Some Characteristics of Endamoeba Histolytica Essentials in Egg Yolk

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SOME CHARACTERISTICS OF
ENDAMOEBIA HISTOLYTICA ESSENTIALS
IN EGG YOLK

by

Miguel Kouarny

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction and Statement of Problem</td>
<td>1</td>
</tr>
<tr>
<td>II. History of Problem</td>
<td>5</td>
</tr>
<tr>
<td>A. Culture Media</td>
<td>6</td>
</tr>
<tr>
<td>B. Growth Requirements</td>
<td>9</td>
</tr>
<tr>
<td>C. Bacterial Flora</td>
<td>14</td>
</tr>
<tr>
<td>III. Material and Methods</td>
<td>17</td>
</tr>
<tr>
<td>A. The Amoeba Strain</td>
<td>18</td>
</tr>
<tr>
<td>B. Preparation of Pipettes</td>
<td>19</td>
</tr>
<tr>
<td>C. Inoculation</td>
<td>19</td>
</tr>
<tr>
<td>D. Transfers</td>
<td>20</td>
</tr>
<tr>
<td>E. Incubation</td>
<td>20</td>
</tr>
<tr>
<td>F. Recording of Growth</td>
<td>21</td>
</tr>
<tr>
<td>IV. Experimental Procedures and Results</td>
<td>23</td>
</tr>
<tr>
<td>Exp. No. 2. Stability of Base Medium Prepared From A Single Yolk; Heated Intermittently</td>
<td>26</td>
</tr>
<tr>
<td>Exp. No. 3. Dialysis and Fractionation of the Yolk-Salt Extract (No. 15)</td>
<td>31</td>
</tr>
<tr>
<td>A. Extraction of Dialysed Yolk Extract With Chloroform</td>
<td>33</td>
</tr>
<tr>
<td>B. Freeze-Drying of the Dialysed Yolk Extract (No. 16)</td>
<td>36</td>
</tr>
<tr>
<td>C. Treatment of the Chloroform Extract in Alcohol With Acetone</td>
<td>36</td>
</tr>
<tr>
<td>D. Separation of the Clear Supernatant Liquid From the Formed Precipitate of Solution (No. 17b)</td>
<td>39</td>
</tr>
<tr>
<td>Exp. No. 4. Fractionation of Yolk-Salt Extract of Twelve Yolks</td>
<td>41</td>
</tr>
<tr>
<td>A. Separation of the Two Components of the Ether-Extract Solution Dissolved in Alcohol (No. 21)</td>
<td>46</td>
</tr>
</tbody>
</table>
B. Possible Precipitation of Acetone-Insoluble Fraction from Clear Ether-Extract (No. 21c) . . . . . 46
C. Dialysis of Ether-Treated Remains of the Twelve-Yolk Extract .................. 51
D. Chloroform Extraction of the Dialysed Yolk Solution (No. 23) ........................ 52
E. Treatment of the Chloroform Extract with Ether ........................................... 53

V. Discussion and Conclusion ................................................................. 57

SUMMARY ...................................................................................................... 71a

BIBLIOGRAPHY .......................................................................................... 72

APPENDIX ...................................................................................................... 76
LIST OF TABLES

Table  Page

I. Amoebae Count Per mm$^3$ For The Symbols Used ................. 22
II. Stability of Hartman's Yolk Preparation In The Dry Phase ..... 25
III. Stability of Yolk-Salt Extract From A Single Yolk In The Wet Phase .................................................. 27
IV. Sample of Tabulated Data Appearing In Writer's Notebook . 28
V. Amount of Growth on Yolk-Salt Extract Powder (No. 16) At End of Six Transfers ........................................... 29a
VI. Amount of Growth on Medium No. 16 (The Dialysed Yolk Solution of a Single Yolk) ........................................... 33
VII. Amount of Growth on Media Prepared After Treatment of The Dialysed Yolk Extract Solution With Chloroform .......................... 35
VIII. Amount of Growth on Medium No. 19 (The Freeze-Dry Process of Dialysed Yolk Extract) ........................................... 37
IX. Amount of Growth on Media Prepared After Treatment of the Alcohol-Dissolved Chloroform Extract With Acetone .................. 39
X. Amount of Growth on Media Prepared After Centrifugation of Medium No. 17b ......................................................... 40
XI. Amount of Growth on Media No's. 20, 21, and 22 At The End of Six Transfers ......................................................... 45
XII. Amount of Growth on Media Prepared After Filtration of Medium No. 21 ................................................................. 48
XIII. Amount of Growth on Media After Precipitation of an Acetone Insoluble Fraction From No. 21c ................................. 50
XIV. Amount of Growth on Medium No. 23 (Dialysed Yolk Extract) 52
XV. Amount of Growth on Media Prepared From The Extraction of Dialysed Yolk With Chloroform Followed by Ether Extraction of the Chloroform Extract.......................... 55

LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flow Sheet For The Fractionation of a Single Yolk</td>
<td>50</td>
</tr>
<tr>
<td>2.</td>
<td>Flow Sheet For The Fractionation of Yolk-Salt Extract Of Twelve Yolks</td>
<td>42</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION TO THE PROBLEM

The development and preparation of a better medium for the cultivation of Endamoeba histolytica will give an opportunity for obtaining more precise information on the physiology, metabolism, and bio-chemical characteristics of this amoeba. A better medium will also permit the preparation of better specific antigens, the development of a suitable primary isolation medium, and the study of possible differences between E. histolytica and other related amoebae.

Since the development of the first medium for the cultivation of E. histolytica in a bacterial mixed culture by Bosak and Drbohlav (1925), many other media have been prepared. The constituents which have gone into the preparation of these media have come from varied and widely distributed sources in the animal kingdom, the most abundant source being found in liver and in egg yolk. Since the factor which enhances growth of E. histolytica most profusely is found in egg yolk, it is the purpose of this problem to study the possible ways of extracting and concentrating this material found in egg yolk. The egg yolk is to be submitted to a series of fractionations, suitable media prepared from each fraction, and biological assays made on each preparation.

At present a sustained cultivation of E. histolytica free from its
bacterial associates has not been achieved. Many workers have stated that a bacterial-free culture of amoebas had been attained, but further research in the literature has revealed this to give rise to conflicting and dubious conclusions.

Rees and his associates (1944) have been able to grow *E. histolytica* in the presence of a single species of bacteria. Shaffer and Frye (1946) have been able to cultivate *E. histolytica* in the presence of a streptobacillus, and Phillips (1950) substituted *Trypanosoma cruzi* for bacteria in an amoeba culture. Although these successful methods of producing growth and multiplication of amoeba in vitro have been realized, it is common knowledge that the amoeba grows better in the presence of a mixed bacterial culture. It is evident, therefore, that the bacterial population present in an amoeba culture plays an important role in conditioning the environment of the amoebas in some way.

It is the belief of the writer that in order to develop a medium that will support growth of *E. histolytica* free from any associates, it is necessary to determine the nutritive requirements of this amoeba in the presence of a bacterial flora, since the flora remains unchanged in media which will not support amoeba growth. For example; the writer prepared a medium which supported growth of the amoeba with a mixed bacterial culture. When the amoebae were growing quite heavily, the mixed bacterial culture was growing scantily. Upon dilution of the medium, the medium no longer supported growth of the amoeba while the mixed bacterial population grew luxuriantly. This leads the writer to believe that some factor or factors other than the bacteria present in the medium was responsible for maintenance of the amoeba culture,
since dilution of the medium diluted this factor to such an extent that not enough was available to the amoebae—hence they died out.

Leoff (1951) in his book summarizes the works and contributions of the various investigators through the years on the amoeba growth factor. In his bibliography, Leoff lists many references which he considers to be important. Most of these papers have been read by the writer and, on the basis of their conclusions, the writer believes that there is still not a full clear-cut elucidation of the factor necessary for the growth or enhancement of E. histolytica. However, from all this the following can be said: The factor(s) necessary for amoebic growth are present throughout the animal kingdom; they are widely distributed and present in animal tissues but seem to be most abundant in egg yolk and liver; and the quantity of this factor(s) required by the amoeba seems to be minute and probably can be expressed in the order or range of milligrams percent or less.

Since experiments have shown that there exists in egg yolk something which is essential for the growth of E. histolytica, and not present in egg white, and this substance is found widely distributed in animal tissue, I have designated the following symbol, "AGF", to mean amoeba growth factor and it is to be used hereafter in this paper.

In experiments designed to determine the nature of, or to cast light upon, some of the conditions or materials which influence or enhance amoeba multiplication, the following procedure was undertaken:

The AGF can be extracted from egg yolk by the use of 5% NaCl solution and heat. This extraction process seems to give better enhancement of amoeba growth than the process used by Balamuth (1944). This salt extract is dried
to a powder (process of Hartman, and unpublished). This salt extract gives a material which is suitable for fractional separation. The procedure then is to submit the dried extract to a series of fractional separations by use of fat solvents such as chloroform, alcohol, and ether. Each separated fraction is then concentrated and prepared into a medium for the growth of E. histolytica. Biological assays are made on each fraction by cultivating and subculturing a strain (NRS) of E. histolytica in a bacterial-mixed culture.

The purpose of this problem then is to attempt to study and to obtain more evidence as to the methods of concentration and purification of the material in egg yolk essential to growth and multiplication of E. histolytica in a bacterial-mixed culture.
CHAPTER II

HISTORY

Numerous attempts have been made by investigators to devise a culture medium suitable for growth and multiplication of Endamoeba histolytica, but, up to the present, a culture medium free from the influence of organisms other than the amoebae has not met with success. During the past two decades, hundreds of media have been prepared, tested, and discarded, as media suitable for amoeba cultivation. The components for these media have come from varied and diversified sources. Even at the present, some of these media are modified and tested again in a search for suitable artificial environmental conditions that would satisfy the requirements of a bacteria-free amoeba culture. A search through the literature will reveal that several investigators have claimed success, but when the medium prepared by them was repeated by others, or even by the originators at later times, they gave irregular or unsatisfactory results. At present, the best medium suitable for good growth has been accompanied by either a bacterial-mixed culture, a monobacterial culture, or a culture of Trypanosoma cruzi. Therefore, to give light to the many types of media prepared for the cultivation of E. histolytica since its first successful cultivation in a bacterial-mixed culture by Roock and Drbohlav, a history follows.
As Culture Media

Boeck and Drbohlav (1925) were the first to cultivate *E. histolytica* in a bacterial-mixed culture. Many culture media were prepared by these investigators and experiments performed to determine the most suitable or favorable medium for the cultivation of the amoeba. They said that a Locke-egg-serum and Locke-egg-albumin media would give a more dependable and consistent media for the maintenance of *E. histolytica*. They recommended that a coagulated egg alant covered with either egg white or serum diluted with Locke's solution be used for a medium. A temperature of 37°C Centigrade was most favorable for the multiplication of the amoeba in culture. In experimenting with the medium, they observed that an initial alkaline reaction of pH 7.2 - 7.8 was necessary but that a buffer substance in the media seemed inadvisable.

Dobell and Laidlaw (1926) used horse serum instead of human serum in the liquid portion of the Boeck and Drbohlav medium and demonstrated that addition of sterile rice starch produced more abundant growth of amoeba and prolonged the life of the culture. A few years later, Cleveland and Collier (1930) employed large numbers of substances in attempts to improve cultivation methods of *E. histolytica*. All media used contained sterile rice flour and consisted, for the most part, of a slant or slope covered with a liquid. Liver infusion agar slants covered with serum-saline (1-6 parts) and a small amount of sterile rice flour added were specific for *E. histolytica*. These investigators also observed that Ioeffler's dehydrated beef serum slants covered with serum-saline (1-6 parts) gave excellent results. Another of their preparations which consisted of an autoclaved liquid medium composed of
hydrolyzed hemoglobin sustained a good crop of amoebae. In 1932, a medium suitable for the cultivation of *E. histolytica* was provided by adding whole wheat flour to a 0.1% extract of heart muscle by St. John (1932).

The use of fecal material in media devised for the purpose of cultivating human protozoa has been mentioned in the literature. Andrews, Johnson, and Schwartz (1939) describe an aqueous extract of human feces with Ringer's or Locke's solution plus serum, for the cultivation of *E. histolytica*. However, previous to this publication, Boseck and Drbohlav had tested the effects of aqueous extracts of human feces in Locke-egg-serum medium and found that the bacteria multiplied so rapidly that the medium soon ceased to be a favorable environment for the amoeba.

Yeast appeared in the literature as a possible enhancement constituent in a medium for the cultivation of *E. histolytica* in a paper by Craig (1939). Results of Craig showed that yeast extract prepared by Difeo, in the lower concentrations accelerated growth of *E. histolytica* in culture. Frye and Meloney (1939) utilized liver extract as a substitute for serum in the culture medium for *E. histolytica*. They dissolved commercially prepared liver extract powder in normal saline and sterilized by autoclaving at 15 pounds for thirty minutes. This solution was then added to sterile medium of Dobell and Laidlaw with a small amount of rice flour. The commercial powder they utilized was Lilly's liver extract No. 343. Best growth was observed in 0.5% dilution of liver extract. Reardon and Rees (1939) cultivated *E. histolytica* in a medium without the utilization of serum. Locke's solution, without addition of serum or any other supplement, was used as an overlay for egg slants in the cultivation of the amoeba.
Balamuth and Sandza (1944) prepared an infusion of coagulated egg yolk in a phosphate buffered salt solution. This medium remained relatively clear owing to the poor growth of accompanying bacterial flora, permitting more accurate study of the amoeba themselves. Doubling the initial quantity of egg yolk and/or adding 0.3% Wilson liver concentrate powder (1-20 parts) to the medium accelerated the growth of the amoebae.

Rees, Besicevich, Reardon, and Daft (1944) stated that very high yields of E. histolytica in culture with a single species of bacteria designated as organism J were obtained from a simplified Locke-egg-serum medium prepared from egg white enriched with vitamins and cholesterol, the vitamins and cholesterol being lacking though present in whole egg. They also stated that medium prepared from egg yolk did not support growth of the amoeba and, as was expected, that the enrichment of yolk medium with the vitamins and cholesterol still failed to give growth properties to the yolk medium. A year later, Rees and Reardon (1945) put out a publication which said that their experiments with egg white fractions failed to indicate whether amoeba nutrients were localized in components of egg white, since their methods to obtain this information were inadequate. However, they said that their data on stored medium indicated that oxygen-free stored overlay containing ovomucoid supported good growth of amoebae. This data supported a theory that growth of E. histolytica is favored by a reduced oxygen potential.

Again Balamuth (1946) modified his medium (Balamuth & Sandza, 1944) by preparing an improved egg yolk infusion for cultivation of E. histolytica and other intestinal protozoa. He proposed that dehydrated egg yolk be used as a substitute for fresh eggs. Nelson (1947) utilized an alcoholic extract
medium for the diagnosis and cultivation of *E. histolytica*. His media was prepared as a 1% extract from tissue or egg preserved in 95% ethyl alcohol. The alcohol was boiled off after the extraction was made. Modification of the medium consisted of incorporating the extract in 2% agar and making a steep slant with buffered saline overlay and rice starch. This, according to Nelson, appeared to be a good medium and *E. histolytica* will keep growing for several transfers while other species of parasitic amoeba would tend to die out. Liver and egg extracts gave good growth of *E. histolytica* while egg white extract failed to support growth. Delemeter and Hallman (1947) demonstrated that a heat stable and dialyzable substance in the protein-free fraction of human serum was essential to the growth of the NPS strain of *E. histolytica* in egg white buffer infusion medium containing starch. Hallman, Michaelson, and Delemeter (1950) prepared a defined medium for the cultivation of *E. histolytica*. This medium consisted of a mixture of about twenty amino acids, buffers, cholesterol, most of the vitamins, minerals, and miscellaneous constituents such as nucleic acids, creatinine, and urea. Schaffer and Sieniewicz (1952) experimented with the propagation of a strain of *E. histolytica* in tissue-bearing cultures. Their results appeared to indicate that amoebae could be transplanted indefinitely in the presence of chick embryo tissue in tissue culture nutrient fluid.

**B. Growth Requirements**

Factors which influence growth and multiplication of *E. histolytica* in culture other than the amoeba's source of energy, are factors such as: substances which enhance growth of amoebae, i.e., cholesterol, reduced oxygen
tension, the effect of age of culture, size of inoculum, amount of rice starch added, variation in vitality of the culture, the effect of pH on the culture, influence of density of population, rate of multiplication, and the relationship of population growth to in vitro encystation of *E. histolytica*.

Snyder and Naleny (1943) after a series of experiments concluded that anaerobiosis and cholesterol were essential for the growth and propagation of an amoeba culture. Shaffer, Ryden, and Frye (1949) made studies on the growth requirements of *E. histolytica* in a clear medium without demonstrable bacterial multiplication, and also observed that the amoeba cultured in the preconditioned medium required anaerobiosis for growth and multiplication. These workers, however, did not conclude from their observation that the amoebae would not multiply in the presence of free oxygen in another medium. In their discussion, they state that there are indications that some factor or factors present in the medium is sensitive to free oxygen and oxidation may render these factors toxic to the amoeba. If removal of these products of oxidation is possible, they state, then perhaps by removal of them the medium might become sustainable for amoebic growth under aerobic conditions. However, up to the present, this is not yet possible, and all cultures are made up to contain an environment in which the oxygen tension is reduced.

Rice starch or rice powder is another component that has to be included in the preparation of medium for the most profuse growth of *E. histolytica*. Delameter and Hallman (1947) included rice starch in their preparation and said that starch appears to act as a temporary inhibitor to the multiplication of bacteria in an amoeba culture. This suppression leads to more favorable growth of the amoebae present. The bacterial flora present in their
culture of the NFS strain were: Escherichia coli, Staphylococcus aureus, a diptheroid, a gamma streptococcus, and a gram-variable, branching bacillus which at that time was not identified.

Shaffer, Walton, and Frye (1948) discuss the effect of rice in an amoeba culture containing one associate organism - a gram negative streptobacillus. If too much rice flour is added to the culture, the amoeba tends to die out. This suggests to the investigators certain possibilities. One possibility is that the starch might be injurious to the amoeba in high concentrations. The bacteria present might inactivate this injurious substance when rice starch is present in optimal amounts, but once the concentration of rice starch is too high, then there will be more injurious substance present then the bacteria can neutralize. A second and more likely possibility is that the bacterial flora may attack the rice in such a manner that the products of metabolism liberated from the rice flour become beneficial or even essential to the growth and multiplication of the amoeba. Reardon and Bartgis (1949) describe a phenomenon noted before by other investigators. They observed that when a culture of E. histolytica with a single species of bacteria is examined microscopically, the rice starch appeared as coarse particles composed of intracellular starch grains enveloped by gluten at the beginning of cultivation. Very few discrete starch grains were visible in the medium but after two days, examination revealed a profusion of discrete starch granules in the medium. This is evidence of enzymatic activity on the part of the amoeba to liberate the starch grains from the protein envelope. In cultures in which only organism j is growing, only the former was observed.
Spingara and Edelman (1950) were two other workers who studied the effect of rice powder on growth of *E. histolytica*. In cultures containing no rice powder, amebae increased moderately and survived 48-96 hours. They note that this was due to the small amount of rice starch present as carry-over in their cultures. Then rice powder was included in the medium; the amebae were seen to improve and persisted for longer periods of time, between four and twenty-four days. It was apparent to these workers that the amount of rice powder added to a culture was correlated to the degree of stimulation of amebic growth. Spingara and Edelman state that a maximum population was obtained when 50 milligrams of rice powder was included; larger amounts of rice powder failed to augment yield of amebae beyond values obtained with 50 milligrams. They were unable to detect any significant changes in the bacterial flora growing with each strain of amebae by varying the amount of rice powder in the medium. As the amebae grew and multiplied in the culture, the grains of rice disappeared from the culture leaving behind an amorphous sediment. After the starch granules were split, presumably by enzymatic action of the amebae, the particles of the split starch granule were phagocytized by the active trophozoite and could actually be seen in the cytoplasm of the feeding amebae.

In studies on the effect of the hydrogen ion concentration on encystation of *E. histolytica* in culture, Chang (1942) determined the optimum total salt concentration and optimum pH value for cyst production of *E. histolytica*. He believed that the maintenance of a favorable pH range throughout the period of incubation depended not only on the initial pH value, but also on the accompanying bacterial flora.
Many investigators have studied the relationship of population growth of *E. histolytica* to encystation and excystation. Everritt (1949) made the observation that agitation of a culture at frequent intervals increased the rate of multiplication and the total population, but inhibited excystation to a considerable extent. At a later date, again Everritt (1950) attributes the increase in the percentage of cysts in undisturbed cultures to the logarithmic increase in the size of the population. These two, he states, are directly proportional to each other. Excystation he also attributes to the phenomena of crowding or accumulation of metabolic products of the multiplying amoebae. In studying the factors which influence excystation of *E. histolytica*, Snyder and Meleeney (1941) describe a method for obtaining amoebic cysts free of living bacteria. These bacteria-free cysts were not seen to excyst when inoculated into a bacteria-free medium. Excystation was observed only in the presence of reducing agents, such as cysteine or neutralized thioglycollic acid, or under conditions of reduced oxygen tension. However, continued cultivation of the bacteria-free excysted forms could not be affected without the addition of living bacteria to the culture. It is apparent from these failures to obtain continued cultivation of *E. histolytica* in the absence of bacteria, that "the association between the amoeba and bacteria is not merely incidental; this association might more correctly be described as a commensalism, in which one of the associates is dependent on the other for some essential factor or factors."

Some sources responsible for variability of entococci amoebae in cultures were studied by Griffin and co-workers (1950). The effects of age of culture, the size of inoculum, the amount of starch, the effect of age of
insolium, and variation in vitality, all were factors which were presumed to be correlated to variation in the culture. Meloney and Zuckerman (1948) previously to Griffin (above) had postulated that perhaps the size range of strains of _E. histolytica_ was not a fixed characteristic but may change from a small to large or vice versa under environmental conditions. Microscopic observations by Balamuth and Brent (1951) demonstrated that amoebae enlarge in hypotonic media and become smaller in hypertonic media indicating a direct dependence of size upon salt concentration.

**C. Bacterial Flora**

Cleveland and Sanders (1930) obtained pieces of bacteria-free abscesses which contained active trophozoites of _E. histolytica_ and inoculated culture media employed in the cultivation of amoebae, but in no instance did they notice that the medium sustained growth and multiplication longer than fourteen days. They also attempted to culture these bacteria-free amoebic abscesses with strains of pure culture of bacteria. After the amoebae were inoculated into suitable medium, various bacteria were added. Here they observed that there were certain strains which did support amoebic growth for a time while other strains did not. China, Jacobs, Reardon, and Rees (1942) studied the influence of the bacterial flora on the cultivation of _E. histolytica_. Numerous strains of bacteria were tested for their ability to sustain growth of _E. histolytica_ from micro-isolated cysts. A table on those strains which support or enhance growth and those strains which do not support growth of the amoeba appears in their paper.

Rees and Reardon (1944) established that the organism _E._ utilized
Dextrose, vitamins, and nitrogenous materials occurring in all media that were tested. The organism is the single bacterial strain found associated in the amoeba cultures of Rees and co-workers (1944). Biological studies on E. histolytica growth cycles in a mixed bacterial flora were made up by Balamuth and Heward (1946) and they offer two possible explanations on the role of the bacterial flora on the amoeba. One explanation is that the products of bacterial metabolism are utilized by the amoebae. The other explanation is that perhaps the media was adjusted through bacterial activity to a physio-chemical range in which E. histolytica can survive. Shaffer and Frye (1948) declared in their paper that the results obtained by them in the maintenance of a strain of E. histolytica through one hundred transplanting in the absence of an actively multiplying bacterial flora indicated that the NRS strain of E. histolytica is capable of active multiplication in a medium with very few, if any, multiplying bacteria. There was good evidence, they said, that the bacteria present in the NRS complex are rendered static by the treatment to which they have been submitted.

Jacobs (1950) published results on the substitution of bacteria in cultures of E. histolytica. He lists twenty-five different strains of bacteria which are capable of supporting growth of E. histolytica and twenty different strains of bacteria which are incapable of supporting growth of the amoebae. Phillips and Rees (1950) maintained through fifty serial transfers bacteria-free cultures of E. histolytica with active Trypanosoma cruzi, and through twenty-five serial transfers similar cultures with T. cruzi heated treated at 48 degrees Centigrade for ten minutes. Balamuth and Weiboldt (1951) compared growth cycles of E. histolytica with different combinations of
bacteria and observed that the highest growth response of amoebae was obtained when they were in the presence of a mixed bacterial flora. In mono-bacterial cultures, amoebic growth was greatest when associated with the common intestinal flora, i.e., *Escherichia coli* and *Aerobacter aerogenes*. Phillips (1951) in a continuation of his work, compared the effects of certain species of *Trypanosoma* on the growth of *E. histolytica* in the absence of bacteria. His conclusions were that *T. cruzi* could indefinitely sustain good growth of *E. histolytica* through serial transfers while the amoebae failed to grow when associated with *T. conorhini*, *T. pinicintelli*, *Leishmania donovani*, *L. tropica*, or *L. braziliensis*.

It is apparent from the above review of the literature that the problem of the amoeba is not yet completely elucidated. Some of the literature is conflicting and might give rise to erroneous conclusions. It is therefore the belief of the writer that the problem of growth and multiplication of *E. histolytica* can come to be readily understood when all factors pertaining to the amoeba are worked out completely.
CHAPTER III

MATERIALS AND METHODS

The procedure for the preparation of yolk-salt extract (process of Hartman, unpublished) is essentially a modification of the process of Balamuth (1944). The yolk is extracted with a 5% solution of sodium chloride, instead of 0.8% NaCl solution, and the addition of a phosphate buffer is eliminated. The yolk-salt infusion is further treated by the Hartman process in order to dry it down to a powder. The infusion preparation is placed in a dehydrating jar and dried under vacuum. The heating temperature is between 70°-75° Centigrade. The pressure in the system is reduced to about 100 mm. of Hg. When the water disappears and the extract is dry, it is pulverized with a mortar and pestle and stored in a jar.

To make media from this base, 0.6 gms. of the extract powder and 0.3 gms. of yeast extract (BBL or Difco) are weighed out and dissolved in 100 ml. of distilled water, adjusting the pH to 7.2. This solution is equivalent to an 0.8% salt solution in respect to the amount of NaCl in the solution. Throughout this study, the preparation of media will follow the general principles of the above procedure. The only known variable will be the subsequent treatment of the yolk extract and the dilution of the media which will be mentioned below.

The media as prepared above has been arbitrarily designated as a media of 100% strength, meaning the amount of yolk extracted in a given batch.
In other words, a medium designated as 100% contains the extracted yolk material in 16 ml. of the extraction liquid or in 0.8 gm. of dried salt. (The NaCl present is not more than 0.8% and not less than 0.7% concentration.) In diluting down a medium prepared as in the above procedure, an appropriate amount of a solution containing 0.3 gm. yeast extract and 0.8 gm. NaCl per 100 ml. of solution is added to the base medium. For example, if the base medium is to be diluted to a 50% medium, all that is necessary to do is to mix equal parts of the base and the yeast-extract-NaCl solution.

All media prepared is tubed in 7 ml. amounts in Pyrex rimless tubes, 15 x 125 mm., plugged, autoclaved at 15 pounds for 15-20 minutes, and stored in the refrigerator. Prior to use, a loopful of sterile rice powder (Difeo) is added to each media tube.

Hereafter, all media will be prepared in the same manner as the above procedure states, except where it is otherwise mentioned. All media prepared will be the same with the only variables being the mode of treatment of the yolk and in the dilutions of the media as explained above.

THE AMOEBA STRAIN

The amoeba strain used in all of the experiments in this problem is the strain designated as the NRS strain of _E. histolytica_. This particular strain, which has been maintained in culture in this laboratory for a number of years by Dr. Ernest Hartman, was obtained from Dr. William Balamuth of Northwestern University. This strain of amoebae had originally been isolated from a Macacus monkey in 1926 by Dr. Clifford Dobell.

The method utilized in partial identification of the bacterial
associates accompanying the HRS culture was made by growing on Blood Agar, Nutrient Agar, and Deconyleolate Agar, and picking different colonies to Nutri-
ent Agar slants. A gram stain was made to determine that the colony picked
was pure. Each colony isolated in this manner was inoculated into a series of
carbohydrate media and other bio-chemical tests were also employed to identify
the genus of the isolated strain and its bio-chemical properties. A table of
the characteristics appears in the Appendix.

PREPARATION OF PIPETTES

Long capillary Pasteur pipettes were used in inoculations and trans-
fers to eliminate stirring of the cultures. The pipettes were made from glass
tubing (5 mm. inside diameter) by drawing to a long capillary. They could
deliver from 6 to 10 drops of a suspension which was equivalent to 0.2 to
0.4 ml. Prior to the use of a pipette, a rubber bulb was attached to it and
the operation carried out with the employment of sterile techniques. All
pipettes were plugged and sterile. A different pipette was employed for each
tube in the transfer of cultures, but not in the inoculation of new media
since the inoculum came from the pooled sediments of stock cultures.

INOCULATIONS

In inoculating media tubes for the first time, the following pro-
cedures were followed: The sediments from several two to three day old cul-
tures of amebae were pooled by transferring to an empty sterile tube. The
pooled sediments were mixed thoroughly by rotating the tube in the palms of
the hands. A sterile pipette was inserted and the suspension further mixed
by drawing up into the pipette and releasing back the suspension into the tube
a few times. The thoroughly mixed suspension was then drawn into the pipette and a new tube of media inoculated with 6 drops of suspension (0.2 to 0.3 ml.). With each series of new media tubes inoculated, a parallel inoculation of stock medium was made to serve as a control. The stock medium grows the amoebae well and is employed in this laboratory for the maintenance of stock cultures.

**TRANSFER**

Transfer were made at 48 hour or 72 hour intervals. It was observed that the maximum growth appeared usually between 48 and 72 hours. A pipette was inserted into the culture and the sediment from the bottom of the tube drawn up and 6 to 8 drops of this sediment transferred to a fresh tube of the same medium. Amoebae were seen to grow profusely on the bottom and on the sides of the tube, so that it was safe to transfer the sediment to a fresh tube and be sure that amoebae had been transferred if they were present at all.

**INCUBATION**

All culture tubes were placed in a mason jar for the purpose of increasing the carbon dioxide pressure. This was accomplished by placing a tablet of sodium bicarbonate (.65 gm.) in the jar with the tubes and then adding 3 ml. of 25% hydrochloric acid, keeping the jar tilted in such a manner that the acid did not come in contact with the bicarbonate. After the jar was sealed it was tilted so that the acid came in contact with the bicarbonate. The carbon dioxide became about 20 per cent of the gas in the jar (theoretically 20.1 per cent, taking the atmospheric carbon dioxide as 2 per cent).

Hartman (1951) had reported enhanced growth of *E. histolytica* with increased
carbon dioxide. The mason jar was incubated in the 37° Centigrade incubator and reclined against a rack at a 45° angle. This inclination allows the amebae to climb the sides of the culture tube and thus be readily observed for degree of growth.

Very strict precautions were taken throughout the experiments to insure against contamination of the amebae cultures with other kinds of bacteria.

**RECORDING OF AMOEBA IN THE CULTURES**

A method of recording growth of amebae used by most workers is by the use of plus signs. A more accurate way would be to make hemacytometer counts on the basis of an aliquot mixture of the contents of the tube. Such a method would require a great amount of time and would make transfer of a sufficient number of amebae very uncertain. It is felt by the writer that the method used in this problem is sufficiently accurate to give meaning to different degrees of recorded growth. The readings as recorded in this paper consist of symbols which express and compare the degree of growth of amebae and are indicated in Table I.
### TABLE I

**AMOEBAE COUNT PER MM³ FOR THE SYMBOLS USED**

<table>
<thead>
<tr>
<th>Degree of Growth Expressed in Symbols</th>
<th>Approximate Number By Haemocytometer Count (Plus or Minus 10 per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>++++ = luxuriant growth</td>
<td>80/mm³</td>
</tr>
<tr>
<td>+++ = heavy to moderate growth</td>
<td>50/mm³</td>
</tr>
<tr>
<td>++ = scantily growth</td>
<td>12/mm³</td>
</tr>
<tr>
<td>+ = very poor growth</td>
<td>5/mm³</td>
</tr>
<tr>
<td>+ = doubtful growth</td>
<td>less than 5/mm³</td>
</tr>
<tr>
<td>_ = no visible growth</td>
<td></td>
</tr>
</tbody>
</table>

The method for reading each culture tube is made by taking out each tube from its slanted jar, wiping the bottom free from any moisture or liquid that might be present, and placing the tube under the low objective of the microscope (100x). The microscope is tilted at an angle so that the tube reclines upon the tilted stage. In this way, the amebae growing and clinging to the sides of the tube can be readily observed without any difficulty.
CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

The general plan of experiments was a process of working with points already known and with these points as a basis, planning an experiment which would seem to give promise of yielding the most useful results for determining the characteristics and methods of purifying the factor(s) responsible for enhancing the growth of E. histolytica. It was necessary to assay each material biologically after a given treatment. Side issues, such as the relation to bacterial growth, were not pursued further even though such points might be intriguing.

From the standpoint of chemical characteristics and the desirability of obtaining a quicker and less cumbersome method for the preparation of the dry yolk-salt extract, it seemed advisable to obtain information on the heat stability of Hartman's dry yolk-salt extract with the possibility in mind that if this dry extract proved to withstand high temperatures without undergoing destruction or deterioration of its amoeba growth properties, the possibility of further modifying the Hartman process of drying the liquid extract by boiling at atmospheric pressure might prove feasible. This method would eliminate the time-consuming and cumbersome method of drying the liquid extract under reduced pressure. At the same time, the information obtained would give light to the chemical characteristics of the yolk-salt extract from the standpoint
of heat stability. With this in mind, a series of experiments were devised to study the effects of long, continuous heating of the dry extract and intermittent heating of the liquid extract before drying.

EXPERIMENT II

Temperature Studies On The Dried Powder Of Hartman's Yolk-Salt Extract

The dried yolk-salt extract that was subjected to high temperatures had been stored in a glass bottle in this laboratory for more than a year. Ten samples of 0.8 gms. of this extract were placed in separate small specimen bottles. To each of five samples 0.3 gm. yeast extract (BBL or Difeo) was added and the contents mixed well. The bottles were then subjected to a constant temperature of 90°-104° Centigrade in a hot air oven used for sterilizing glassware. At different time intervals of 30, 60, 120, and 240 minutes, one sample with and one without yeast were taken out and allowed to cool to room temperature. The contents of each bottle was made up into media as already described. The experiment was repeated a second time, using a higher temperature of 135°-139° Centigrade. Media were prepared from the above substances and these were inoculated with amoebae and subcultured for no less than six transfers.

The results obtained from the experiment on temperature studies of the dried powder of Hartman's yolk-salt extract appear below in Table II. There was no destruction of AGF at these temperatures and times, and there was no effect on the yeast extract.
### TABLE II

**STABILITY OF HARTMAN'S YOLK PREPARATION IN THE DRY PHASE**

<table>
<thead>
<tr>
<th>Time of Heating in Minutes</th>
<th>Density of Population at End of Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20°-104° Centigrade</td>
</tr>
<tr>
<td></td>
<td>With Yeast Extract</td>
</tr>
<tr>
<td>0</td>
<td>++++</td>
</tr>
<tr>
<td>30</td>
<td>++++</td>
</tr>
<tr>
<td>60</td>
<td>++++</td>
</tr>
<tr>
<td>120</td>
<td>++++</td>
</tr>
<tr>
<td>240</td>
<td>++++</td>
</tr>
</tbody>
</table>

In experiments planned to answer questions brought forth in the above discussion, it was thought feasible and practical to test the stability of yolk-salt extract in the liquid phase and on a preparation of a single yolk. It was believed that experiments on a single yolk for the heat treatment and for subsequent treatment, providing the essential is not destroyed in the operation, would tend to strengthen or support the results of Experiment Number I.

It is recalled that in the experiment mentioned previously, the dried extract powder used was prepared by a modification of Balamith's process and consisted of the extracts of twelve yolks.
EXPERIMENT #2

Stability of Base Medium Prepared from A Single Yolk Heated Intermittently

A single fresh egg was broken carefully and the yolk separated from the albumin and placed in a beaker containing a solution of 3% NaCl. The yolk and the salt solution were agitated and stirred to break up the yolk and bring it into suspension with the solution. The suspension was then placed in a 5 liter Pyrex bottle and enough 3% NaCl solution added to bring the volume up to 3 liters. The bottle was then placed in the autoclave and heated for different intervals of time at 15 pounds pressure. After each of four different time intervals, 30, 45, 165, and 195 minutes, the bottle was taken out and allowed to cool. A sample of the extract infusion was withdrawn and made up into media as described below, and the rest was stored in the refrigerator for later use.

The sample of infusion was strained and filtered through a Buchner funnel before being made up into media. In this particular case, since we were dealing with a liquid, it was calculated that 16.0 ml. of the liquid, plus 84.0 ml. of distilled water, plus 0.3 gms. of yeast extract, mixed and adjusted to a pH of 7.2, would be equivalent to a 0.6% base medium described previously. This also would be the same as the 100% medium arbitrarily set by the writer and also described previously. The media were inoculated with the culture of E. histolytica and subcultured for not less than six transfers.

Table III shows the results obtained from the above experiment.
### TABLE III

**Stability of Yolk-Salt Extract From A Single Yolk In The Wet Phase**

<table>
<thead>
<tr>
<th>100% Medium Supporting Growth After Six Transfers</th>
<th>Time in Minutes of Heat Treatment Using Autoclave at 15 pounds.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density of Population At End of Six Transfers</td>
<td>30</td>
</tr>
<tr>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

In displaying the results obtained from experimental observations in the preceding tables as well as in any future tables, it is well to draw attention to the fact that in all tables only one set of observations appear; those made on the sixth and last transfer. The detailed readings are given only where it seems there is meaning in the details. In other cases, only the final results are being tabulated. All observations appear in the writer's laboratory notebook and as an example of how they are recorded the following table is cited as being representative of all tabulated data in the notebook.
<table>
<thead>
<tr>
<th>Date of Inc.</th>
<th>Date of Obser.</th>
<th>Date of Trans.</th>
<th>Concentration and Dilution of Base Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>400 %</td>
</tr>
<tr>
<td>3/14</td>
<td>3/16</td>
<td>3/16</td>
<td></td>
</tr>
<tr>
<td>3/18</td>
<td>3/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/20</td>
<td>3/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/23</td>
<td>3/23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/25</td>
<td>3/25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Remarks: All tubes appearing Negative were subsultured at end of experiment to stock media; no growth observed.
As an aid to the following section on procedures, it is recommended that reference be made to the flow sheet (Figure 1).

Since it was established that neither adverse conditions of aging or heating to high temperatures destroyed the essential found in egg yolk either in the dry state or the wet state, the Hartman process of drying the extract was modified further.

The remaining yolk-salt extract in the liquid phase which had been stored in the refrigerator from the preceding procedure was placed in a stainless steel vessel and boiled at atmospheric pressure, stirring occasionally to keep the forming salt from being scorched or burned. When most of the water had evaporated off, the moist salt was transferred to a large beaker and this beaker kept over a water bath until the yolk-salt extract was completely dry. The dry extract was pulverized in a mortar and stored at room temperature.

A code number (No. 15) was assigned to the dry extract as a means of identification. All media prepared hereafter have been assigned a code number for their identification and as an aid in following the flow sheet. Media were prepared on the basis of an 0.6% solution being arbitrarily assigned as a medium of 100% strength. Other dilutions were also prepared and the media were inoculated with amoebae and subcultured for at least six transfers. The observations were recorded and the effect of dilution on the media appears below in Table V.

Up to this point, it has been demonstrated that in the preparation of yolk-salt extract the essential does not undergo any destruction and still seems to retain its amoeba growth properties. Since it would be desirable to concentrate and isolate the essential fraction found in egg yolk, a series
### TABLE V

**AMOUNT OF GROWTH ON YOLK-SALT EXTRACT POWDER (MEDIUM NO. 15)**  
**AT THE END OF SIX TRANSFERS**

<table>
<thead>
<tr>
<th>Dry Yolk-Salt Extract From One Yolk</th>
<th>100%</th>
<th>20%</th>
<th>10%</th>
<th>5%</th>
<th>2%</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density of Population</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>At End of Six Transfers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++++</td>
</tr>
</tbody>
</table>

**Remarks:** All tubes appearing negative were subcultured at end of experiment to stock media: Negative.
Flow Sheet for the Fractionation of a Single Yolk

Yolk-Salt Extract
  Liquid Infusion
    Dried
  Dry Extract (No. 15)
    Dialysed
    NaCl (Discarded)
    Dialysed Solution (No. 16)
      Freeze-Dry
        Powder (No. 19)
        CHCl₃ Extract
          Dissolved in ROH
          ROH-CHCl₃ Extract (No. 17)
            Precipitated with acetone
            Precipitate (No. 17b)
            ROH-Acetone-Clear Solution
              Boiled off and dissolved in ROH
              Above Solution (No. 17c)
              Upon Standing
                Precipitate (No. 17d)
                Clear Solution (No. 17e)
of fractionations were devised employing some of the common fat solvents. However, in a series of fractionations carried out on the dry yolk-salt extract by the process known as "continuous extraction" in a Soxhlet column, the data obtained was not conclusive since some AGF was found in the solvents used and some remained in the salt. On the basis of the data obtained from the Soxhlet extractions, it was seen necessary to rid the dry extract of the accompanying salt in the hopes that the salt-free yolk extract would yield more consistent results when subjected to fractionation.

EXPERIMENT 49

Dialysis and Fractionation of the Yolk-Salt Extract (No. 15)

Fifty grams of the yolk-salt extract (No. 15) were placed in a thin visking sac and the sac, with its contents, placed in a five liter glass cylinder with distilled water. The cylinder was placed and stored in the refrigerator during the dialysing process to keep bacteria from growing in the dialysate. The water was changed twice daily until dialysis was complete. At intervals, the silver nitrate test was carried out on the solution outside of the visking sac for presence of the chloride ion. The test was made by acidifying 3 ml. of the solution to be tested with three normal nitric acid. To this mixture two drops of a solution of 10 per cent silver nitrate was then added. Presence of the chloride ion was demonstrated by the formation of a white precipitate. After it was ascertained that the chloride ion was absent from the water outside of the sac, the contents of the sac were emptied into a bottle and the silver nitrate test employed on a few milliliters of this to
verify that all the sodium chloride had been removed from the yolk extract. The dialysed extract (No. 16) was bottled and stored in the refrigerator when not in use.

In preparing media from the dialysed yolk extract, an appropriate amount of this salt-free yolk extract (No. 16) was pipetted out and on the basis of the following calculation, media prepared:

315 ml. (No. 16) recovered from 50 gms. (No. 15)

Therefore:

\[
\frac{50 \text{ gms.}}{315 \text{ ml.}} = \frac{0.8 \text{ gms.}}{x} \quad x = 5.1 \text{ ml.}
\]

"5.1 ml. is equivalent to 0.8% ≈ 100% medium"

To prepare media (No. 16), 5.1 ml. salt-free extract, 94.9 ml. distilled water, 0.3 gms. yeast extract, and 0.8 gms. sodium chloride were mixed and the pH adjusted to 7.2. This media would be equivalent to one prepared with 0.8 gm. dry salt extract if none of the AGF dialysed thru the membrane. The concentration of the media was also doubled, as well as a dilution of 50% prepared. It seemed impractical to prepare lesser dilutions than the 50%, since previous assay showed that any concentration less than 50% would not support growth in the case of the single yolk. The media were inoculated with the amoeba culture and subcultured for six transfers. Table VI shows the results.

The essential has thus far been isolated from accompanying salt by dialysing the salt from the extracted matter. This brings forward the assumption that if the essential is soluble in one of the common fat solvents, the
sodium chloride having now been eliminated, the dialysed yolk extract could be treated with chloroform to fractionate the complex. The resulting fraction could then be assayed for activity.

**TABLE VI**

Amount of Growth on Medium No. 16
(The Dialysed Yolk Solution of a Single Yolk)

<table>
<thead>
<tr>
<th>Density of Population at End of Six Transfers</th>
<th>200%</th>
<th>100%</th>
<th>50%</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>++++</td>
<td><strong>%/</strong></td>
<td><strong>%/</strong></td>
<td><strong>%/</strong></td>
<td>++++</td>
</tr>
</tbody>
</table>

Remarks: All Tubes Supported Growth of the Amoebae.

**Extraction of Dialysed Yolk-Extract with Chloroform**

A volume of 165 ml. of dialysed yolk-extract (No. 16) was extracted with five volumes of 25 ml. of chloroform and the chloroform fractions pooled. The total volume of the chloroform with the soluble matter extracted was measured to be 255 ml. This was given the number (No. 17). The dialysed yolk solution remaining after the treatment with chloroform measured 165 ml. The number given this solution was (No. 18).

Appropriate amounts of the chloroform extract (No. 17) and of the solution remaining after extraction with chloroform (No. 18) were measured out and prepared into media on the basis of the following calculations:
It was assumed that all extractable matter soluble in chloroform was
extracted by the solvent from (No. 16). In preparing media, the chloroform
was evaporated off by placing the chloroform solution under vacuum and applying
very gentle heat. The yellow waxy substance remaining in the flask was assumed
to have been extracted from 165 ml. of the original dialysed yolk extract
(No. 16), but this 165 ml. had previously come from a total volume of 315 ml.
when 50 gms. of (No. 15) were treated.

Therefore:

\[
\frac{50 \text{ gms.}}{315 \text{ ml.}} = \frac{0.8 \text{ gms.}}{X} \]

and \[
\frac{165 \text{ ml.}}{5.1 \text{ ml.}} = 32.4
\]

It is evident that from this 100 ml. of media can be prepared 32.4 times.

Further, if the waxy substance can be dissolved in 32.4 ml. of absolute alcohol
it stands to reason that 1.0 ml. of this alcohol-complex will prepare an 0.8% solution
equivalent to media 100% strength. But, since the waxy substance was
dissolved in only 16.2 ml. of alcohol, 1.0 ml. of the alcohol solution would
be equivalent to 200% strength. In preparing media (No. 17, 1.0 ml. of this
solution, 99.0 ml. of distilled water, 0.3 gms. yeast extract, and 0.8 gms.
sodium chloride were mixed and the pH adjusted to 7.2. Dilutions were made
with appropriate amounts of a solution containing 0.3 gms. yeast extract and
0.8 gms. of sodium chloride per 100 ml. of solution.

The preparation of media from the solution remaining after being
extracted with chloroform (No. 16) was prepared in the same way that media
No. 16 was prepared.
Insculations with amoebae were done on all the tubes prepared and subcultured for six transfers. Table VII shows the observed results.

**TABLE VII**

Amount of Growth on Media Prepared after Treatment of the Dialysed Yolk Extract Solution with Chloroform

<table>
<thead>
<tr>
<th>Density of Population At End Of Six Transfers</th>
<th>200%</th>
<th>100%</th>
<th>50%</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform Extract, Alcohol Evaporated (No. 17)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Chloroform Extract, Alcohol Retained (No. 17)</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Solution Remaining After CHCl₃ Treatment (No. 18)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

Remarks: All tubes appearing Negative were subcultured at end of experiment to stock media. All tubes Negative, except the Doubtful tube No. 16, which gave a density of amoebic growth of ++ in the stock medium.

In an alternative method to prepare media (No. 17) the addition of the one milliliter of extract dissolved in the alcohol was subjected to heat over a water bath before addition of the rest of the materials. This treatment was undertaken with the purpose of evaporating off the alcohol. To recognize any difference in growth, if any at all, between this alternative method and the common method employed, 100 ml. of distilled water were added instead of the 99.0 ml. Information was desirable as to which of the two techniques would prove better. The results are also shown in Table VII after inoculation and subculturing.
It was demonstrated that the remaining solution after being treated with chloroform no longer retained its growth promoting properties and that the actual essential could be separated out of the water fraction. The question arose on whether the dialysed solution (No. 16) could be treated in such a way as to obtain the essential in a dry form. If the process of freeze-drying could be applied to (No. 16) and the dry form of the waxy substance could be obtained without showing any decrease of activity, the step taken would be a profitable one.

Freeze-Drying of the Dialysed Yolk-Extract (No. 16)

A measured quantity of 75 ml. of dialysed yolk-extract (No. 16) was delivered into a round-bottom flask and the liquid frozen as a thin layer on the sides of the flask by twirling the flask rapidly in an ethyl alcohol dry ice bath. The flask was then attached to the freeze-dry system in the biochemistry laboratory and the pressure inside reduced to about 0.001 mm. The contents were dried down to a fluffy powder. This powder was assigned a code number (No. 19) and the total substance recovered was 40.1 milligrams. An appropriate amount was weighed out and media prepared from this amount on the basis of the following calculations:

From the same mathematical treatment used above, it follows that 40.1 mg. was obtained from 75 ml. and since the latter figure originally came from 50 gms. of salt which had given 315 ml. of the same solution:

\[
\frac{75 \text{ ml.}}{40.1 \text{ mg.}} = \frac{5.1 \text{ ml.}}{X} \quad \Rightarrow \quad X = 2.7 \text{ mg.} \approx 0.8\% \approx 100\%
\]
To prepare media No. 19

2.7 mg. + 0.3 gms. yeast extract + 0.8 gms. NaCl +
100 ml. distilled H<sub>2</sub>O = 100% solution

Media in the following concentrations were prepared: 400%, 200%,
100%, and 50%, and bio-assay tests run on them. The observations appear in
Table VIII.

TABLE VIII

Amount of growth on medium No. 19
(The freeze-dry process of dialysed yolk extract)

<table>
<thead>
<tr>
<th>Density of Population at End of Six Transfers</th>
<th>Growth after Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400%</td>
</tr>
<tr>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

Remarks: Negative tubes transferred to stock media at end of experiment: Negative.

It would appear from the results of the test that, while most of the essential was either destroyed or deteriorated in the presence of a high vacuum, there was slight activity only when media of high concentrations were used.

Having established thus far that the essential found in yolk extract was still active after the preceding treatments, it was thought desirable to see if the chloroform extract dissolved in alcohol (No. 17) could be treated with acetone to precipitate any fraction insoluble in this solvent.
Treatment of the CHCl₃ Extract in Alcohol with Acetone

Eleven milliliters of CHCl₃ extract in alcohol (No. 17) were put into a separatory funnel. Six volumes of acetone were added to precipitate the acetone insoluble fraction. After shaking the funnel at intervals, it was allowed to sit overnight. The next day the solution was filtered and the filtrate and the sediment collected in separate containers. The filtrate, composed of alcohol-acetone solution, was evaporated off under vacuum and gentle heat to get rid of the acetone and alcohol. The remaining waxy substance was re-dissolved in 11.0 ml. (same volume originally started with) of absolute alcohol. The slight precipitate was also re-dissolved in 44.0 ml. (four times original volume) of an 0.6% NaCl solution. The code numbers assigned were the following: The precipitate after acetone treatment (No. 17a), and the remaining solution without acetone precipitate (No. 17b).

Media (No. 17a) was prepared as follows: Since the volume in which the precipitate was dissolved was four times its original volume, it holds that:

1.0 ml. was equivalent to 0.465 = 50% solution

The same procedure was employed in the preparation of media as previously mentioned.

In preparation of media (No. 17b) the waxy substance remaining after evaporating the alcohol-acetone solution was redissolved into its original volume; therefore, it was safe to assume that:

1.0 ml. was still equivalent to 1.465 = 200% solution.

The same procedure was employed in the preparation of media as previously mentioned. Media were inoculated with amoebae and subcultured for six transfers.
see Table IX for results.

**TABLE IX**

**AMOUNT OF GROWTH ON MEDIA PREPARED AFTER TREATMENT OF THE ALCOHOL-DISLOVED CHLOROFORM EXTRACT WITH ACETONE**

<table>
<thead>
<tr>
<th>Growth For Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Density of Population</strong></td>
</tr>
<tr>
<td>At End Of Six Transfers</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Remarks: All tubes appearing Negative at end of experiment were subcultured to stock media: Negative

After a few days it was noticed that a very slight sediment began to form in the bottle where the acetone treated clear solution (No. 17b) was being stored. This raised the question of whether both fractions were necessary for growth of the amebae, or only one of the fractions. With this in mind, it was thought worth while to separate the fractions and submit them to a bio-assay test.

Separation of the Clear Supernatant Liquid from the Formed Precipitate of Solution (No. 17b)

The bottle containing the solution (No. 17b) was shaken well and 6.0 ml. of the suspension transferred to a heavy-wall centrifuge tube and centrifuged for 10 minutes at 6000 R.P.M. The clear supernatant was drawn off very carefully with a long capillary pipette and after being put into a
suitable container, numbered (No. 17d).

The precipitate in the bottom of the centrifuge tube was dissolved in 6.0 ml. of an 0.8% sodium chloride solution by stirring and application of gentle heat. The number allocated this fraction was (No. 17c).

In the preparation of media (No. 17c), it was assumed that 1.0 ml. of this solution would still be the equivalent of a 1.6% base medium or 200% culture medium since the precipitate had come out of an original volume of 11 ml. and each 1.0 ml. was equivalent to 1.6% base medium or 200% culture medium.

In preparation of media (No. 17d) it can be observed that 1.0 ml. of this solution is also equivalent to a 200% culture medium. Employing the same procedure mentioned elsewhere in this paper, appropriate media were prepared and inoculated with amoebae, subcultured for six consecutive transfers. Table X exhibits the results obtained.

**TABLE X**

**Amount of Growth on Media Prepared after Centrifugation of Medium #17d**

<table>
<thead>
<tr>
<th>Density of Population At End Of Six Transfers</th>
<th>Precipitate (No. 17c)</th>
<th>200%</th>
<th>100%</th>
<th>50%</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear Solution (No. 17d)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

Remarks: All tubes Negative when subcultured to stock media.

It would appear from the above observations that both fractions are needed.
Having obtained certain relative information in reference to the
problem under study from the consistency of results on the fractionation of a
single yolk, and having also demonstrated the apparent stability of the yolk
essential accompanying each subsequent fraction, it was in order to supplement
the established facts with further experimental data. Therefore, it seemed
advisable to duplicate the above prototype (original procedures) modifying the
procedures slightly. It was reasoned that if the original quantity of yolk
material is increased, a finer degree of fractionations could be accomplished,
in reference to the dilution of the quantity of yolk extracted in a given batch.
An increase of original material could also give a wider range of media dil-
utions appropriate for bio-assay.

EXPERIMENT #

Fractionation of Yolk-Salt Extract of 12 Yolks

It is recommended that the Flow Sheet (Figure 2) be referred to in
following the experimental procedures that are to be described.

Twelve yolks were broken up and stirred in 6 liters of a 3% sodium
chloride solution and base medium prepared by the Hartman process. One-half of
the yolk infusion recovered was dried down to the dry yolk-salt extract. The
amount of dry extract obtained was 142.5 gms. It was numbered (No. 20) and
bio-assay tests were made on the media prepared from this powder as has been
described elsewhere. Table XI shows the results obtained.

The remaining one-half of yolk infusion was extracted with di-ethyl
ether until the infusion lost its yellow-brown color, imparting to the ether
a greenish tinge of color. It was in question to test the effect of ether on
FLOW SHEET FOR THE FRACTIONATION OF YOLK-SALT EXTRACT OF TWELVE YOLKS

Yolk-Salt-Extract Liquid Infusion

Dried

Extracted with ether

Remaining Solution (No. 22)

Ether Extraction (No. 21)
dried
dissolved in alcohol

Dry Extract (No. 22)

Alcohol-dissolved Ether Extract (No. 21)
dialysed

Ether Extract (No. 22)

standing and filtering

Dialysed Yolk Solution (No. 23)

NaCl Dialysate (Discarded)

extracted with CHCl₃

Clear Solution (No. 21a)

Sediment (No. 21p)

precipitated with acetone

Remaining Solution (No. 24)

CHCl₃ Extract (No. 25)

Precipitate (No. 21a)

Clear Solution (No. 21b)
evaporated to dryness and extracted with ether

Ether Solution

CHCl₃ Extract after ether extraction
dried and dissolved in alcohol
dried and dissolved in alcohol

Second Extraction of ether (No. 25a)

CHCl₃ Extraction in alcohol (No. 24r)
the yolk-salt extract while still in the liquid phase, before subjecting the
dry extract to CHCl₃ extraction. The reasoning for this treatment prior to
CHCl₃ extraction was based on the premise that if there were substances soluble
in both ether and CHCl₃ and there existed a slight difference in their solu-
ibilites in relation to the solvents, then it would be possible to separate
out these different fractions by taking advantage of their solubilities.
Hence, "what the ether will not extract, the CHCl₃ will".

The ether with its soluble extracted yolk substances was numbered
(No. 21) while the solution remaining after being extracted with the ether was
numbered (No. 22). The ether was then evaporated off from (No. 21) by reduc-
ing the pressure and the application of very gentle heat. A yellowish oily
fraction remained in the flask after all the ether was evaporated off. After
the water was similarly evaporated, the ether extract remaining appeared as
a yellow, liquid, oil-like substance.

To make an appropriate medium from this substance, the procedures
for calculating and preparing suitable media were essentially the same as
used in the single yolk counterpart. Hence, if we assume that everything was
extracted by the ether from the yolk infusion (which would have given 142.5
gms. of the dried extract) the basis for calculation would be:

\[
\frac{142.5 \text{ gms.}}{0.8 \text{ gms.}} = 176
\]

\( \text{The amount of extract recovered would prepare 100 ml. of a 100\% medium 176 times.} \)

\[
\frac{176}{2} = 89
\]

\( \text{100 ml. of a 200\% medium 89 times.} \)

The extract was dissolved into 89.0 ml. of absolute alcohol and stored as
such; (No. 21). 1.0 ml. of the alcohol-dissolved ether extract would be
equivalent to a 200% medium. Therefore, to prepare medium:

1.0 ml. of ROE-dissolved ether extract + 99.0 ml. distilled H₂O + 0.9 gms. yeast extract + 0.8 gms. NaCl = 200% medium.

Media were prepared from the above ether extract dissolved in alcohol. Since the amount of yolk essential was assumed to be much greater than the quantity found in the single yolk extract, it was appropriate to prepare and test media of high dilutions. At the same time, it was desirable to test the effect of too much essential in a medium; therefore, media of 200% and 400% concentrations were prepared. All media were inoculated with amoebae and subcultured for six transfers. The observations appear in Table XI.

The yolk-salt infusion remaining after ether treatment (No. 22) was dried to a powder by the process already mentioned elsewhere, and stored at room temperature for further use. Media was prepared from this dried extract by measuring out 0.8 gms. (No. 22), and 0.9 gms. yeast extract, mixing them with 100 ml. distilled water and adjusting the pH to 7.2. The strength of the media thus prepared equals 100 per cent. The media were inoculated with amoebae and subcultured for six transfers. Table XI shows the results observed.

The observations noted in Table XI imply that augmenting the quantity of yolk material in the initial preparation of the Hartman extract give indications that an increased amount of AHF can be extracted per given batch and, consequently, preparation of a wider range of media dilutions could prove fruitful. The observations further show the degree of amoebic growth attain-
ed in the highest dilution—moderate, at 2% concentration. These findings throw considerable light on the fact that the AGF, when properly concentrated, is required only in a very minute amount. Treatment of the liquid infusion with ether demonstrated that the solvent as used was able to extract most, but not all of the AGF.

**TABLE XI**

**AMOUNT OF GROWTH ON MEDIA NO.'s 20, 21, AND 22 AT THE END OF SIX TRANSFERS**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Salt-Yolk Extr. of 12 yolks (No. 20)</th>
<th>Ether Extr. of 12 Yolks (No. 21)</th>
<th>Solution Remaining After Ether Treatment (No. 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400%</td>
<td>media not prep.</td>
<td>-</td>
<td>media not prep.</td>
</tr>
<tr>
<td>200%</td>
<td>media not prep.</td>
<td>-</td>
<td>media not prep.</td>
</tr>
<tr>
<td>100%</td>
<td>+++</td>
<td>++/+++</td>
<td>+++</td>
</tr>
<tr>
<td>20%</td>
<td>+++/++++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>10%</td>
<td>+++</td>
<td>+++/+++</td>
<td>-</td>
</tr>
<tr>
<td>5%</td>
<td>+++/+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>2%</td>
<td>+++</td>
<td>+/++</td>
<td>-</td>
</tr>
</tbody>
</table>

**Remarks:**

1. Media No. 22 was also prepared at 30% strength: +++/+++
2. All controls at first subculture were: ++++
3. All negative tubes at end of experiment transferred to stock media: Negative.

For the sake of clarity, the subsequent treatment of the alcohol-
dissolved ether-extract (No. 21) shall be taken up first. It will be followed
by discussion of the subsequent procedures made on the dried extract (No. 22).

It is interesting to note that upon standing for a few days, a very
light, but visible, precipitate began to form in the ether-extract dissolved
in alcohol (No. 21). The question immediately raised was whether a separation
of these components would show the ACF to disappear or deteriorate when media
prepared from them were bio-assayed.

Separation of the Two Components of the Ether-Extract Solution
Dissolved in Alcohol (No. 21)

Fifty milliliters of (No. 21) were filtered through a Buchner funnel
under reduced pressure and the components recovered in different receptacles.
The clear yellow-like solution was numbered (No. 21a), while the brownish
precipitate was numbered (No. 21b). It is recalled that 1.0 ml. of (No. 21)
was originally equivalent to a 200% solution so that no significant error
would be introduced if we assume that 1.0 ml. of solution (No. 21a) is also
equivalent to a 200% solution. The only difference between (No. 21a) and
(No. 21) is that the former lacks the precipitate filtered out in the opera-
tion just described. Therefore, to prepare media from (No. 21a) 1.0 ml. of
the solution, 99.0 ml. of distilled water, 0.3 gms. yeast extract, and 0.8 gms.
sodium chloride, are mixed and the pH adjusted to 7.2. This gives a solution
of 200% concentration. Dilutions of 100%, 20%, 10%, 5%, and 2% were prepared
as well as a concentration of 400%. It should be mentioned here that, here-
after, the above concentrations and dilutions are to be prepared for all bio-
assay of media. This is done with the purpose in mind to test for growth at
high dilutions, as well as for the effects of too much essential in a medium.

The precipitate (No. 21p) was dried and weighed; 212 mg. were recovered. In preparing media from this precipitate, the following calculations were used in arriving at the number of milligrams which would have to be added to a solution to make it a 100 per cent solution.

212 mg. recovered from 50 ml. solution, and the concentration of the solution was: 1.0 ml. \( \equiv \) 200%. On this basis, 1.0 ml. of solution would be equivalent to 1.6 gms. of its dry form so that 50 ml. \( \times \) 1.6 gms. \( \equiv \) 80.0 gms.

The original solution would have come from 80.0 gms. of its dry form.

\[
\frac{80 \text{ gms.}}{212 \text{ mg.}} = 0.38 \text{ gms.} \quad x = 2.1 \text{ gms.} \equiv 100\%
\]

On the basis of the above calculations, the following concentrations were prepared: 400%, 200%, 100%, 20%, 10%, 5%, and 2%. Media (No. 21a) and (No. 21p) were inoculated with amoebae and subcultured for six transfers. The end results appear in Table XIII.

The data presented in Table XII establishes the AGF in the clear solution of (No. 21a) and no apparent major deterioration of the essential taking place. Further fractionation of this solution was considered with the intention of purifying and concentrating the essential. Acetone was chosen as a possible agent, taking into consideration that acetone is at times used to precipitate certain phospholipids from solution, and since the ether extract seemed to contain an oily fraction, it was thought that perhaps some fraction of this oily substance would be insoluble in acetone, thus precipitating out and being removed by filtration.
### TABLE XII

**AMOUNT OF GROWTH ON MEDIA PREPARED AFTER FILTRATION OF NO. 21**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Density of Population at End of Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No. 21a) clear soln.</td>
</tr>
<tr>
<td>40%</td>
<td>-</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
</tr>
<tr>
<td>10%</td>
<td>++++</td>
</tr>
<tr>
<td>5%</td>
<td>++++/+++</td>
</tr>
<tr>
<td>2%</td>
<td>-</td>
</tr>
</tbody>
</table>

**Remarks:** All controls at first subculture were: ++++.  
All negative cultures at end of experiment transferred to stock media: Negative.

---

**Possible Precipitation of Acetone-Insoluble Fraction From Clear Ether-Extract Solution (No. 21a)**

Ten ml. of the clear filtered ether-extract solution (No. 21a) were placed in a separatory funnel and six volumes of acetone added to the liquid. After shaking, the funnel was allowed to sit overnight. The next day, a white
precipitate was filtered off through a Buchner funnel and the precipitate re-
dissolved in 10.0 ml. absolute alcohol (the volume from which it was origin-
ally filtered). The number allocated to this precipitate was (No. 21a). Since
1.0 ml. of the original liquid (where the precipitate was in solution before
addition of acetone) was equivalent to a 200% solution, it was feasible to
assume that 1.0 ml. of (No. 21a) would also be equivalent to a 200% solution
without introducing too much error. Media were prepared from this solution
and bio-assay made on all concentrations.

The clear yellow solution from which the acetone insoluble precip-
itate was filtered off was placed over a water-bath and, with very mild heat,
the mixture of alcohol and acetone was evaporated off. To the remaining
yellow oil enough absolute alcohol was added to make a volume of 20.0 ml.
This volume, it is recalled, is twice the original volume that was placed in
the separatory funnel at the beginning of the experiment. In the original
concentrations, 1.0 ml. of solution (No. 21a) was equivalent to a 200% solution
so that having now doubled the original volume, the new concentration of
(No. 21b) would be: 1.0 ml. equivalent to a 100% solution. Appropriate con-
centrations were prepared from this liquid to make the customary dilutions of
media. Bio-assay tests were run on all media. The observations on the assay
of media (No. 21a) and (No. 21b) appear in Table XIII.

Having established the AGF in the supernatant liquid after treatment
of (No. 21a) with acetone, the essential seemed to remain in the acetone-
soluble fraction while no significant change had been suffered by it. Some
purification might have been accomplished by the removal of the acetone-
insoluble precipitate which was not able to support growth.
<table>
<thead>
<tr>
<th>Concentration</th>
<th>Density of Population at End of Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No. 21a) precipitate</td>
</tr>
<tr>
<td>400%</td>
<td>++</td>
</tr>
<tr>
<td>200%</td>
<td>+</td>
</tr>
<tr>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
</tr>
<tr>
<td>10%</td>
<td>-</td>
</tr>
<tr>
<td>5%</td>
<td>-</td>
</tr>
<tr>
<td>2%</td>
<td>-</td>
</tr>
</tbody>
</table>

Remarks: All controls at first subculture were: ++++  
All negative cultures at end of experiment were transferred to stock media and found: Negative.

We now turn our attention to extract (No. 22), the 12-yolk infusion remaining after the treatment with ether. A glance at the flow sheet (Fig. 2) will bring forward the assumption that ether treatment of the original yolk-salt extract (No. 20) in the liquid phase extracted most, but not all, of the
essential from this preparation, the remaining essential being left as a residual in the dry (No. 22) extract and having growth promoting properties only when prepared at high concentrations.

It follows that if the essential is still retained after any subsequent treatment of (No. 22) the growth promoting properties of this would appear only when prepared at concentrations of 100% or more. In following the same procedures employed in the experiments on the single yolk, dialysis of (No. 22) was contemplated to test the extent of the AGF remaining in this substance.

Dialysis of The Ether-Treated Remains of the 12-Yolk Extract

Fifty grams of (No. 22) were placed in a vashing bag and the salt dialysed out by the same procedure as was used on the single yolk. The amount of dialysed yolk solution recovered was 200 ml. A portion of this liquid was prepared into media and assayed for growth essential. The following calculations establish the amount of liquid that would be equivalent to a 100% medium. The dialysed yolk solution was numbered (No. 23).

\[
\begin{align*}
50 \text{ gm. extract} &= 200 \text{ ml. solution} \\
1 \text{ gm. extract} &= 4.0 \text{ ml. solution} \\
0.2 \text{ gm. extract} &= 3.2 \text{ ml. solution}
\end{align*}
\]

Therefore, to prepare media, 3.2 ml. of (No. 23), 96.8 ml. distilled water, 0.3 gm. yeast extract, and 0.5 gm. sodium chloride are mixed and the pH adjusted to 7.2. This is equivalent to a medium of 100% strength. Concentrations of 400%, 200%, 100%, 50%, 10%, 5%, and 2% were also prepared. The media prepared were assayed and the observations appear in Table XIV. As was
expected, only media prepared at high concentrations proved reactive while dilutions below 100% gave no visible anaerobic growth.

TABLE XIV

AMOUNT OF GROWTH ON MEDIUM NO. 23 (DIALYSED YOLK EXTRACT)

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>Media Supporting Growth After Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40%  20%  10%  20%  10%  5%  2%</td>
</tr>
<tr>
<td>Density of Population at End of Six Transfers</td>
<td>++++  ++++  ++++  -   -   -   -</td>
</tr>
</tbody>
</table>

Remarks: Control at First Subculture: ++++
All negative cultures at end of experiment were transferred to stock media and found: Negative.

The next step undertaken was the attempt to extract the remaining essential out of the dialysed extract. This step in essence was to determine whether the CHCl₃ was capable of extracting out the fraction that the ether was unable to take out initially.

Chloroform Extraction of the Dialysed Yolk Solution

The extraction of 154 ml. of the dialysed yolk solution (No. 23) was made with five separate volumes (50 ml.) of CHCl₃, and the CHCl₃ portions pooled. The dialysed solution lost its yellow color. The CHCl₃ with its extractable soluble substance was numbered (No. 25), while the dialysed solution remaining after the extraction was numbered (No. 24). In preparing media from (No. 24), 2.2 ml. of this solution was taken to be equivalent to a 100%
solution. It was reasoned that if nothing was extracted by the CHCl₃ treatment, the original concentration of the dialysed yolk (No. 29) would still hold, i.e., 3.2 ml. of (No. 29) was equivalent to a 100% media. Media were prepared on the basis of this concentration and all media were assayed.

It would be of interest to see if the substance that the chloroform extracted out of the dialysed yolk could be subsequently treated, after evaporation of the chloroform, with ether with the referred insinuation that the ether at this point could now take out the remaining AGF that it did not take out originally. On the other hand, it may very well be that no apparent essential was taken out by the chloroform even though a waxy fraction came off in the solvent.

Treatment of the Chloroform Extract with Ether

The pooled CHCl₃ solution with its soluble fraction (No. 25) from the preceding treatment, was placed in a flask with a side arm. The chloroform was evaporated off by placing the flask over a water-bath and under reduced pressure. A waxy substance remained in contrast to the oily fraction remaining in the ether treatment. Di-ethyl ether was added to this waxy fraction to see if the ether would penetrate it and take out any other soluble substance. Gentle shaking of the flask while letting hot water run over it only imparted a slight coloration to the ether solvent. The ether solution was filtered through filter paper and the solution collected in a bottle. This was numbered (No. 25e) while the remaining waxy material was numbered (No. 25r).

In preparing media from (No. 25e) the ether extract was concentrated to six times its supposed concentration and media prepared on this basis.
This arbitrary concentration was fixed at this figure as a matter of general interest since the amount of ether extractable substances extracted out by the ether was insignificant and could not possibly support amebie growth. Media were prepared on the basis that 1.0 ml. of (No. 25e) dissolved in alcohol would be equivalent to 6(100%) concentration and each subsequent dilution or concentration would be presumably six times as great as generally would have been. Media of 6(400%, 200%, 100%, 20%, 10%, 5%, and 2%) concentration were inoculated with amebies and subcultured for six transfers.

In preparing media from the wax-like material (No. 25r) remaining after the ether treatment, it is recalled that the substance prior to the addition of ether had come originally from 154 ml. of dialyzed yolk solution (No. 23) and that 3.2 ml. of this was equivalent to a 100% solution. But since addition of ether extracted only a very minute amount of the oily substance from the GCH3 saline sediment (No. 25), it was assumed that this second ether extraction did not take out any appreciable amount of material. Therefore, if we postulate that the material (No. 25r) came from the apparent 154 ml. volume of the original dialyzed solution, not too much error would be introduced.

\[
\frac{154 \text{ ml.}}{3.2 \text{ ml.}} = 48.1 \text{ times}
\]

Therefore, addition of 48.1 ml. of absolute alcohol to the waxy substance (No. 25r) will make 1.0 ml. of this equivalent to a 100% solution. However, all of the substance did not dissolve in this volume so that another volume of alcohol had to be added. Now 2.0 ml. of the mixture was equivalent to a 100% solution. Media were prepared in all concentrations and assayed. The experimental results of the assaying of media
(No. 24), (No. 25a), and (No. 25b) appear in Table XIV.

### Table XIV

**Amount of Growth on Media Prepared from the Extraction of Dialysed Yolk with CHCl₃ Followed by Ether Treatment of the CHCl₃ Extract**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Density of Population at End of Six Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dialysed solution after CHCl₃ Extract</td>
</tr>
<tr>
<td>400%</td>
<td>++++</td>
</tr>
<tr>
<td>200%</td>
<td>++++</td>
</tr>
<tr>
<td>100%</td>
<td>++++</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
</tr>
<tr>
<td>10%</td>
<td>-</td>
</tr>
<tr>
<td>5%</td>
<td>-</td>
</tr>
<tr>
<td>2%</td>
<td>-</td>
</tr>
</tbody>
</table>

**Remarks:** All controls at first subculture were: ++++

All negative cultures were transferred to stock media: Negative.

* All concentrations in this column are presumably six times as great as mentioned.
From all appearances, it seems that even though CHCl₃ extracted a moderate amount of wax-like substance from the dialyzed yolk solution, apparently a very minute amount of AGF was found in this extract, (No. 25r), which in turn was extracted out by the ether. This ether extract (No. 25e), when prepared into a medium of very high concentration (six times as great as originally prepared) still proved to be a very poor supporter of amoebic growth. The dialyzed yolk solution after CHCl₃ treatment retained the growth essential as can be seen in Table XV.
CHAPTER VI

DISCUSSION AND CONCLUSION

It was mentioned in the beginning of Chapter IV that the general plan was to start this study with points already known and, with these points as a basis, plan experiments which would seem to give promise of yielding the most useful results for determining the characteristics and methods most suitable for purifying the factor(s) responsible for enhancing or supporting the growth of E. histolytica.

The points already known at the beginning of this study and which formed the basis of this plan were the following: (1) The constituents which had gone into the preparation of media for the support and growth of E. histolytica had come from varied and widely distributed sources in the animal kingdom. Beek and Drbohlav (1925), Dobell and Laidlaw (1936), Cleveland and Collier (1930), Craig (1939), Frye and Holeney (1939), and Balamuth and Sandza (1944). (2) The factor which enhanced growth of E. histolytica most profusely was apparently found in egg yolk. (3) The factor(s) found in egg yolk could be extracted out of the yolk by the use of salt and heat, Balamuth (1944). (4) The yolk-salt extract of Balamuth could be dried and stored at room temperature without undergoing any deterioration, (Process of Hartman, unpublished). (5) The Hartman preparation of base medium seemed to indicate that if this yolk essential could be concentrated and purified, high dilution of the base
medium giving amoeba growth properties might be attained.

From the standpoint of chemical stability in the presence of high temperatures, both in the dry form and in the wet form, the yolk-salt seemed to withstand high temperatures without any apparent deterioration of the essential. When the heat-treated extract was prepared into media and assayed, the amoebae seemed to do well and were very active, being motile and exhibiting great variations in their size and shape as they constantly changed form. The observations of this experiment showed that the Hartman process of preparing yolk extract could be modified by drying the extract over a direct flame at atmospheric pressure or in an oven. The results also showed that whatever the essential was, it was heat stable at the temperatures described. In preparing the yolk-salt extract from a single yolk, heating at 15 pounds pressure for as much as 165 minutes gave moderate growth of the amoebae when media were prepared. An extra 30 minutes heating, however, gave a heavier crop of amoebae (Table III) than heating for 165 minutes. In the wet phase, the essential seemed to be favored by a prolonged heating time, at 15 pounds pressure.

From these two experiments, it was established that the ASF present in egg yolk is heat stable under the conditions mentioned and in preparing the extract, a longer heating time favors more extraction of the essential. At the same time, it can be concluded that after drying down the yolk-salt infusion to a salt, or while in the process of drying, temperatures as high as 135°-139° Centigrade still fail to destroy the yolk fraction responsible for amoebic growth. And lastly, the dry extract form proved stable after storage for more than a year at room temperatures.
Having established the heat stability of the yolk essential, the fractionation of the yolk extract was attempted by the process known as "continuous extraction" in a Soxhlet column. A weighed quantity of yolk-salt extract was consecutively treated with fat solvents, such as ether, chloroform, and alcohol. Each subsequent procedure was subjected to from five to six hours of continuous extraction with a solvent. Each fraction separated was prepared into media as described previously and the media assayed for growth. However, the results obtained from the experimental procedures did not appear to be conclusive in regard to complete separation. Repeated extraction procedures continued to give inconsistent results so that the question arose as to whether the NaCl present limited the extraction of yolk essential by the solvent. It was theorized that in the formation of the salt crystal during the drying procedure, a part of the AGF became enveloped in the crystal as the salt formed from solution. Extraction of the dry yolk-salt preparation with adequate solvents failed to take out all of the AGF, leaving behind enveloped in or about the salt crystal enough of the essential so that when the separate fractions were put in solution in the preparation of media, the NaCl crystal dissolved and thus released the remaining AGF, which resulted in giving inconsistent experimental data.

Having thus speculated on the interference by salt on the extraction of AGF when in the yolk-salt phase, it was thought best to free the yolk-salt preparation from any salt for any extraction procedure. The mode of action then was to employ a visking sac and dialyze out the NaCl present so that subsequent treatment of the salt-free yolk extract could be achieved without interference by the sodium chloride. A more consistent series of experimental
data could then be obtained.

**Dialysis and Fractionation of the Yolk-Salt Preparation From a Single Yolk**

The dry yolk-salt extract from the single yolk appeared to give growth to amoebae at preparations of 100% strength and no growth at dilutions of 20%, 10%, 5%, and 2%. This observed growth, in the 100% media, showed amoebae to grow quite luxuriantly, but no visible growth was apparent at 20% or any dilution below this strength. No media had been prepared at 50% strength so that at this stage, information on this dilution was incomplete. It will be taken into account below. A check on the apparently negative cultures of the lesser dilutions of this media by subsampling all negative tubes to stock media confirmed the absence of amoebae, since these also did not show any visible amoebae present.

In the preparation of media from the dialysed yolk solution only three concentrations were prepared. One at 50%, one at 100%, and one at 200%, to test the effects of high concentrations of essential on the amoebae. It was determined that in this particular series (single yolk), only these three preparations would be sufficient, since no growth of amoebae resulted in the previous preparation (No. 15) at 20%, 10%, 5%, and 2%. A glance at Table VI will show the observed results of the dialysed yolk solution. Doubling the concentration, i.e. 200%, grew the amoebae luxuriantly, while the other two concentrations gave moderate growth. It is interesting to note here, that at concentration of 50% apparently the same degree of amoebae growth occurred as in the 100% media. It is also interesting to note the dropping in the degree
of growth in the 100% (No. 16) media in relation to the same concentration of
the undialysed yolk-salt extract (No. 15). It seems that in dialysing out the
NaCl, some fraction of the essential was lost, however, the primary interest
is the question concerning the feasibility of dialysing out the salt from the
yolk essential and from the observations recorded in Table VI, one can con-
clude that even though a little of the AGF was lost, the operation was feas-
ible and practical and does purify and concentrate the AGF. The yolk essent-
ial, salt free, could then be subjected to extraction with fat solvents.

In the course of the present study, the Biuret Test was employed to
test for presence of proteins in the yolk extracts. The test consisted of
adding an equal volume of 10% sodium hydroxide solution to two to three ml. of
extract solution in a test tube. After mixing thoroughly, a 0.5% copper
sulfate solution was added, drop by drop, until 2 ml. had been added. The
appearance of a blue color would indicate the presence of a protein and a
reddish-brown would indicate shorter chain compounds such as polypeptides.
Only a very faint blue color developed. The protein test was so slight as to
make it seem improbable that it was the fraction that played a major role in
the growth promoting properties of the yolk essential. The oily fraction and
waxy material, on the other hand, extracted by ether and chloroform, made it
appear more probable that the important fraction was tied up in a lipid
complex.

In treating the dialysed yolk extract (No. 16) with chloroform, a
moderate amount of waxy substance was extracted out. An alternative method
of preparing media from this extract has been mentioned in the previous chapter.
This method in which the alcohol-dissolved CHCl3 extract is gently heated to
drive off the alcohol prior to incorporating it into media, and the method generally employed of dissolving the alcohol-dissolved CHCl₃ extract into water without boiling off the alcohol showed a difference in the degree of amoebae growth. Table VII records this difference. A referral to Table VII will substantiate that the remaining solution (No. 16) after chloroform treatment of (no. 16) no longer supported amoebic growth. It is therefore demonstrated that chloroform was able to extract out of the dialyzed yolk solution (No. 16) a wax-like substance in which AGF was present. Most, if not all, of the AGF was taken out by the solvent. Doubling the concentration of the media did not favor better growth, since the same degree of growth was obtained as that obtained in the 100% media. The reason for dissolving the oily-like or wax-like substances in alcohol in the above fractionations, as well as in the subsequent fractionations, was that it offered a better way to work with the waxy fractions. The fraction was soluble in alcohol and in this way measurements and dilutions could be accomplished with ease.

Freeze-drying the dialyzed yolk extract (No. 16) to obtain the essential in a dry, salt-free form did not meet with too much success. The observations recorded in Table VIII show that there was poor growth in the 100% media prepared from the freeze-dried powder, while scant growth at the higher concentrations. While it cannot be positively determined from the present data that most of the essential either was destroyed or deteriorated in the presence of a high vacuum, this process, in light of the present observations, could stand more experimentation since the slight activity recorded was present only in media of high concentrations.

The alcohol-dissolved CHCl₃ extract (No. 17) was treated with acetone
to see if any acetone-insoluble fraction would precipitate out and both fractions assayed for growth. The acetone precipitate (No. 17a) filtered out of the solution was not able to support growth at any of the concentrations prepared while the remaining solutions (No. 17b) supported growth. The observations recorded (Table IX) show that at 200% concentration doubtful growth appeared which would inevitably become negative with sufficient subcultures. The referred implications in this case is that elimination of the acetone insoluble precipitate purified the essential to some extent and concentrated the AGF in such a manner that media prepared at 200% strength appears to be inhibitory to the amebae. In addition to this, media prepared at 50% strength was not able to support amoebic growth, implying that not enough essential was present to sustain amoebic growth. It was rather surprising to note that at 100% concentrations, the degree of growth was recorded as being between "scant and moderate", presumably also due to high amounts of essential, leading the writer to presume that heavy growth of amebae would probably occur at a concentration between 60% and 90%, instead of at 100%.

The alcohol-dissolved CHCl₃ extract (No. 17b), less the acetone precipitate (No. 17a) separated from it previously, began to separate into two components when stored for several days. A slight sediment began to form and, at separation of the two components from each other and assaying each for growth, it was concluded from the observations recorded in Table X that both fractions are needed since neither the sediment alone supported growth nor the supernatant by itself. It appears therefore that both fractions are needed to support and sustain growth of the amebae.

It would seem appropriate at this time to characterize the types of
amoeba growth observed in the above experiments as well as in the subsequent experiments. In tubes which showed luxuriant, heavy, or moderate degrees of growth, the amoebae were all in the trophozoite stage, motile and climbing the side of the tube to the very top. Most amoebae were devoid of any starch granules in the cytoplasm and showed a varied degree of cytoplasmic activity.

Microscopic observations (100x) of the rice starch particles showed that these were no longer large masses or aggregates, but instead were seen to be very minute, discrete, and single granules on the bottom of the tube, leaving the medium clear and transparent. In tubes of scant and poor amoebic growth, the amoebae were either all in the cyst stage, partially full or completely full of rice starch particles, or they were in the trophozoite stage, large, not active but motile, and with the cytoplasm full of starch particles. The amoebae were mostly on the bottom of the tube or just one or two centimeters from the bottom. Microscopic observations of the starch particles revealed these to be quite large masses or aggregates and being in suspension throughout the medium imparting a turbid-like effect upon the medium. In media which did not support growth at all, the starch particles appeared as large masses or aggregates, very coarse and in suspension throughout the medium. In media which supports the amoebae very poorly or in media which could not sustain amoebae, the first and perhaps the second transfers showed a slight degree of growth, but upon further subculture, no visible amoebae growth occurred. These observations noted demonstrated that amoebae were able to survive one or two subcultures due to "carry over" of nutrients in the transfer. This also accounted for the subculturing of the amoebae for at least six transfers before a media was finally credited as supporting growth of amoebae.
Fractionation of the Yolk-Salt Extract of Twelve Yolks (No. 21)

Certain general impressions from the data in Table XI can be deduced. First, that increasing the amount of yolks in the initial preparation of Hartman's yolk-salt extract gives rise to the assumption that an increased amount of ASF can be adequately extracted out. This presumably greater quantity of essential taken out can be put into media and the media diluted down to only 2% concentration, which still proves favorable for amoebic growth. Attention is called to the fact that active and moderate growth occurred at this dilution, while at 5% concentration the degree of growth was between heavy and luxuriant. This manifestation established the ASF as being required only in minute amounts when properly concentrated. Second, as the concentration of a media is increased, and hence the concentration of the essential, the degree of amoebic growth increases up to a certain limit, then tapers off and begins to decrease. Third, it is thus evident that at too high a concentration of essential, growth decreases or is inhibited completely, as can be seen at the higher concentrations of media (No. 21).

Treating the liquid phase of the 12 yolk infusion with ether disclosed that most, but not all of the essential was extracted out of the yolk-salt infusion, (No. 20). Media prepared from (No. 22), the solution remaining after (No. 20) was treated with ether, showed heavy amoebas growth at 100% concentration, scant growth at 20%, and no growth at the dilution below 20%. On the other hand, the ether extract (No. 21) from the 12 yolk-salt extract (no. 20) showed a rising degree of growth as the concentration of the media increased from 2%, reaching its peak at the 10% concentration and dropping down at continued high concentrations, establishing again the inhibitory
effects of high concentrations of AGF on the amebae. While no evidence of an inhibitor or a lethal material has been found, the possibility of such material cannot be ruled out by the data secured.

When stored for several days the alcohol-dissolved ether extract solution (No. 21) began to separate into two components, a slight precipitate and the supernatant. From general appearances, the precipitate was presumably an alcohol-insoluble fraction of the ether extract. Separation of these two fractions and assay of each disclosed that the alcohol insoluble precipitate (No. 21p) did not support growth (Table XII). Appearance of scant growth at the highest concentrations makes it seem that enough of the AGF was carried down with the precipitate to make it slightly reactive at the highest concentration. This might also explain the reason why growth was lacking at 2% concentration of media (No. 21c), while the same dilution of media (No. 21) sustained growth. It would not be in accord unless the loss of activity of 2% concentration of (No. 21c) could be correlated with the slight reactivity of (No. 21p) at 400%. Elimination of this precipitate most likely purified and concentrated the AGF to some extent so that the observations of media (No. 21c) recorded in Table XII are in line with what was expected. Namely, that purification of the essential allowed the prolific and luxuriant growth of amebae at 5%, 10%, 20%, and 100% concentration. The fact stands out that media (No. 21) which contained the precipitate mentioned above did not give as luxuriant a growth as the media without this precipitate. In concentrations above 100%, amebae were inhibited.

In attempting to further purify the precipitate-free alcohol-dissolved ether extract (No. 21e) mentioned above, this solution was treated with
acetonc to precipitate out any acetonc-insoluble fraction, if present at all. It is recalled at this point that this same procedure was undertaken on the single yolk CHCl₃ extract and that the acetonc-insoluble fraction did not support any degree of amoebic growth. As was the case in the single yolk acetone treatment, the acetonc-insoluble fraction of the 12 yolk series proved unreactive in all media except at the higher concentrations 200% and 400%. Media prepared from the acetonc precipitate (No. 21a) at 200% and 400% showed only poor to scarce amoebic growth. The precipitate-free solution (No. 21b) seemingly retained the essential with only a slight loss of activity. Table XIII shows the recorded degree of growth. Heavy growth of amebas occurred at 20% concentration, while at 100% growth was scarce. Moderate growth occurred at 5% and 10%. Referral to Tables XIII and XII will make this slight loss of essential apparent, nevertheless it is not a significant loss since most of the essential remained in the acetonc-soluble fraction.

We turn our attention now to the dried extract (No. 22), the yolk-salt infusion remaining after most of the ether-soluble substances were extracted by the solvent. No growth of amebas was demonstrated in media prepared from this at 10%, 5%, and 2% concentrations. There was luxuriant growth at 100%, while 20% only gave meager growth. It was inferred from these observations that the ether did not take all of the AGF out of the yolk infusion and that the remaining residual essential remained in (No. 22). It was presumed that this remaining essential would be soluble in chloroform since chloroform extraction in the case of the single yolk demonstrated the AGF in that extract. However, before fractionation with CHCl₃ could be effected the NaCl had to be dialysed out of the extract by the process mentioned else-
The dialysed yolk extract from the above preparation again confirmed that the AGF is retained by a membrane and that no apparent deterioration takes place when it is freed from the sodium chloride. The dialysed yolk extract further established that the AGF in this particular phase does not prove inhibitory to amebes when high concentrations of media are prepared. Table XIV presents the experimental observations of 400%, 200%, and 100% media prepared from the dialysed yolk solution (No. 23) giving evidence of luxuriant amebic growth. No growth of amebes at preparations of 20%, 10%, 5%, and 2% is in the line since the previous extraction of the essential with ether accounted for this deficiency.

The treatment of the dialysed yolk solution (No. 23) with chloroform to establish whether this solvent is able to extract the residual AGF left behind by the first extraction by ether met with failure. The yolk solution (No. 24) remaining after being treated with chloroform retained most, if not all, of the residual essential left behind by the ether. The three higher concentrations of this media sustained amebic growth luxuriantly. The remaining concentrations of this media, as expected, did not support growth since medium (No. 23) at these same concentrations had been established as being lacking in AGF.

It is interesting to note that even though the chloroform was able to extract certain moderate amounts of a wax-like substance from the dialysed yolk solution, any appreciable amount of essential failed to appear in this waxy material; in fact, the amount that did appear was insignificantly small to be able to support amebic growth at very high concentrations. Moreover,
subsequent treatment of this chloroform extract (No. 25r) with ether, extracted this minute essential and only when this ether extract (No. 25s) was concentrated to six times the original concentrations, did the three highest concentrations support the amoebae very poorly. Although the observations noted in Table XIV indicated that poor growth occurred in (No. 25s) at 6(100%, 200%, and 100%) concentrations, the writer doubts that these growths would have survived two more transfers.

In an attempt to obtain more evidence as to the methods of extracting and concentrating the essential found in egg yolk responsible for amoebae growth properties, as put forth by this problem, the following conclusions can be drawn: From the apparent validity of the experimental results obtained in this study, it was demonstrated that di-ethyl-ether extracts more AGF than chloroform. It was demonstrated that if a greater quantity of yolk material is used in the preparation of Hartman’s dried extract powder, a greater and richer batch of AGF per given treatment would be yielded. Obviously, twelve yolks are better than one. The results proved that partial purification of the AGF could be accomplished by dialysing out the NaCl, the essential being retained by a visking membrane. In the ether treatment of the yolk-salt extract, some ether-soluble fraction not important to growth was apparently extracted out of the yolk infusion. This could later be removed by dissolving the oily ether extract into alcohol and separating the alcohol-insoluble precipitate formed after a few days storage. The remaining extract could be further purified by treating with acetone and removing the acetone-insoluble precipitate; but in the process of purification, some of the essential was lost. It is suggested that this treatment should not be carried out since
the acetone-insoluble precipitate is not harmful to the amebae and the AGF yield can be augmented if this last step is eliminated. The remaining solution of the ether-treated yolk infusion can be dried down to a salt and dialyzed to remove the sodium chloride. The dialyzed yolk solution can then be treated with chloroform to purify this extract. The essential in a partially purified state will be found, not in the chloroform extract sediment, but in the remaining solution. The chloroform-soluble fraction apparently has no appreciable amount of essential so that in effect what the chloroform did was to purify the remaining AGF in the dialyzed yolk infusion. It must be remembered, however, that this chloroform purified extract preparation at 100% strength or greater will support amebae growth.

As another point of interest, the results of this study appear to indicated that the AGF is also soluble in chloroform and if the yolk infusion extract is treated initially with chloroform, some essential can be taken out which will support amebae growth. However, this amount is not too high. This might seem to imply that the amebae essential is both ether and chloroform soluble, but more so in the former solvent. On the other hand, there is also the implication that this essential is found in two components, either one being able to support and sustain amebae. It is not infrequent that one finds in nature a mixture of similar, but not identical, fractions of an essential necessary for growth and that one of the fractions can "spare" the other.

In a study of this kind, speculation is sometimes permissible and in line with this statement, the author wishes to delve into this a little. To elucidate on the matter, and taking into consideration that the following is
merely speculation, it appears that the ACF found in egg yolk seem to include
substances having physical properties like those of the fats, such properties
as: insusceptibility with water or nearly so; solubility in ether and similar
"fat solvents"; having a waxy or oily texture; and being utilizable by an
animal organism, are properties exhibited by the essential at various stages
in the experimental procedures described previously. Fats are defined chemi-
cally as the triglycerides of fatty acids and are found widely distributed
throughout the animal and vegetable kingdoms. Approximately 32% of egg yolk
is made up of "fats", so that it can be speculated that the extractable matter
was part of this "fat" present in the yolk. Since by chemical definition
fats are triglycerides of fatty acids, we can expect to have an extract conta-
ingen one or a mixture of triglycerides. The fatty acids making up the
natural fats and oils of animals and plants, as a general rule and almost with-
out exception, are composed only of fatty acids having an even number of carbon
atoms and consist almost exclusively of straight chain acids. We might have
just that composition in the ACF separated out from the yolk, mainly a straight
chain with an even number of carbon atoms. We also established that the other
extract was an oil at room temperature, while the chloroform extract was a
solid. It is recalled that the lower members of the fatty acid triglycerides
are liquid at ordinary temperatures. This may mean that we might have a fatty
acid belonging in the lower members of the fatty acids, such as the butyric
and Lauric groups, and another belonging to the higher members, such as palme-
ate and stearate, since the chloroform extract was solid at room temperatures.
However, the possibility also exists that the essential is a fraction contain-
ing an unsaturated fatty acid such as oleic. Introduction of unsaturated
linkages in an acid results in a lower melting point of an acid or triglyceride.

This study has cast light on some of the characteristics of AGF found in yolk. The essential can be extracted in a partly purified form and concentrated. Adequate media can be prepared from this fractionation. The amount of essential necessary for growth of amebae is very minute; experiments have substantiated this claim. The AGF seems to be tied up in a lipid complex, with the implication of being a triglyceride or a mixture of triglycerides of low temperature melting point.
SUMMARY

1. The extracted material from egg yolk which supports growth of *Endamoeba histolytica* was found to be heat stable at various times in both the dry and wet phases.

2. The growth promoting factor for *Endamoeba histolytica* can be extracted from the yolk material with 5 per cent sodium chloride and with autoclave temperatures.

3. The material necessary for growth of *Endamoeba histolytica* can be extracted from salt by ethyl ether more readily than by chloroform.

4. Only slight traces of protein and appreciable fatty material appeared in the extracted yolk fractions; these were soluble in the fat solvents used. The growth promoting factor appears to be fat rather than protein in nature.

5. The material necessary for growth of *Endamoeba histolytica* does not dialyse thru a membrane.

6. The necessary material may be volatile at high vacuum.

7. There is an optimum concentration of ether soluble material for amoebic growth.
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APPENDIX

The bacterial organisms found in the NRS strain of the [illegible] histolytica culture were: A gram positive, alpha hemolysis type Streptococcus and a gram negative rod, identified as *Proteus mirabilis*. The following table characterizes the gram negative *Proteus* organisms.

<table>
<thead>
<tr>
<th><strong>Proteus mirabilis bio-chemical characteristics at 48 hours growth</strong></th>
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<tbody>
<tr>
<td>adenitol</td>
<td>urea</td>
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<tr>
<td>asselin</td>
<td>indol</td>
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<td>VP</td>
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<td>0.5% lactose</td>
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<tr>
<td>xylose</td>
<td>A</td>
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</tbody>
</table>

+ = positive
- = negative
A = acid
AG = acid and gas
APPROVAL SHEET

The thesis submitted by Miguel Kourany has been read and approved by three members of the Department of Microbiology. The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 27, 1953

Date

Ernest Hartman

Signature of Adviser