))</td>
<td>Not detectable</td>
</tr>
<tr>
<td>A-15 base medium (No added ( \text{FeCl}_3 ))</td>
<td>&quot;</td>
</tr>
<tr>
<td>Penicillin G potassium solution</td>
<td>&quot;</td>
</tr>
<tr>
<td>Coenzyme A and pyridoxal phosphate solution</td>
<td>&quot;</td>
</tr>
<tr>
<td>Total iron content of amino acid base media (No added ( \text{FeCl}_3 ))</td>
<td></td>
</tr>
<tr>
<td>Total iron content of acid hydrolyzed casein medium</td>
<td></td>
</tr>
</tbody>
</table>
and bacterial cells. Aliquots of horse serum were analyzed for total calcium and magnesium employing a modification of the method described by Schwarzenbach et al. (1946). The determination of calcium alone is based on the principle that in very alkaline solutions insoluble magnesium hydroxide is formed which will not react with EDTA (ethylenediamine tetraacetic acid). The magnesium concentration was then obtained by subtracting the value found for calcium alone from that found for the total of calcium plus magnesium. On the basis of these analyses it was found that the calcium and magnesium content of horse serum was approximately 3.4 and 0.78 mM per liter, respectively. Calculated on the basis of 14 ml culture fluid the concentrations of calcium and magnesium contributed by horse serum (0.25 ml per culture) are $6.1 \times 10^{-5}$ and $1.4 \times 10^{-5}$ M, respectively.

A 5 ml aliquot of a 10 X suspension of bacterial cells was ashed in a platinum crucible. A small amount of ethanolamine was added and the residue dissolved in an excess of standard Na$_2$EDTA (2 meq/l) and quantitatively transferred to a 50 ml Erlenmeyer flask by rinsing with water. When excess EDTA was titrated with a standard calcium solution a variable and indistinct end point was observed. It was suspected that this interference was due to the presence of the iron which was found in the ash of the bacteria and is known to interfere in the
estimation of calcium and magnesium by the EDTA method. Thus, no values were obtained for these cations in the bacteria.

D. Extraction of iron impurities

Recrystallization of thiomalic acid. It was found that recrystallization of thiomalic acid was necessary to remove the iron impurities present in the commercial product. To five hundred gm thiomalic acid (mercapto-succinic) was added sufficient boiling water to completely dissolve the acid. The hot solution was then filtered and allowed to cool. The crystals were removed by filtration and the procedure was repeated. The 2 X recrystallized thiomalic acid was spread out on filter paper to air dry, and then bottled.

Dialysis of horse serum. Horse serum (10 ml) was placed in a cellophane membrane and allowed to dialyze against saline (250 ml) containing 50 mg chiniofon at 3 to 5 C. The saline solution of chiniofon was replaced several times during a two day period. On the third day the horse serum was dialyzed against saline to remove the chiniofon. The dialyzed serum required resterilization by Millipore or Seitz filtration and examination for microbial contaminants, thus increasing the number of manipulations prior to use in amebae cultures. For this reason, dialyzed serum was not routinely employed in amino acid media although its ability to support amebae growth in acid-hydrolyzed casein medium was
satisfactory. In one experiment employing dialyzed and undialyzed horse serum the amebae harvests were 248,000 and 270,000, respectively.

E. Initial investigation of cation requirements for amebae growth

The amino acid media were prepared in the usual manner but the calcium, magnesium and iron salts were not added until after the media were tubed in order to facilitate the proper setting up of the experiment. Solutions of calcium, magnesium and ferric chloride were prepared in 100 X molar concentrations, that is, 2.5 x 10^{-2}, 5 x 10^{-2} and 4 x 10^{-3} M, respectively, and 0.125 ml aliquots could then be added to the tubed amino acid medium individually and in various combinations. A medium containing none of these added salts was also prepared. These media were autoclaved and stored in the usual manner.

The amebae inoculum for experiments with three-amino acid media was usually taken from a control amino acid culture, that is, one which contained the standard amounts of all of the cations being tested. In the case of the six- and fifteen-amino acid media, the inocula were taken from a culture containing none of the added cations whenever possible. Two or three serial transfers were necessary to dilute out the calcium, magnesium and iron carried over with the first amebae inoculum.
F. Iron studies

Varying the iron concentration in three-amino acid medium. In order to determine the optimal iron concentration for amebae growth in three-amino acid medium, the concentration of iron in the medium was varied and the amebae cultures were continued serially for two or more transfers. The background iron concentration, consisting of that present in some of the ingredients of the medium was $4 \times 10^{-6}$ M. Other iron levels tested were $8 \times 10^{-6}$, $1.2 \times 10^{-5}$, $4.4 \times 10^{-5}$, and $4 \times 10^{-4}$ M. Solutions of ferric chloride were used in preparing the last four media.

Iron chelating agents employed in amino acid-amebae cultures. In Table 5 are listed the ferrous and ferric iron chelators which were selected for this study. The structures of these compounds are shown as well as their stability constants, when known. CHEL DP$^4$ and Versene Fe-3 Specific$^5$ are especially effective for chelating ferric iron and have little tendency to coordinate with calcium and magnesium. o-Phenanthroline forms a very stable ferrous iron chelate. The stability constants of

$^4$Available from Geigy Industrial Chemicals, Ardsley, New York.

$^5$Available from Versenes Incorporated, Framingham, Massachusetts.
Table 5
Iron Chelators Used in Culture Fluids

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Log stability constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHEL DP ethylene diamine di(o-hydroxyphenyl acetic acid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image1.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Versene Fe-3 Specific (N,N dihydroxyethylglycine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image2.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 10 α-Phenanthroline</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image3.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Chiniofon (Ferron) (7-iodo, 8-hydroxyquinoline, 5-sulfonic acid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image4.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>
chiniofon⁶ were not found in the literature; however, it is presumed that they would be of the same relative magnitude as those of 8-hydroxyquinoline, 5-sulfonic acid which forms a stable ferrous iron chelate.

Solutions of chelating agents used in cultures were sterilized by Millipore filtration. In all of the experiments with chelating agents a control ameba culture with no added chelator was set up in the medium being tested.

**CHEL DP**

A 0.01 M solution of CHEL DP was prepared by dissolving the accurately weighed powder in a small volume of N HCl followed by dilution with water and adjustment to pH 7 with sodium hydroxide. Graded amounts of this solution ranging from 0.1 to 0.6 ml were added to three-amino acid medium containing 4.4 x 10⁻⁵ M iron and the ameba growth was estimated. Calculated on the basis of moles per liter the concentrations of CHEL DP tested increased from 6.7 x 10⁻⁵ and doubled for each successive culture to a maximum level of 1.1 x 10⁻³ M.

**Versene Fe-3 Specific**

Versene Fe-3 Specific is supplied as the sodium salt in solutions containing 47% solids. A 5% solution was prepared and neutralized to pH 7 with concentrated HCl

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⁶Available from The Matheson Company, Incorporated, East Rutherford, New Jersey.
and utilized in three-amino acid medium containing 4.4 x 10^{-5} M iron. Beginning with 1.25 mg per culture tube the amount was doubled for each successive culture until a maximum of 40 mg was reached. On the basis of moles per liter the concentration of chelating agent increased from 5.2 x 10^{-4} to 1.7 x 10^{-2} M.

**o-Phenanthroline**

Solutions of o-phenanthroline containing 1 mg/ml and 10 mg/ml were made. Increasing amounts of the more dilute solution were added to three-amino acid medium containing 4.4 x 10^{-5} M iron. Similar experiments using increasing amounts of both of these solutions were carried out at higher iron levels, namely, 1.6 x 10^{-4}, 4 x 10^{-4} and 8 x 10^{-4} M. The concentrations of o-phenanthroline tested at the 4.4 x 10^{-5} M iron level were 0.1, 0.2, 0.3 and 0.4 mg/culture tube (4.3 x 10^{-5} to 1.7 x 10^{-4} M). Those tested at the 1.6 x 10^{-4} M iron level were 0.2, 0.4, 0.8, 1.6 and 2.4 mg/culture tube (8.5 x 10^{-5} to 1.0 x 10^{-3} M). At the 4 x 10^{-4} M iron level the amounts employed were 1.0, 2.0, 2.4 and 3.0 mg/culture (4.3 x 10^{-4} to 1.3 x 10^{-3} M). Finally, the amounts employed at the highest iron level tested (8 x 10^{-4} M) were 1.0, 2.0, 4.0 and 8.0 mg/culture (4.3 x 10^{-4} to 3.4 x 10^{-3} M).

**Chiniofon**

A solution of chiniofon containing 10 mg/ml was made after adjusting to pH 7 with sodium hydroxide a
suspension of a weighed sample of the acid. A 1:10 dilution was made with 1 ml of this solution to obtain a solution containing 1 mg/ml. These two solutions were then utilized in the following experiments. Graded amounts were added to three-amino acid media containing three different concentrations of iron, namely, $4.4 \times 10^{-5}$, $1.6 \times 10^{-4}$ and $8 \times 10^{-4}$ M. At the $4.4 \times 10^{-5}$ M iron level the amounts of chiniofon employed were 0.4, 1.0, 2.0 and 4.0 mg/culture ($8.5 \times 10^{-5}$ to $8.5 \times 10^{-4}$ M). At the $1.6 \times 10^{-4}$ M iron level those first three amounts tested at the lower iron level were again employed as well as two other amounts namely, 5.0 and 10.0 mg/culture ($1.1 \times 10^{-3}$ and $2.1 \times 10^{-3}$ M). The quantities of chiniofon employed at the highest iron level were 4.0, 8.0, 9.0, 10.0 and 12.0 mg/culture ($8.5 \times 10^{-4}$ to $2.5 \times 10^{-3}$ M).

The complete concentration series were not done for the six-amino acid medium. Instead $4.2 \times 10^{-4}$ M chiniofon was used in this medium containing two iron concentrations. One was the background iron ($4 \times 10^{-6}$ M), the other was the amount found to be optimum in the cultures ($4.4 \times 10^{-5}$ M). In order to reverse the amebicidal effect of the chelator, the iron level was raised to $3.2 \times 10^{-4}$ M without changing the dose of chelator. In addition, half of the surviving amebae after the first transfer in the culture with $4.4 \times 10^{-5}$ M iron plus chiniofon was continued serially and the other half
subcultured into medium with adequate iron available for
growth \((3.2 \times 10^{-4} \text{ M iron plus } 4.2 \times 10^{-4} \text{ M chiniofon})\).

**Chelating agents employed in acid-hydrolyzed and**
**MS-F amebae cultures.** Concentration series with chiniofon and \(\alpha\)-phenanthroline were carried out in acid-
hydrolyzed casein medium whose iron content had been
previously determined as \(1.4 \times 10^{-5} \text{ M}\) (Table 4) and in
MS-F medium with added yeast extract (0.2%). The amebae
growth obtained at each concentration (moles/liter) of
chelating agent for these two media was determined. In
acid-hydrolyzed casein medium the concentrations of
chiniofon employed ranged from \(4.3 \times 10^{-5}\) to \(1.1 \times 10^{-3} \text{ M}\);
those of \(\alpha\)-phenanthroline ranged from \(4.1 \times 10^{-6}\) to
\(3.0 \times 10^{-5} \text{ M}\). In yeast-enriched MS-F medium the concen-
trations of chiniofon employed ranged from \(1.1 \times 10^{-4}\) to
\(6.0 \times 10^{-4} \text{ M}\); those of \(\alpha\)-phenanthroline ranged from
\(4.1 \times 10^{-5}\) to \(3.1 \times 10^{-5} \text{ M}\).

In another experiment \(8.4 \times 10^{-4} \text{ M}\) chiniofon was
used in an amebae culture employing acid-hydrolyzed
casein medium. The surviving amebae after the first
transfer were divided into two equal portions and one
inoculum continued serially in chiniofon-containing
medium and the other added to fresh medium without the
chelator. Another serial transplant was made of each
of these cultures. In other amebae cultures employing
this medium Versene Fe-3 Specific and CHEL DP were
utilized at concentrations of \(1.7 \times 10^{-2}\) M and
For the experiments in yeast-enriched MS-F medium, subcultures from those amebae cultures having the growth-inhibitive doses of o-phenanthroline or chiniofon were made into fresh medium containing 0.2 ml of 1.5% penicillinase. After incubation for 48 hours the subcultures were examined for bacterial growth.

Iron chelating agents employed in bacterial cultures. Rather poor bacterial growth occurred in the acid-hydrolyzed casein medium and in three-amino acid medium. Since abundant growth could be attained in MS-F medium with added yeast extract (Reeves et al., 1957), this was chosen as the test medium to which was added various amounts of chiniofon or o-phenanthroline. The inocula consisted of 0.2 ml of a suspension of stored bacterial cells. The amounts of chiniofon used ranged from $1.1 \times 10^{-4}$ to $6.4 \times 10^{-4}$ M and those of o-phenanthroline ranged from $4.3 \times 10^{-5}$ to $1.0 \times 10^{-4}$ M. Bacterial growth was estimated and graded by visual comparison with a control tube after incubation for 48 hours.

Bacterial inocula (0.1 ml) were then exposed to $3.2 \times 10^{-3}$ M chiniofon or $2.1 \times 10^{-3}$ M o-phenanthroline which were equivalent to 5 and 20 times, respectively, the growth-inhibitive doses of these agents as determined in the previous experiment. After five days exposure, subcultures were made into fresh media in order to
ascertain whether there were any viable bacteria remaining. Subcultures were examined for bacterial growth after incubation for 48 hours and tested for the presence of contaminants.

Versene Fe-3 Specific \((1.7 \times 10^{-2} \text{ M})\) or CHEL DP \((1.2 \times 10^{-3} \text{ M})\) were also tested in this medium employing 0.1 ml bacterial inocula. The cultures were examined for bacterial growth after 72 hours.

**Substitution of other cations for iron in amebae cultures.** The divalent cations of cobalt, copper, manganese, zinc, nickel, barium and vanadium in the form of their chloride salts as well as aluminum chloride and molybdic acid were substituted individually for the added ferric chloride in three-amino acid medium at a concentration of \(4 \times 10^{-5} \text{ M}\). A \(4 \times 10^{-3} \text{ M}\) solution of each of these cations was prepared and added in 0.125 ml aliquots to the tubed three-amino acid medium lacking the added iron salt. These media were then autoclaved in the usual manner. The amebae inocula were taken from a regular three-amino acid culture with the standard amounts of calcium, magnesium and iron. Each of the cultures was carried serially for two or three transfers to dilute out the traces of iron.

**Substitution of hemin, cytochrome c or peroxidase for iron.** A crude preparation of hemin crystals was substituted for the added iron in the three-amino acid medium. About 1 mg per culture tube was autoclaved with
the base medium, which if completely in solution would be equivalent to \(9.5 \times 10^{-6}\) M iron. However, a large portion of the crystals remained undissolved. Six serial amebae transfers were made in this medium and the amebae growth was compared to a control series containing the iron salt (\(4.4 \times 10^{-5}\) M).

Solutions of cytochrome \(c\)\(^7\) and horse-radish peroxidase\(^8\) containing 3 mg/ml were prepared and sterilized by Millipore filtration. Cytochrome \(c\) with a molecular weight of 12,000 has one heme group or 0.465% iron and horse-radish peroxidase with a molecular weight of 40,000 has one heme group or 0.14% iron (Long, 1961). Beginning with 0.3 mg/culture (equivalent to \(1.9 \times 10^{-6}\) M iron) this amount was doubled to a maximum level of 4.8 mg/culture (equivalent to \(3.1 \times 10^{-5}\) M iron). In the peroxidase experiment the same concentration series was employed except the range was from 0.3 to 2.4 mg/culture, equivalent to a molar iron concentration of \(5.8 \times 10^{-7}\) to \(4.6 \times 10^{-6}\). In addition the cultures had the basic background iron concentration of \(4 \times 10^{-6}\) M. The amebae growth response at the various concentrations of these substances was observed. Attempts were made to continue serially those

\(^7\)Available from Nutritional Biochemicals Corporation, Cleveland, Ohio.

\(^8\)Available from Worthington Biochemical Corporation, Freehold, New Jersey.
cultures having 2.4 and 4.8 mg of cytochrome c.

G. Experiments on glucose utilization

**Glucose utilization by bacterial cells.** Since under the culture conditions employed, the bacterial cells are under penicillin inhibition, it was deemed necessary to study the metabolic process which might continue even when the bacteria were not actively multiplying. Glucose utilization was selected for study using the three-amino acid medium whose glucose concentration had been lowered to 0.1% (1 mg/ml). Experiments were designed to study principally the effect of iron on the rate of glucose uptake by freshly harvested bacterial cells when placed under penicillin inhibition. All of the experimental culture tubes contained in addition to the base medium (lacking the added iron salt) the regular amounts of horse serum and penicillin and 0.2 ml of a suspension of bacterial cells. Various conditions were imposed by adding iron \((4.4 \times 10^{-5} \text{ M})\), 2 B vitamins \((13 \ \mu g \text{ each})\), chiniofon \((8.4 \times 10^{-4} \text{ M})\), iron plus vitamins or iron plus chiniofon. The added iron was sterilized by autoclaving in the base medium; the vitamins and chiniofon by Millipore filtration. The bacteria were incubated for 68 hours and the culture fluids were analyzed for glucose at 0,18,42 and 68 hours. One ml aliquots were removed for analysis, diluted to 20 ml with water and 1 ml of the dilute sample used in the glucose determination. Total carbohydrate was determined employing
the method described by Montgomery (1957). A glucose solution was used to obtain the standard curve.

Comparison of glucose utilized by bacteria alone versus amebae plus bacteria. It was then elected to study glucose uptake of the amebae-bacteria complex and to compare this with the bacterial contribution under the same cultural conditions. The complete three-amino acid medium was used; however, the glucose concentration was again lowered (mean concentration, 10.7 mg/culture tube). Glucose was determined in the culture fluid initially and after every two days incubation at which time amebae were counted and serial transfers were made. During this experiment the age of the stored bacterial cells ranged from two to 16 days when inoculated into the penicillin-containing three-amino acid medium.
III PRELIMINARY STUDIES ON REQUIREMENTS FOR ADDED CALCIUM, MAGNESIUM AND IRON IN AMINO ACID MEDIA

In the following studies it should be pointed out that in those experimental cultures which contained none of the added cations under investigation there was present some background iron, calcium and magnesium equal to approximately $4 \times 10^{-6}$, $6.1 \times 10^{-5}$ and $1.4 \times 10^{-5}$ M, respectively. These background concentrations are due, chiefly, to the horse serum which it was necessary to employ. The approximate molar concentrations of calcium, magnesium and iron after addition of their salts to the test media were $3.1 \times 10^{-4}$, $5.1 \times 10^{-4}$ and $4.4 \times 10^{-5}$ M, respectively. Amebae growth reported in the results following is expressed in generations\(^9\). Since each of the cultures (with only a few exceptions) received an inoculum of 10,000 amebae, 1 generation would yield 20,000 amebae; 2 generations, 40,000 amebae; 3 generations, 80,000 amebae; 4 generations 160,000 amebae; 5 generations, 320,000 amebae; and so forth. The greatest growth obtained in these experiments was slightly less than 6.5 generations. A value of

\[ \text{Generations} = \frac{\log(\text{amebae harvested})}{\text{amebae inoculated}} \times 3.32 \]

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\(^9\)Generations = $\frac{\log(\text{amebae harvested})}{\text{amebae inoculated}} \times 3.32$
indicates an amebae harvest ranging anywhere from none to 10,000 amebae.

A. Three-amino acid medium (A-3)

In Table 6 are shown results of a typical experiment in A-3 medium designed to study the effect of iron, calcium and magnesium on amebae growth. The serial transfer numbers are given in the first column. In the other columns are listed the amebae harvests in generations for each cation test. It appears that good, continuous amebae growth occurred only in those cultures having the added iron. Those cultures with no added cations or added calcium alone, magnesium alone, or calcium plus magnesium did not support continuous multiplication. Occasionally the first transfer for these cultures showed sparse growth but more often only a few amebae survived and these usually died out by the second or third transfer.

For those cultures containing the added iron the mean amebae harvests are shown at the end of the appropriate columns (Table 6). It can be observed that growth in A-3 medium containing the three added cations is decidedly better than that in medium with iron alone, calcium plus iron, or magnesium plus iron. For instance, the average difference in amebae harvests between the iron group (4.60 G) and the complete medium (6.05 G) is 317,000 amebae. The average difference in amebae harvests between the calcium plus iron group (5.33 G) and
Table 6

The Effect on Amebae Growth of Added Calcium, Magnesium and Iron in Three-Amino Acid Medium

<table>
<thead>
<tr>
<th>Transfer number</th>
<th>Added cations</th>
<th>none</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Ca,Mg</th>
<th>Ca,Fe</th>
<th>Mg,Fe</th>
<th>Ca,Mg,Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.26</td>
<td>5.20</td>
<td>0</td>
<td>6.08</td>
<td>4.15</td>
<td>6.36</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0*</td>
<td>0</td>
<td>0.68</td>
<td>4.06</td>
<td>0*</td>
<td>5.35</td>
<td>4.96</td>
<td>6.10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0*</td>
<td>0</td>
<td>4.54</td>
<td>0</td>
<td>0*</td>
<td>4.55</td>
<td>5.00</td>
<td>5.68</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.60</td>
</tr>
</tbody>
</table>

The concentrations of calcium, magnesium and iron added to the medium were $2.5 \times 10^{-4}$, $5 \times 10^{-4}$ and $4 \times 10^{-5}$ M, respectively. Amebae growth is given in generations. Each culture received an inoculum of 10,000 ameba except those designated with an asterisk. These cultures received the entire ameba harvest of less than 10,000 ameba from the preceding culture. The mean growth for the three serial transfers is given at the end of the columns for those cultures which supported continuous growth.
the complete medium (6.05 G) is 261,000 amebae.

B. Six-amino acid medium (A-6)

The results of an experiment carried out in A-6 medium are presented in Table 7. The serial transfer numbers are listed in the first column. The amebae harvests in generations for each cation test are given in the remaining columns with the average growth shown at the end of the column for those cultures which usually supported continuous growth. The serial transfers were discontinued when the culture in a particular series showed no surviving amebae. For some unexplained reason amebae growth in the medium containing added magnesium plus iron ceased abruptly on the fourth serial transfer in the experiment presented. In similar experiments this medium supported amebae growth about as well as did the medium with added calcium plus iron. In medium containing no added cations as well as that with only added calcium there were less than 10,000 amebae (zero generations) for three consecutive transfers.

The A-6 media with added calcium, or magnesium or without added cations supported fairly good amebae growth for 3 to 5 serial transfers which then declined to only a few surviving amebae or ceased altogether. These results are in contrast to those obtained for the A-3 media which frequently gave poor harvests on the first transfer. In the presence of both calcium and magnesium growth was supported a longer period of time; hence, some improve-
Table 7

The Effect on Amebae Growth of Added Calcium, Magnesium and Iron in Six-Amino Acid Medium

<table>
<thead>
<tr>
<th>Transfer number</th>
<th>Added cations</th>
<th>none</th>
<th>Ca</th>
<th>Mg</th>
<th>Ca,Mg</th>
<th>Fe</th>
<th>Ca,Fe</th>
<th>Mg,Fe</th>
<th>Ca,Mg,Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>4.64</td>
<td>5.35</td>
<td>5.50</td>
<td>4.50</td>
<td>6.25</td>
<td>6.15</td>
<td>5.54</td>
<td>5.68</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.32</td>
<td>2.40</td>
<td>3.66</td>
<td>1.26</td>
<td>4.62</td>
<td>4.55</td>
<td>5.21</td>
<td>5.80</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.28</td>
<td>2.74</td>
<td>3.64</td>
<td>3.86</td>
<td>5.06</td>
<td>5.04</td>
<td>5.40</td>
<td>5.74</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.90</td>
<td>4.35</td>
<td>0</td>
<td>4.30</td>
<td>4.64</td>
<td>5.10</td>
<td>0</td>
<td>5.20</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0</td>
<td>2.09</td>
<td>disc.</td>
<td>0.68</td>
<td>2.82</td>
<td>4.25</td>
<td>disc.</td>
<td>5.40</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0*</td>
<td>0</td>
<td>4.55</td>
<td>4.60</td>
<td>4.52</td>
<td></td>
<td>4.86</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0*</td>
<td>0*</td>
<td>3.97</td>
<td>2.66</td>
<td>3.95</td>
<td></td>
<td>3.18</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.38*</td>
<td>0*</td>
<td>5.24</td>
<td>5.58</td>
<td>5.46</td>
<td></td>
<td>5.56</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.55</td>
<td>4.53</td>
<td>4.88</td>
<td>5.38*</td>
<td>5.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of calcium, magnesium and iron added to the medium were about $2.5 \times 10^{-4}$, $5 \times 10^{-4}$ and $4 \times 10^{-5}$ M, respectively. Amebae growth is given in generations. Each culture received an inoculum of 10,000 amebae except those designated with an astericks. These cultures received the entire amebae harvest of less than 10,000 amebae from the preceding culture. The mean growth for the eight serial transfers is given at the end of the columns for those cultures which usually supported continuous growth.

* Average growth for first three serial transfers.
ment can be attributed to their being added together although the average growth obtained in the first five transfers with calcium alone (3.39 G) is not significantly different from that obtained for the medium with calcium plus magnesium (3.55 G). A pronounced enhancement of growth occurred when iron was added to the media. The medium which supported the best amebae multiplication contained all three added cations.

C. Fifteen-amino acid medium (A-15)

The results of an experiment designed to study the effect of calcium, magnesium and iron on amebae growth in A-15 media are presented in Table 8. Here again as in the two preceding tables, the transfer numbers are shown in the first column. In the remaining columns are listed the amebae growth in generations for each cation test with the average growth given at the end of each column. The 0 generation obtained on the third transfer in the medium lacking the added cations represents an amebae harvest of 8,000 which when continued serially showed fair amebae multiplication. The differences in ability of the cations or their various combinations to promote growth are not as pronounced as in A-3 and A-6 media. However, on careful examination of the data it appears that in those cultures lacking the added iron salt amebae growth was slightly depressed.

D. Statistical analysis of experiments on calcium, magnesium and iron in amino acid media
Table 3

The Effect on Amebae Growth of Added Calcium, Magnesium and Iron in Fifteen-Amino Acid Medium

<table>
<thead>
<tr>
<th>Transfer number</th>
<th>Added cations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>none</td>
</tr>
<tr>
<td>1</td>
<td>1.72</td>
</tr>
<tr>
<td>2</td>
<td>3.10</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>3.37*</td>
</tr>
<tr>
<td>5</td>
<td>3.28</td>
</tr>
<tr>
<td>6</td>
<td>3.52</td>
</tr>
<tr>
<td>Mean</td>
<td>2.50</td>
</tr>
</tbody>
</table>

The concentrations of calcium, magnesium and iron added to the medium were about $2.5 \times 10^{-4}$, $5 \times 10^{-4}$ and $4 \times 10^{-5}$ M, respectively. Amebae growth is given in generations. Each culture received an inoculum of 10,000 amebae except the one designated with an asterisk which received the entire amebae harvest of less than 10,000 amebae from the preceding culture. The mean growth for the six serial transfers is given at the end of the columns.
Statistical tests using standard testing methods (Bancroft, 1957) were employed to establish the reliability of results obtained. Amebae growth was expressed as generations and the \( t \) test for significance applied to these values. All decisions were made at the 0.05 level of significance.

Comparison of amebae growth in amino acid media with and without added iron. In Table 9 are listed the probability levels and the values for the mean and standard deviation for the groups compared. Groups were selected for comparison as indicated in column one with the number of different cultures examined given in parenthesis below the particular group. In forming the iron group for each of the amino acid media all cultures containing added iron were included regardless of what other cations were present. The other group with no added iron included experiments in which the cations of calcium and magnesium were either excluded as far as possible, added separately, or added together.

The data presented in Table 9 shows excellent amebae growth for A-15, A-6 and A-3 media with added iron. This was not the case when the iron salt was not added to the media. Amebae growth was very poor in A-3 medium without iron, but increased to 2.97 and 3.63 generations for A-6 and A-15 media, respectively. The results clearly indicate that iron stimulates the multiplication of \( E. \) histolytica in association with penicillin-inhibited cells of
Table 9

Comparison of Amebae Growth in Amino Acid Media with and without Added Iron

<table>
<thead>
<tr>
<th>Groups compared (n) no. of cultures</th>
<th>Mean ± s.d. (Generations)</th>
<th>Level of probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (24) A-15</td>
<td>4.71 ± 1.09</td>
<td>.001 &lt; P &lt; .01</td>
</tr>
<tr>
<td>No added Fe (24)</td>
<td>3.63 ± 1.38</td>
<td></td>
</tr>
<tr>
<td>Fe (90) A-6</td>
<td>4.70 ± 1.42</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>No added Fe (75)</td>
<td>2.97 ± 1.90</td>
<td></td>
</tr>
<tr>
<td>Fe (62) A-3</td>
<td>4.62 ± 0.99</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>No added Fe (54)</td>
<td>0.82 ± 1.34</td>
<td></td>
</tr>
</tbody>
</table>
B. symbiosus in all of the amino acid media tested. The probability level obtained for the three- and six-amino acid media were less than 0.001; that for the fifteen-amino acid medium was between 0.01 and 0.001. All of these probabilities are highly significant.

Comparison of amebae growth in cultures containing added calcium, magnesium and iron versus that in cultures with only added iron. Table 10 shows clearly that the presence of calcium and magnesium significantly enhanced amebae multiplication when added with the iron in three- and six-amino acid media. The probability level obtained for the A-3 medium was between 0.01 and 0.001; that for A-6 medium was between 0.05 and 0.02. The data for the A-15 medium did not meet the test for significance at the 0.05 level of probability. The evidence presented does not distinguish between calcium and magnesium but simply shows that in combination they stimulate amebae growth and are essential for optimal multiplication. Further studies would be needed to show quantitative requirements for these cations.

Comparison of the ability of the various amino acid media with no added cations to support amebae growth. The amino acid media examined in Table 11 contained none of the added cations. The values for the mean growth obtained for the A-15, A-6, and A-3 media were 2.50, 1.94 and 0.39 generations, respectively. There was a significant difference in amebae multiplication in the A-15.
Table 10

Comparison of Amoebae Growth in Amino Acid Media with Added Calcium, Magnesium and Iron versus That with Only Added Iron

<table>
<thead>
<tr>
<th>Groups compared (n) no. of cultures</th>
<th>Mean ± s.d. (Generations)</th>
<th>Level of probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca,Mg,Fe (6)</td>
<td>5.00 ± 0.82</td>
<td>.6 &lt; P &lt; .7</td>
</tr>
<tr>
<td>Fe (only) (6)</td>
<td>4.65 ± 1.55</td>
<td></td>
</tr>
<tr>
<td>Ca,Mg,Fe</td>
<td>5.07 ± 1.15</td>
<td>.02 &lt; P &lt; .05</td>
</tr>
<tr>
<td>Fe (only) (17)</td>
<td>4.02 ± 1.83</td>
<td></td>
</tr>
<tr>
<td>Ca,Mg,Fe (16)</td>
<td>5.22 ± 2.44</td>
<td>.001 &lt; P &lt; .01</td>
</tr>
<tr>
<td>Fe (only) (16)</td>
<td>4.13 ± 1.22</td>
<td></td>
</tr>
</tbody>
</table>
Table 11

Comparison of the Ability of the Different Amino Acid Media with No Added Cations to Support Amebae Multiplication

<table>
<thead>
<tr>
<th>Groups compared (n) no. of cultures</th>
<th>Mean ± s.d. (Generations)</th>
<th>Level of probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-15 (6) No cations added</td>
<td>2.50 ± 1.39</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>A-3 (15)</td>
<td>0.39 ± 1.09</td>
<td></td>
</tr>
<tr>
<td>A-15 (6) No cations added</td>
<td>2.50 ± 1.39</td>
<td>.4 &lt; P &lt; .5</td>
</tr>
<tr>
<td>A-6 (20)</td>
<td>1.94 ± 1.93</td>
<td></td>
</tr>
<tr>
<td>A-6 (20) No cations added</td>
<td>1.94 ± 1.93</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>A-3 (15)</td>
<td>0.39 ± 1.09</td>
<td></td>
</tr>
</tbody>
</table>
or A-6 media as compared to that in A-3 medium. Probabilities of less than 0.001 were obtained in comparing A-15 or A-6 media with A-3 medium. Since these media all contained the same background calcium, magnesium and iron the growth enhancement must have been due to the presence of the additional amino acids. The probability level obtained when comparing A-15 medium versus A-6 medium did not meet the test for significance at the 0.05 level of probability.
A. Influence of various iron concentrations on amebae growth

Table 12 shows results of several experiments carried out in which the total iron in the three-amino acid medium was directly varied. The approximate concentrations of calcium and magnesium were $3.1 \times 10^{-4}$ and $5.1 \times 10^{-4}$ M, respectively. In the second column are listed the mean amebae harvests on the first transfer for a given number of experiments at the various iron concentrations. The mean amebae harvests for a given number of serial transfers after the first transfer are shown in the third column. The amount of iron which was brought into the medium with the serum and bacterial cells is represented by the lowest value, $4 \times 10^{-6}$ M. As was found previously (Chapter III, Table 6), continuous amebae growth was not possible at this low iron level. Doubling this concentration by adding an equivalent amount of iron to the medium still did not maintain growth. Increasing the concentration to $1.2 \times 10^{-5}$ M iron gave improved, though suboptimal growth. But, by raising the iron level by approximately one order of magnitude good and continuous amebae growth was obtained which was not
Table 12

Amebae Growth Response at Various Iron Concentrations in Three-Amino Acid Medium Containing Added Calcium and Magnesium

<table>
<thead>
<tr>
<th>Conc. of iron in culture fluid moles/liter</th>
<th>Amebae harvest mean of (n) expts. 1st transfer</th>
<th>Mean amebae harvest for (n) serial transfers after the 1st</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 x 10^{-6}</td>
<td>36,000 (6)</td>
<td>0 (1)</td>
</tr>
<tr>
<td>8 x 10^{-6}</td>
<td>131,000 (2)</td>
<td>0 (1)</td>
</tr>
<tr>
<td>1.2 x 10^{-5}</td>
<td>309,000 (5)</td>
<td></td>
</tr>
<tr>
<td>4.4 x 10^{-5}</td>
<td>475,000 (5)</td>
<td>429,000 (4)</td>
</tr>
<tr>
<td>4 x 10^{-4}</td>
<td>555,000 (5)</td>
<td>428,000 (4)</td>
</tr>
</tbody>
</table>
significantly improved by further increasing the iron concentration. According to these results the optimal iron level for amebae growth in this medium was approximately $4.4 \times 10^{-5}$ M.

B. Attempts to replace the added iron in the medium with other cations

In Table 13 are presented results of experiments in which various cations were substituted for the added iron in the three-amino acid medium at a concentration of $4 \times 10^{-5}$ M. In addition to the cation tested the medium contained about $3.1 \times 10^{-4}$ M calcium, $5.1 \times 10^{-4}$ M magnesium and a background concentration of $4 \times 10^{-6}$ M iron. The enhanced growth that occurred for the first transfer in the case of cobalt, copper, manganese, nickel and zinc was nil or practically so by the second or third transfer. It should be pointed out that two or three transfers are necessary to dilute out the traces of iron carried over with the amebae inoculum. In the case of aluminum, barium, molybdenum and vanadium, there were no surviving amebae or only a few hundred even on the first transfer which might suggest a toxic effect. It is evident from the data presented that none of the cations tested could be successfully substituted for iron in the three-amino acid medium.

C. Attempts to replace iron with hemin, cytochrome c or peroxidase

When hemin crystals (1 mg/culture) were substituted
Table 13

Substitution of Other Cations for Iron in Three-Amino Acid Medium Containing Added Calcium and Magnesium

<table>
<thead>
<tr>
<th>Added cation 4 x 10^{-5} M</th>
<th>Amebae harvest, transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Iron</td>
<td>435,000</td>
</tr>
<tr>
<td>Cobalt</td>
<td>160,000</td>
</tr>
<tr>
<td>Copper</td>
<td>176,000</td>
</tr>
<tr>
<td>Manganese</td>
<td>258,000</td>
</tr>
<tr>
<td>Nickel</td>
<td>216,000</td>
</tr>
<tr>
<td>Zinc</td>
<td>152,000</td>
</tr>
<tr>
<td>Aluminum</td>
<td>500</td>
</tr>
<tr>
<td>Barium</td>
<td>165</td>
</tr>
<tr>
<td>Molybdenium</td>
<td>0</td>
</tr>
<tr>
<td>Vanadium</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 14

Amebae Growth with Hemin Substituted for the Added Iron in Three-Amino Acid Medium

<table>
<thead>
<tr>
<th>Amebae growth in generations</th>
<th>Added iron 4 x 10^-5 M</th>
<th>Hemin 1 mg/culture tube (9.5 x 10^-6 M Fe)</th>
<th>Transfer number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.98</td>
<td>3.96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6.02</td>
<td>4.52</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.15</td>
<td>2.43</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5.70</td>
<td>3.68</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5.70</td>
<td>4.80</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5.23</td>
<td>4.16</td>
<td>6</td>
</tr>
</tbody>
</table>
for the added iron in three-amino acid medium, 6 serial transfers yielded 23.6 generations as compared to 32.8 for the iron-containing control for an equivalent number of subcultures (Table 14). A probability level between 0.01 and 0.001 was obtained which is a statistically significant difference in growth. Calculated on the basis of its iron content 1 mg hemin (8.3% iron) if completely dissolved would increase the iron level of the base medium by $9.5 \times 10^{-6}$ M. This amount added to that present as background iron ($4 \times 10^{-6}$ M) in the components of the medium would give a total of $1.4 \times 10^{-5}$ M iron. However, much of the hemin remained undissolved in the bottom of the culture tube, and since amebae are capable of ingesting particulate matter the effective iron concentration may have been considerably greater than $1.4 \times 10^{-5}$ M.

Cytochrome c and peroxidase were also substituted for the iron salt in three-amino acid medium and results of these experiments are shown in Tables 15 and 16. The amounts of these substances added to each culture tube are listed. Also shown are the molar iron concentrations contributed by each quantity of cytochrome c or peroxidase. The values listed do not take into consideration the background level of iron. Calculated on the basis of 13 ml culture fluid per tube the iron concentrations contributed by the various amounts of cytochrome c
<table>
<thead>
<tr>
<th>Cytochrome c</th>
<th>Moles/liter as Fe</th>
<th>Amebae harvest, transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>3,670</td>
</tr>
<tr>
<td>1.9 x 10^{-6}</td>
<td></td>
<td>3,350</td>
</tr>
<tr>
<td>3.9 x 10^{-6}</td>
<td></td>
<td>23,000</td>
</tr>
<tr>
<td>7.7 x 10^{-6}</td>
<td></td>
<td>23,500</td>
</tr>
<tr>
<td>1.5 x 10^{-5}</td>
<td></td>
<td>24,200</td>
</tr>
<tr>
<td>3.1 x 10^{-5}</td>
<td></td>
<td>80,500</td>
</tr>
<tr>
<td>Control (4 x 10^{-5} M added Fe)</td>
<td>327,000</td>
<td>605,000</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Amebae harvest</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>Moles/liter as Fe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3,670</td>
<td></td>
</tr>
<tr>
<td>$5.8 \times 10^{-7}$</td>
<td>37,600</td>
<td></td>
</tr>
<tr>
<td>$1.2 \times 10^{-6}$</td>
<td>20,200</td>
<td></td>
</tr>
<tr>
<td>$2.3 \times 10^{-6}$</td>
<td>55,600</td>
<td></td>
</tr>
<tr>
<td>$4.6 \times 10^{-6}$</td>
<td>68,000</td>
<td></td>
</tr>
<tr>
<td>Control (4 x $10^{-5}$ M added Fe)</td>
<td>327,000</td>
<td></td>
</tr>
</tbody>
</table>
ranged from $1.9 \times 10^{-6}$ to $3.1 \times 10^{-5}$ M; those contributed by peroxidase ranged from $5.8 \times 10^{-7}$ to $4.6 \times 10^{-6}$ M. It is evident that amebae growth in those cultures containing cytochrome c or peroxidase was markedly less than that of the control cultures with $4.4 \times 10^{-5}$ M iron. Growth in those cultures containing 2.4 and 4.8 mg cytochrome c did not improve on the second transfer.

D. Effect of increasing the arginine, glutamic acid and cysteine concentrations in three-amino acid medium

Amebae growth in three-amino acid medium with and without the added iron salt was compared with that obtained in this medium having doubled (2 X) amino acid concentrations under the same conditions. For those cultures with only the background iron level ($4 \times 10^{-6}$ M) the amebae harvests for the standard and 2 X amino acid media in one experiment were 3,700 and 1,300, respectively. For those cultures with $4.4 \times 10^{-5}$ M iron, the amebae harvests for the standard and 2 X amino acid media were 327,000 and 307,000 respectively. This experiment was repeated several times and gave similar results each time indicating that the increased concentrations of arginine, glutamic acid and cysteine did not stimulate amebae growth at the lower iron level nor had any effect at the higher iron level.
A. Effect of iron chelators on amebae growth

Ferric iron chelators in three-amino acid medium. Amebae harvests obtained with increasing concentrations of the ferric iron chelators, CHEL DP and Versene Fe-3 Specific, for A-3 medium with $4.4 \times 10^{-5}$ M iron are given in Table 17. There was slight, gradual decline in amebae growth as the CHEL DP concentration was increased from $6.7 \times 10^{-5}$ to $1.1 \times 10^{-3}$ M. The control culture yielded a 430,000 amebae harvest whereas, the highest level of CHEL DP yielded a 107,000 amebae harvest. This effect can be more clearly demonstrated by means of the curve shown in Figure 1. Amebae growth in generations per culture is plotted versus the log M concentration of CHEL DP. The broken line represents the log of the molar concentration of iron present in the medium. Even at the highest concentration tested which corresponds to a 25:1 molar ratio of CHEL DP to iron there was fairly good amebae growth. Table 17 shows that $1.7 \times 10^{-2}$ M Versene Fe-3 Specific corresponding roughly to a 390:1 molar ratio of chelating agent to iron had no effect whatever on amebae growth.

In addition, experiments were repeated using only
Table 17

Amebae Harvests Obtained with Increasing Concentrations of Ferric Iron Chelating Agents in Three-Amino Acid Medium Containing $4.4 \times 10^{-5}$ M Iron

<table>
<thead>
<tr>
<th>CHEL DP</th>
<th>Moles/liter</th>
<th>Amebae harvest</th>
<th>Versene Fe-3 Specific</th>
<th>Moles/liter</th>
<th>Amebae harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amebae harvest</td>
<td></td>
<td></td>
<td>Amebae harvest</td>
</tr>
<tr>
<td>0</td>
<td>430,000</td>
<td>0</td>
<td>352,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$6.7 \times 10^{-5}$</td>
<td>218,000</td>
<td>$5.2 \times 10^{-4}$</td>
<td>356,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.4 \times 10^{-4}$</td>
<td>131,000</td>
<td>$1.0 \times 10^{-3}$</td>
<td>270,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2.8 \times 10^{-4}$</td>
<td>190,000</td>
<td>$2.1 \times 10^{-3}$</td>
<td>349,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5.5 \times 10^{-4}$</td>
<td>114,000</td>
<td>$4.2 \times 10^{-3}$</td>
<td>396,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.1 \times 10^{-3}$</td>
<td>107,000</td>
<td>$8.3 \times 10^{-3}$</td>
<td>425,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.7 \times 10^{-2}$</td>
<td>320,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Influence of increasing concentrations of CHEL-DP on amebae growth in A-3 medium containing $4.4 \times 10^{-5}$ M iron.
the highest amounts of CHEL DP and Versene Fe-3 Specific tested in the above described concentration series. In one such experiment a culture with $1.1 \times 10^{-3}$ M CHEL DP yielded an amebae harvest of 158,000 with the control culture having an amebae harvest of 428,000. The culture with $1.7 \times 10^{-2}$ M Versene Fe-3 Specific had a harvest of 303,000 amebae. These results seem to substantiate the effects noted for the complete concentration series for the ferric iron chelators.

**Ferrous iron chelators in three-amino acid medium.**

The ferrous iron chelators, chiniofon and o-phenanthroline, produced an entirely different effect on amebae growth. The amebae harvests obtained with graded concentrations of o-phenanthroline and chiniofon at different iron levels are shown in Tables 18 and 19, respectively. With these chelating agents it was possible to cause complete cessation of amebae growth at relatively low concentrations. It would appear from examination of these results that increasing the iron concentration required a roughly corresponding increase in the concentration of chelating agent to achieve a 50 percent inhibition of amebae growth as compared to the control cultures.

Figure 2 illustrates the results obtained at the iron level customarily employed in amebae cultures, $4.4 \times 10^{-5}$ M, and at the highest level tested, $8 \times 10^{-4}$ M. Similar results were obtained at two intermediate iron concentrations, namely $1.6 \times 10^{-4}$ and $4 \times 10^{-4}$ M iron, and are
<table>
<thead>
<tr>
<th>o-Phenanthroline (Moles/liter)</th>
<th>Amebae harvests at various iron concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.4 x 10^{-5} M</td>
</tr>
<tr>
<td>0</td>
<td>462,000</td>
</tr>
<tr>
<td>4.3 x 10^{-5}</td>
<td>484,000</td>
</tr>
<tr>
<td>8.5 x 10^{-5}</td>
<td>346,000</td>
</tr>
<tr>
<td>1.3 x 10^{-4}</td>
<td>121,000</td>
</tr>
<tr>
<td>1.7 x 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>3.4 x 10^{-4}</td>
<td>124,000</td>
</tr>
<tr>
<td>4.3 x 10^{-4}</td>
<td>28,000</td>
</tr>
<tr>
<td>6.8 x 10^{-4}</td>
<td>188,000</td>
</tr>
<tr>
<td>8.5 x 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>1.0 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>1.3 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>1.7 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>3.4 x 10^{-3}</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 19

Amebae Harvests Obtained with Graded Concentrations of Chiniofon in Three-Amino Acid Medium at Different Iron Levels

<table>
<thead>
<tr>
<th>Moles/liter</th>
<th>$4.4 \times 10^{-5}$ M</th>
<th>$1.6 \times 10^{-4}$ M</th>
<th>$8 \times 10^{-4}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>388,000</td>
<td>506,000</td>
<td>386,000</td>
</tr>
<tr>
<td>$8.5 \times 10^{-5}$</td>
<td>290,000</td>
<td>480,000</td>
<td></td>
</tr>
<tr>
<td>$2.1 \times 10^{-4}$</td>
<td>34,000</td>
<td>402,000</td>
<td></td>
</tr>
<tr>
<td>$4.2 \times 10^{-4}$</td>
<td>384</td>
<td>43,000</td>
<td></td>
</tr>
<tr>
<td>$8.5 \times 10^{-4}$</td>
<td>0</td>
<td></td>
<td>400,000</td>
</tr>
<tr>
<td>$1.1 \times 10^{-3}$</td>
<td></td>
<td>785</td>
<td></td>
</tr>
<tr>
<td>$1.7 \times 10^{-3}$</td>
<td></td>
<td></td>
<td>105,000</td>
</tr>
<tr>
<td>$1.9 \times 10^{-3}$</td>
<td></td>
<td></td>
<td>70,300</td>
</tr>
<tr>
<td>$2.1 \times 10^{-3}$</td>
<td></td>
<td>406</td>
<td>52,000</td>
</tr>
<tr>
<td>$2.5 \times 10^{-3}$</td>
<td></td>
<td></td>
<td>2,150</td>
</tr>
</tbody>
</table>
Figure 2. Influence of increasing concentrations of chiniofon (O); or O-phenanthroline (●) on amebae growth in A-3 medium with 4.4 x 10^-5 M and 8 x 10^-4 M iron.
illustrated in Figures 3 and 4, respectively. From the curves obtained it can be observed that there is no effect on amebae growth at a 1:1 molar ratio of chelating agent to iron. But when the iron concentration was exceeded by two or three fold, amebae growth abruptly and rapidly declined. It is apparent from examining the curves in Figure 2 that the growth-inhibitive effect of the chelating agents can be completely reversed. For instance, the growth-inhibitive dose in media containing 4.4 x 10^{-5} M iron had no effect on growth when the iron level was increased to 3 x 10^{-4} M.

**Chiniofon in six-amino acid medium.** Table 20 shows that continuous, though suboptimal, amebae growth was possible in this medium at an iron level of 4 x 10^{-6} M. However, amebae multiplication was inhibited by 4.2 x 10^{-4} M chiniofon at this low iron level as well as at 4.4 x 10^{-5} M iron and a complete reversal of this effect occurred when the iron was increased to 3.2 x 10^{-4} M. The reversal action could be accomplished in yet another manner as shown in columns 5 and 6 of Table 20. The amebae exposed to an amebicidal dose of chiniofon for 48 hours gave excellent amebae harvests when subcultured into medium containing adequate iron.

**Ferrous and ferric iron chelators in acid-hydrolyzed casein medium and yeast-enriched MS-F medium.** The object of carrying out these experiments with chelators in these media which have been routinely employed in this
Figure 3. Influence of increasing concentrations of chiniofon (O); or \( O^- \)-phenanthroline (•) on amebae growth in A-3 medium containing \( 1.6 \times 10^{-4} \) M iron.
Figure 4. Influence of increasing concentrations of o-phenanthroline on amebae growth in A-3 medium containing $4 \times 10^{-4}$ M iron.
Table 20

Effect of Chiniofon on Amebae Growth in Six-Amino Acid Medium Containing Various Iron Levels

<table>
<thead>
<tr>
<th>Transfer</th>
<th>Amebae harvests obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 x 10^{-6}M Fe, 4 x 10^{-6}M Fe, 4.2 x 10^{-4}M chiniofon</td>
</tr>
<tr>
<td>1</td>
<td>442,000</td>
</tr>
<tr>
<td>2</td>
<td>57,000</td>
</tr>
<tr>
<td>3</td>
<td>98,000</td>
</tr>
<tr>
<td>4</td>
<td>18,000</td>
</tr>
<tr>
<td>5</td>
<td>198,000</td>
</tr>
</tbody>
</table>

*Each of these cultures received a 5,500 ameba inoculum from the preceding culture in column 5.
laboratory was merely to establish whether they would influence amebae growth in a manner similar to that observed in the amino acid media. It was possible to demonstrate the growth-inhibitive action of chiniofon and \( \text{o-phenanthroline} \) in acid-hydrolyzed casein medium and yeast-enriched MS-F medium as shown in Tables 21 and 22, respectively. The acid-hydrolyzed casein medium containing \( 1.4 \times 10^{-5} \text{ M} \) iron according to iron analyses (Table 4) required a lower concentration of \( \text{o-phenanthroline} \) to achieve complete cessation of growth than did the yeast-enriched MS-F medium. The MS-F medium contains \( 4 \times 10^{-5} \text{ M} \) iron and undoubtedly the yeast extract brings in an additional amount of iron although its iron content was not determined.

Although the amebae failed to survive when sufficiently exposed to adequate doses of these chelating agents, the accompanying bacterial cells multiplied when subcultured into fresh medium with penicillinase. There was no evidence of contamination by direct plating.

Versene Fe-3 Specific (\( 1.7 \times 10^{-2} \text{ M} \)) or CHEL DP (\( 1.2 \times 10^{-3} \text{ M} \)) had little or no effect on amebae growth in the casein media as shown in Table 23.

B. Effect of iron chelators on bacterial growth

In Table 24 are presented the results of experiments showing the influence of chiniofon and \( \text{o-phenanthroline} \) on bacterial multiplication in yeast-enriched
Table 21

Amebae Growth in Acid-Hydrolyzed Casein Medium with Graded Concentrations of Chelating Agents

<table>
<thead>
<tr>
<th>Moles/liter</th>
<th>Amebae growth (Generations)</th>
<th>Moles/liter</th>
<th>Amebae growth (Generations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.78</td>
<td>0</td>
<td>4.52</td>
</tr>
<tr>
<td>4.3 x 10⁻⁵</td>
<td>4.42</td>
<td>4.1 x 10⁻⁶</td>
<td>4.15</td>
</tr>
<tr>
<td>8.5 x 10⁻⁵</td>
<td>4.85</td>
<td>3.2 x 10⁻⁶</td>
<td>3.97</td>
</tr>
<tr>
<td>2.1 x 10⁻⁴</td>
<td>3.42</td>
<td>1.2 x 10⁻⁵</td>
<td>3.62</td>
</tr>
<tr>
<td>4.2 x 10⁻⁴</td>
<td>2.38</td>
<td>1.6 x 10⁻⁵</td>
<td>3.04</td>
</tr>
<tr>
<td>1.1 x 10⁻³</td>
<td>0</td>
<td>2.0 x 10⁻⁵</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 x 10⁻⁵</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 22

Amebae Growth in Yeast-Enriched MS-F Medium with Graded Concentrations of Chelating Agents

<table>
<thead>
<tr>
<th></th>
<th>Chiniofon</th>
<th></th>
<th>o-Phenanthroline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles/liter</td>
<td>Amebae growth (Generations)</td>
<td>Moles/liter</td>
<td>Amebae growth (Generations)</td>
</tr>
<tr>
<td>0</td>
<td>3.15</td>
<td>0</td>
<td>3.75</td>
</tr>
<tr>
<td>$1.1 \times 10^{-4}$</td>
<td>2.74</td>
<td>$4.1 \times 10^{-5}$</td>
<td>3.30</td>
</tr>
<tr>
<td>$2.1 \times 10^{-4}$</td>
<td>2.20</td>
<td>$6.1 \times 10^{-5}$</td>
<td>3.66</td>
</tr>
<tr>
<td>$3.1 \times 10^{-4}$</td>
<td>1.14</td>
<td>$7.1 \times 10^{-5}$</td>
<td>2.43</td>
</tr>
<tr>
<td>$4.1 \times 10^{-4}$</td>
<td>0.39</td>
<td>$8.1 \times 10^{-5}$</td>
<td>0</td>
</tr>
<tr>
<td>$6.0 \times 10^{-4}$</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 23

Iron Chelating Agents in Acid Hydrolyzed Casein Medium (Htmgc) and Their Effect on Amoebae Growth

<table>
<thead>
<tr>
<th>Transfer</th>
<th>Htmgc</th>
<th>Htmgc</th>
<th>Htmgc</th>
<th>Htmgc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>Chiniofon (8.4 x 10^{-4} M)</td>
<td>CHEL DP (1.2 x 10^{-3} M)</td>
<td>Versene Fe-3 Specific (1.7 x 10^{-2} M)</td>
</tr>
<tr>
<td>1</td>
<td>250,000</td>
<td>4,600</td>
<td>177,000</td>
<td>234,000</td>
</tr>
<tr>
<td>2</td>
<td>192,000</td>
<td>0*</td>
<td>25,000*</td>
<td>discontinued</td>
</tr>
<tr>
<td>3</td>
<td>discontinued</td>
<td>0</td>
<td>354,000</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

*Each of these cultures received a 2,300 amoeba inoculum from the preceding culture in column 3.
Table 24

Comparison of Bacterial Growth at Various Concentrations of Chiniofon or o-Phenanthroline

<table>
<thead>
<tr>
<th>Chiniofon</th>
<th>o-Phenanthroline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles/liter</td>
<td>Turbidity (visual)</td>
</tr>
<tr>
<td>0</td>
<td>++++</td>
</tr>
<tr>
<td>$1.1 \times 10^{-4}$</td>
<td>++++</td>
</tr>
<tr>
<td>$2.1 \times 10^{-4}$</td>
<td>++++</td>
</tr>
<tr>
<td>$3.2 \times 10^{-4}$</td>
<td>++++</td>
</tr>
<tr>
<td>$4.2 \times 10^{-4}$</td>
<td>++++</td>
</tr>
<tr>
<td>$6.4 \times 10^{-4}$</td>
<td>+</td>
</tr>
</tbody>
</table>
MS-F medium. The degree of multiplication at the various concentrations of chelating agents was roughly compared to the control bacterial culture by visual examination of the turbidity. The concentrations of chiniofon and \( \text{o}-\text{phenanthroline} \) which appeared to allow no bacterial multiplication were \( 6.4 \times 10^{-4} \) and \( 1.0 \times 10^{-4} \) M, respectively. These bacterial growth-inhibitive doses of chelators are approximately the same as those necessary to completely suppress ameba growth in yeast-enriched MS-F medium (Table 22).

Subcultures from tubes containing bacterial inocula exposed for 5 days to \( 3.2 \times 10^{-3} \) M chiniofon or \( 2.1 \times 10^{-3} \) M \( \text{o}-\text{phenanthroline} \) showed definite signs of multiplication after 48 hours. Tests for the presence of contaminants were negative. Neither \( 1.7 \times 10^{-2} \) M Versene Fe-3 Specific nor \( 1.2 \times 10^{-3} \) M CHEL DP appeared to suppress bacterial multiplication.
VI GLUCOSE UTILIZATION IN BACTERIAL AND AMEBAE CULTURES

A. Stimulation of bacterial glucose utilization by iron

In Table 25 are shown the results of an experiment in which *B. symbiosus* cells under penicillin inhibition were incubated in three-amino acid medium containing 1 mg/ml glucose. The amount of glucose utilized after various incubation periods is expressed as percent of the initial amount present in the culture fluids. The concentrations of calcium (3.1 x 10^{-4} M) and magnesium (5.1 x 10^{-4} M) in the medium were those regularly employed. Two different iron levels were employed in the amino acid medium, namely, the background iron level of 4 x 10^{-6} M and 4.4 x 10^{-5} M. Other test media employing each of these iron concentrations contained pantetheine and pyridoxal phosphate (13 µg each) or 8.4 x 10^{-4} M chiniofon. It was found that glucose utilization reached a maximum of 50% (0.50 mg/ml) after 68 hours in the medium with the higher iron level whereas a maximum of only 12% (0.12 mg/ml) was attained at the lower iron level. The addition of the B vitamins, pyridoxal phosphate and pantetheine, did not influence the glucose utilization at either iron concentration. The relationship of the iron concentration to the glucose
Table 25

Percent of Total Glucose Utilized in Three-Amino Acid Medium by Freshly Harvested Bacterial Cells Under Penicillin Inhibition

<table>
<thead>
<tr>
<th>Condition</th>
<th>18</th>
<th>42</th>
<th>68 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 x 10^{-6} M iron</td>
<td>12%</td>
<td>14%</td>
<td>16%</td>
</tr>
<tr>
<td>4 x 10^{-6} M iron plus panthetheine &amp; pyridoxal phosphate (13 μg each)</td>
<td>0</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>4 x 10^{-6} M iron plus 8.4 x 10^{-4} M chiniofon</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>4.4 x 10^{-5} M iron</td>
<td>26</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>4.4 x 10^{-5} M iron plus panthetheine &amp; pyridoxal phosphate (13 μg each)</td>
<td>26</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>4.4 x 10^{-5} M iron plus 8.4 x 10^{-4} M chiniofon</td>
<td>18</td>
<td>14</td>
<td>20</td>
</tr>
</tbody>
</table>
utilization activity of the bacteria is illustrated in Figure 5. The ordinate represents glucose utilized in mg/ml of culture fluid; the time in hours is given on the abscissa. It appears that the concentration of available iron in the medium does influence the glucose utilization of the penicillin-inhibited bacterial cells, the higher iron concentration having a stimulatory effect.

Binding the available iron with the ferrous iron chelator chiniofon, lowered the glucose utilization from 16% to 8% (0.16 to 0.08 mg/ml) in the case of the lower iron concentration and from 50% to 20% (0.50 to 0.20 mg/ml) for the higher iron level. This effect of the iron chelator on bacterial glucose uptake at $4.4 \times 10^{-5}$ M iron is shown in Figure 6. The results of these glucose utilization experiments at the two iron concentrations and with the iron chelator clearly show the importance of iron for glucose metabolism by the penicillin-inhibited bacterial cells in three-amino acid medium.

B. Comparison of glucose utilization by bacteria and bacteria plus amebae

Table 26 gives the percent of the total glucose utilized by the bacterial cells and parallel amebae-containing cultures in three-amino acid medium as the age of the bacteria increased from 2 to 16 days. These percentages were calculated on the basis of the initial amount of glucose found in the cultures (column 2, Table 26). The percent of glucose uptake fluctuated
Figure 5. The effect of iron concentration on bacterial glucose utilization in A-3 medium containing 1 mg/ml glucose, 
( O = 4.4 x 10^{-5} M iron; ● = 4 x 10^{-6} M iron).
Figure 6. The effect of $3.4 \times 10^{-4}$ M chiniofon on bacterial glucose utilization in A-3 medium containing $4.4 \times 10^{-5}$ M iron and 1 mg/ml glucose, ( $\bullet = 4.4 \times 10^{-5}$ M iron; $\circ = 4.4 \times 10^{-5}$ M iron plus $8.4 \times 10^{-4}$ M chiniofon).
### Table 26

Percent of Total Glucose Utilized in Three-Amino Acid Medium by Bacteria and Amebae plus Bacteria

<table>
<thead>
<tr>
<th>Age of bacterial cells in days</th>
<th>Total glucose in mg/13 ml culture fluid</th>
<th>Percent of total glucose utilized</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacteria</td>
<td>Bacteria &amp; ameba</td>
</tr>
<tr>
<td>2</td>
<td>11.9</td>
<td>47%</td>
<td>56%</td>
</tr>
<tr>
<td>4</td>
<td>10.6</td>
<td>52</td>
<td>57</td>
</tr>
<tr>
<td>6</td>
<td>10.6</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>8</td>
<td>10.9</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>9.7</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>12</td>
<td>10.4</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>14</td>
<td>10.4</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>16</td>
<td>10.5</td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>
somewhat but showed a decline as the age of the bacterial cells increased. The maximum bacterial glucose utilization was 47% (5.6 mg) at the beginning of the experiment and dropped to about 15% (1.6 mg) after 16 days.

The differences in percent glucose utilization by the bacteria alone and parallel amebae-containing cultures are given in the last column. For the first four successive transfers the amebae cultures utilized only a slightly greater amount of glucose than did the parallel bacterial cultures with differences ranging from 5% to 9% (0.5 to 1.1 mg). For the last four transfers the bacterial glucose uptake was somewhat higher than the amebae cultures. However, the differences are so small (1 to 3%) as to be within the accuracy of the techniques used. According to these results obtained by the method of differences it would appear that the amebae are using very little glucose for multiplication. However, one must consider that the bacterial population of the amebae-containing culture has been decreased considerably by the amebae's ingestion of the bacterial cells. Hence, no valid conclusions can be made with regard to amebae glucose utilization from the data presented.

C. Relationship of amebae growth to bacterial glucose utilization.

The changes in glucose utilization in three-amino acid medium by the bacteria for eight successive transfers, and the amebae harvests in parallel amebae-containing
cultures are shown in Figure 7. The ordinate at the left of the graph represents the glucose utilized (mg/culture tube); that at the right, amebae harvest/culture. The age in days of the bacterial cells when employed is given on the abscissa. Amebae harvests ranged from 605,000 to 92,000. An interesting observation was that amebae growth seemed directly dependent on the amount of bacterial glucose utilization. The amebae harvests followed very closely and even fluctuated with the glucose uptake decreasing simultaneously with the reduction in the amount of glucose utilized.
Figure 7. Comparison of bacterial glucose utilization and amebae growth in parallel amebae-containing A-3 cultures with an initial average of 10.5 mg glucose/culture tube, (● = glucose utilized by bacteria; O = amebae harvest).
VII DISCUSSION

Very few critical experiments have been conducted on the inorganic requirements of protozoa and what little knowledge has accumulated deals mostly with the ciliate Tetrahymena. Kidder, Dewey and Parks (1951) demonstrated definite magnesium, potassium and phosphate requirements for Tetrahymena gelei W in a synthetic medium but found it impossible to demonstrate the indispensability of iron in spite of its stimulatory nature. They feel that the probable explanation for this is the contaminations with iron of the various constituents of the medium. Moreover, these authors were unable to demonstrate a calcium requirement for this ciliate in their synthetic medium.

Direct evaluation of metal cation requirements for multiplication and growth of Entamoeba histolytica has not been previously reported undoubtedly because satisfactory control of inorganic constituents in complex undefined media is difficult. In this study a three-amino acid base medium was developed which provided considerably better control over the inorganic constituents of the culture fluids. Employing this medium a rather high iron concentration was found to be necessary for amebae growth. A relatively lower iron
level is sufficient to support amebae multiplication in six- and fifteen-amino acid media since these media supported some growth at a $4 \times 10^{-6}$ M iron level whereas growth could not be maintained at this iron level in the three-amino acid medium. Since some of the plasma proteins and in particular transferrin are capable of binding iron, it is possible that not all of the background iron ($4 \times 10^{-6}$ M) of the media derived chiefly from horse serum and bacterial cells is readily available in cultures.

In comparing the ability of the various amino acid media with no added cations to support amebae growth it was observed that A-15 and A-6 media yielded low but significantly better amebae harvests than did the A-3 medium. Since these media contained the same background concentrations of calcium, magnesium and iron it was concluded that the growth enhancement was due to the additional amino acids. Stimulation of amebae growth did not occur in A-3 medium containing the background iron when the amino acid concentrations were doubled but a maximal amebae growth response occurred when the iron concentration was raised to $4.4 \times 10^{-5}$ M. This result might be interpreted to mean that the higher level of iron stimulated the action of various enzyme systems to transform the three-amino acids into a whole spectrum of different amino acids. That this might happen to a limited extent is not excluded by
the data obtained, but evidence of such transformation was not detected. (Reeves, Latour and Frye, 1960).

It was also shown that calcium and magnesium are necessary for optimal amebae growth in three- and six-amino acid media although stimulation by these cations was not evident in the fifteen-amino acid medium. Inasmuch as no attempts were made to free the components of the culture fluids of calcium and magnesium impurities, the data obtained can only be interpreted to mean that no additional calcium and magnesium were required for maximal amebae growth in this medium. Quantitative requirements for calcium and magnesium in three- and six-amino acid media were not established.

Chelating agents have been widely used in studying metal ion requirements in biological systems. Kun, Bradin and Dechary (1955) studied the effect of metabolic inhibitors known to act by their metal complex-forming properties on the rate of CO\textsubscript{2} and H\textsubscript{2}S evolution by enzyme systems of \textit{E. histolytica}. Both CO\textsubscript{2} and H\textsubscript{2}S evolution were suppressed by such chelators as 3-hydroxyquinoline and \textalpha;\textalpha; bipyridyl suggesting that a metalloprotein plays an important role in the metabolic system of \textit{E. histolytica}. Complete inhibition of anaerobic rumen bacteria has been obtained with o-phenanthroline which was completely overcome by adding sufficient iron to form the chelate complex (McNaught and Owen, 1949).

Iron chelating agents were employed in order to
gain some understanding about the active form of iron in the culture fluids. It is interesting to note that at all of the iron concentrations tested in three-amino acid medium a 2 or 3 to 1 molar ratio of the ferrous iron chelators, chiniofon and o-phenanthroline, showed a profound suppressive effect on amebae growth. Adequate concentrations of these two chelators also inhibited amebae growth in two other media employed. This action could be reversed completely by adding an excess of iron and in some instances by subculture of the exposed amebae to fresh media containing sufficient iron.

CHEL DP and Versene Fe-3 Specific which are especially effective for chelating ferric iron had little or no effect on amebae growth. Since the oxidation-reduction potential of the amebae cultures is very low the iron in the medium is primarily in the ferrous state. Hence, a ferric iron chelator like CHEL DP even with such a great stability constant as $10^{30}$ would be expected to have very little effect in this system. On the other hand, the ferrous iron chelators, with much smaller stability constants were growth-inhibitive.

At levels of chiniofon or o-phenanthroline which were completely inhibitory to the growth and survival of the amebae, the associated organism retained its viability for it multiplied when subcultured into fresh medium containing penicillinase. Experiments in which bacteria were exposed to various amounts of
chiniofon or o-phenanthroline indicated that bacterial multiplication was inhibited by these agents, but that the bacteria were not killed.

Chiniofon has been used with some degree of success in the treatment for amebiasis. Its site of activity is primarily in the lumen of the large bowel; however, its mode of action has thus far not been elucidated. The results presented in this work show that chiniofon exerts its *in vitro* amebicidal effect by binding the iron which is essential for amebae growth. Its growth-inhibitive effect becomes pronounced at the same relative proportions of chiniofon to iron at all of the iron levels tested and its action could be completely reversed by increasing the iron concentration. It is, therefore, plausible to suppose that its *in vivo* mode of action is related to its property of binding iron.

It is conceivable that iron might be essential as a substituent of a more complex organic molecule which is synthesized in culture. If such is the case, then a relatively lower concentration of iron might support good amebae growth if supplied in the form synthesized or of the proper precursor. In an effort to test this possibility several experiments were carried out in which the added iron in the medium was replaced with cytochrome c, peroxidase or hemin. Moderate though suboptimal amebae growth was obtained with hemin in three-amino acid medium. Since much of the hemin added (1 mg/culture)
remained undissolved its concentration in the medium is not known and these results cannot be compared to those of Iralu and Shaffer (1961) who observed a toxic effect on amebae growth at high concentrations of hemin (75 μg/ml) and a stimulatory effect at low hemin concentrations.

The maximum quantity of cytochrome c utilized per culture tube introduced approximately 3.1 x 10⁻⁵ M iron in addition to that which was already present as a contaminant in the base medium. The total quantity approached the iron level present in the regular media. However, these cultures yielded rather poor amebae harvests with no significant improvement in growth occurring in the second transfer. The quantities of peroxidase employed introduced up to a maximum of 4.6 x 10⁻⁶ M iron in addition to the background iron level of 4 x 10⁻⁶ M. The amebae growth in cultures containing peroxidase were significantly less than the control cultures at all concentrations tested. These experiments by no means exhausted all of the possible naturally occurring iron-containing compounds which could be tested. However, the aim of this particular line of investigation was not achieved in the experiments described.

None of the characteristic cytochrome absorption bands were found in suspensions of Clostridium tetani and the fermentation of this organism was relatively insensitive to cyanide (Lerner and Pickett, 1945).
However, inhibition of gas production from glucose by washed cells of *Cl. tetani* was obtained with the chelating agent, α,α-dipyridyl. Pappenheimer and Shaskan (1944) found that lactic acid and gas production by washed cells of *Cl. welchii* as well as growth in defined media were completely inhibited by 0.002 M bipyridine. According to these authors this inhibitory action of bipyridine on *Cl. welchii* stands in contrast to its effect on aerobic organisms known to contain the cytochrome system. This also precludes the existence of hemin or another iron-porphorin compound as the iron-containing enzyme for dipyridyl does not combine with these (Sherman et al., 1934). Since cyanide and azide at concentrations effective against cytochrome oxidase did not inhibit the survival of *E. histolytica*, Yang (1959) reported that the cytochrome oxidase system is not important in the metabolic pathway of the amebae. Hilker and White (1959) could not detect characteristic spectra of the reduced cytochrome compounds in amebal preparations on the addition of hydrosulfite. Since *E. histolytica* is considered to be anaerobic the lack of cytochromes is not unexpected and the failure of cytochrome c to replace the iron salts in the medium is not surprising.

The effect of iron on the glucose utilization of penicillin-inhibited cells of *Bacteroides symbiosus* was investigated. It was found that the glucose utilization activity of this organism was directly related to the
concentration of iron in the medium. Chelating the available iron with chiniofon caused a marked decrease in glucose utilization. It should be pointed out that the conditions imposed on the bacterial cultures in three-amino acid medium which caused minimal rates of glucose uptake were inadequate for supporting amebae multiplication. A comparison of bacterial glucose utilization to amebae harvests in parallel amebae-containing cultures showed that amebae growth varied directly with the amount of glucose utilized. As the age of the stored bacterial cells increased their glucose utilization activity decreased. These results might explain the observation made by Reeves, Latour and Frye (1960) that the stored bacterial cells, at some time ranging between 10 and 15 days after harvest, would cease supporting the multiplication of amebae in three-amino acid medium but without showing the least diminution of amebae multiplication in a medium derived from casein.

It has been found that the products obtained from the breakdown of glucose by *Clostridium welchii* depend upon the iron content of the cells (Pappenheimer and Shaskan, 1944). As the iron content was decreased the reaction shifted from a predominantly acetic-butyric acid type with production of large amounts of CO₂ and H₂ towards a more purely lactic type of fermentation with slight gas formation. The authors suggest the existence of two separate mechanisms for breakdown of glucose, one of
these an iron-containing enzyme. Lerner and Pickett (1945) found that the breakdown of glucose by washed suspension of *Cl. tetani* was far more complete when the organism was grown in a medium containing an excess of iron than when they were grown in an iron-deficient medium.

As mentioned earlier, the investigation of biochemical activities of parasitic amebae have been seriously hampered by the presence of associated organisms in amebae cultures. In the present study glucose utilization of amebae-bacteria mixtures showed no significant differences when compared to that of the bacteria alone. However, caution should be exercised in interpreting these results to mean that the amebae made little or no contribution to total glucose uptake of the system. It is uncertain whether it is valid to measure the separate contributions of the associated organism by this method of differences.

On the basis of the results obtained, one might postulate that the amebae use the products of bacterial glucose metabolism or that the bacteria may provide enzymic systems which the amebae require for glucose metabolism.

In conclusion it should be emphasized that a definite iron requirement for amebae growth was readily demonstrated in A-3 medium for unless iron salt was added to this medium amebae multiplication usually ceased by the second transfer. A growth stimulatory effect for iron was shown by statistical methods for the A-6 and A-15 media. These experiments indicate that the iron enters
into some phase of the residual glucose metabolism of the penicillin-inhibited bacterial cells, but they do not answer the question of whether the iron enters directly or only indirectly into the metabolism of the amebae when cultivated in association with the bacteria.
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Title of Thesis: Studies on the cation requirements of Entamoeba histolytica

Approved:

Richard E. Reeves
Major Professor and Chairman

Max Goodrich
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