1960

A Study of the enzymes of Entamoeba histolytica

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A STUDY OF THE ENZYMES

OF

ENTAMOEBA HISTOLYTICA

by

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A.B., Montana State University, 1958

Presented in partial fulfillment of the requirements

for the Degree of

Master of Science

1960

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Dean, Graduate School

MAR 7 1960

Date

UMI Number: EP40120

UMI®

Dissertation Publishing

UMI EP40120

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ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Relatively little is known about the metabolism of
Entamoeba histolytica, the etiological agent of amoebiasis.
Studies dealing with the physiology of this protozoan
parasite have been hindered by the inability to cultivate
this organism in axenic culture. Metabolic studies are
difficult to perform in biological systems containing more
than one living species. However, with adequately con-
trolled experiments vital information can be obtained which
may directly or indirectly lead to the cultivation of the
amoebae in pure culture (free of other living organisms).
Studies on the enzymology of E. histolytica will shed in-
formation on the metabolism of this organism. Knowledge of
the enzymes and metabolic processes of the amoebae could
lead to a rational approach to the chemotherapy of amoe-
biasis and possibly to the elucidation of the mechanism of
pathogenicity.

This approach to the physiology of E. histolytica
has remained largely unexplored (50), mainly because of
the difficulties encountered in ruling out the biochemical
contributions of the bacterial associates (44). Many
workers (8, 21, 42, 50) feel that it is possible to obtain
preparations of amoebae relatively free of the associated

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bacterial flora by repeated washing procedures; in such
preparations the bacteria are present in such small numbers
that they contribute little or nothing to the observed
amoebic activity.

A number of workers have reported on the existence
of certain enzymes in E. histolytica in vitro. Anderson
and Hansen (1) and Reardon and Bartgis (12) reported that
the amoebae were capable of hydrolyzing the gluten sur-
rounding rice powder granules. A hemosiderin mass was
observed in amoebae and this was thought to be due to
residue from the dissolution of hemoglobin (1). Rees and
co-workers found gelatinase in E. histolytica (64). They
inoculated a gelatin medium with amoebae growing in associa-
tion with bacteria unable to hydrolyze gelatin and
obtained liquefaction. Harinasuta and Maegraith (34, 35)
reported that a washed culture of E. histolytica destroyed
the gelatin on an exposed and developed photographic film
when incubated at 37° C for two hours. Neal in 1956 (57)
reported that extracts of washed amoebae were capable of
hydrolyzing both gelatin and casein. Nakamura (53), in a
preliminary report, could not detect any casein-hydrolyzing
activity in the amoebae using a plate method. Nakamura and
Klein (55) found that actively growing cultures of E. his-
tolytica did not produce collagenase; the assay system used
was the rat-tail tendon dissolution technique. Bradin
showed that amoebae isolated from hamster liver abscesses

produced hyaluronidase, but did not do so in cultures (11).

DeLamater and co-workers (16) were unable to detect intra-
cellular or extracellular hyaluronidase production from
amoebae in culture, nor could they detect hyaluronidase
activity in preparations of washed amoebae obtained from
hamster liver abscesses. Carrera and Changus (13) demon-
strated histochemically that there was an acid phosphatase
in the amoebae, and Carrera (14) also showed that tissues
surrounding the invading amoebae in the intestinal lesions
were high in acid phosphatase activity. The presence of acid
phosphatase in the amoebae was also reported by Balamuth
(6). Hara and co-workers (33), utilizing a histochemical
technique, found an alkaline phosphatase in E. histolytica.
Blumenthal and co-workers (9) confirmed the presence of
both acid phosphatase and alkaline phosphatase in the amoe-
bae. These workers used p-nitro-phenyl-phosphate, a chro-
mogenic substance, and measured the amount of color produced
due to the hydrolysis of the chromogen-phosphate linkage.

The first and only report of any aspect of the amino
acid metabolism of E. histolytica was published in 1957.
Nakamura and Goldstein (56) were able to demonstrate a phos-
phate activated glutaminase in washed preparations of
amoebae. Succinic dehydrogenase was shown to exist in the
amoebae by Seaman in 1953 (68). He stated that the enzyme
of the amoebae differed from the mammalian counterpart in
that it was inhibited by certain arsono and phosphono

analogues of succinic acid. The existence of an esterase
in E. histolytica was shown by Hallman et al. (31). They
titrated the acid produced from methyl-n-butyrate by
washed, lysed cells of E. histolytica. The only report
on the presence of an oxidase-type enzyme in the amoebae
was presented by Hara and his group in 1954 (33). They
were able to demonstrate cytochemically a strong peroxi-
dase reaction in this protozoan.

Other observations dealing with the enzymology and
metabolism of the amoebae have been primarily concerned
with the carbohydrate metabolism of the organism. Amylase
production has been noted by many workers (3, 29, 38).
Hilker and co-workers (38) found a maltase, but no lactase
nor sucrase in E. histolytica. In 1943, von Brand (50)
demonstrated that the amoebae did not produce gas from
glucose. Hallman et al. (30) and Loran and co-workers (45)
also have reported that the amoebae do not directly utilize
glucose. However, others have reported that the amoebae do
utilize glucose (8, 22, 42, 49, 51). Kun and Bradin (42)
presented evidence for the utilization of nine sugars by
washed preparations of the amoeba, and in addition showed
that E. histolytica produced H_2S from sulfur-containing
compounds such as cysteine.

Hilker and White (39), utilizing disrupted cell
preparations of washed amoebae, claimed to have demonstrated
the presence of phosphoglucomutase, phosphohexokinase,

aldolase, and glucose-6-phosphate dehydrogenase. They could not, however, demonstrate glyceraldehyde-3-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase. From these observations they concluded that the hexosemonophosphate shunt did not exist in E. histolytica, and that the Entner-Doudoroff pathway does exist in the amoebae (39). Recently, Entner (23) has confirmed the work of Hilker and White (39) with regard to the existence of the Entner-Doudoroff pathway in E. histolytica.

The enzymes reported to be present in E. histolytica are listed in Table I. Included also are some reactions that could be due to enzymes or enzyme systems. It can be seen that the metabolic studies on E. histolytica have been very meager and little progress has been made compared to the advances in the metabolism of other microorganisms. One of the main reasons for this is the lack of a pure culture of the amoebae. The technical difficulties so often encountered in working with E. histolytica might also be a reason for the lack of progress (42, 44). This would account for the comparatively crude methods so far used in the studies of the enzymes of E. histolytica.

TABLE I

DATA FROM LITERATURE ON ENZYMES OF ENTAMOEBA HISTOLYTICA

Reference	Observation	Conclusion
Craig, 1927 (c.f. Anderson et al) (2)	lysis of red blood cells and tissue cells by an alcoholic extract of E. histolytica	E. histolytica possesses an endotoxin capable of lysing red blood and tissue
Morita, 1938 (c.f. Anderson et al) (2)	lysis of tissue-cell suspensions by actively growing cells of E. histolytica	E. histolytica produces a cytolytic substance
Hambly, 1947 (c.f. Anderson and Hansen) (1)	cytochemical demonstration of a hemoriderin mass in E. histolytica	E. histolytica produces a hemolysin
Anderson and Hansen, 1947 (1)	digestion of glutin coat of rice starch	E. histolytica produces a proteolytic enzyme
Carrera and Changus, 1946 (13)	cytochemical demonstration of an acid phosphatase in E. histolytica	E. histolytica elaborates acid phosphatase
Reardon and Bartgis, 1949 (12)	digestion of glutin coat of rice starch	E. histolytica produces a proteolytic enzyme
Carrera, 1950 (14)	cytochemical demonstration of an acid phosphatase in tissues of an amoebic ulcer	E. histolytica elaborates an acid phosphatase

TABLE I (continued)

Reference	Observation	Conclusion
Balamuth, 1950 (6)	cytochemical demonstration of acid phosphatase in E. histolytica	E. histolytica produces an acid phosphatase
Seaman, 1953 (68)	followed the rate of reduction of cytochrome C by washed preparations of E. histolytica in the presence of succinic acid	E. histolytica elaborates succinic dehydrogenase
Hallman and DeLamater, 1953 (29)	noted the ability of washed preparations of E. histolytica to cause starch to become unreactive with iodine	E. histolytica produces an amylase
Rees et al., 1953 (64)	noted liquefaction of conventional gelatin tubes by E. histolytica in association with a non-gelatin hydrolyzing bacterium	E. histolytica produces a gelatinase
Bradin, 1953 (11)	decrease in turbidity of a potassium hyaluronidate solution caused by washed cells of a freshly isolated strain of E. histolytica	E. histolytica produces a hyaluronidase that seems to be lost upon cultivation of the amoebae
DeLamater et al., 1954 (16)	a freshly isolated strain of E. histolytica was not capable of reducing the turbidity of a solution of potassium hyaluronidate	E. histolytica does not produce a hyaluronidase

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TABLE I (continued)

Reference	Observation	Conclusion
Hara et al., 1954 (33)	histochemical demonstration of peroxidase and alkaline phosphatase in E. histolytica	E. histolytica elaborates an alkaline phosphatase and a peroxidase
Baernstein et al., 1954 (3)	breakdown of starch to reducing sugars by acetone powders of washed E. histolytica	E. histolytica secretes an amylase
Harinasuta and Maegraith, 1954 (34)	extracts of E. histolytica were able to hydrolyse the gelatin on exposed, developed photographic film	E. histolytica produces a gelatinase
Blumenthal et al., 1955 (9)	washed cell preparations of E. histolytica were able to hydrolyze p-nitrophenyl-phosphate	E. histolytica produces an acid and an alkaline phosphatase
Hallman et al., 1955 (31)	acid production from the action of washed preparations of E. histolytica on methyl-N-butyrate	E. histolytica elaborates an esterase
Neal, 1956 (57)	casein was hydrolyzed, and the viscosity of gelatin solutions was reduced by extracts of E. histolytica	E. histolytica elaborates a casease and a gelatinase

TABLE I (continued)

Reference	Observation	Conclusion
Nakamura and Goldstein, 1957 (56)	preparations of E. histolytica were capable of releasing ammonia from glutamine	E. histolytica produces a glutaminase
Hilker et al., 1957 (38)	extracts of E. histolytica were able to split starch into components that would not react with iodine---- chromatographic analysis showed maltose, but not lactose or sucrose to be metabolized	E. histolytica produces an amylase and a maltase, but not sucrase or lactase
Nakamura and Klein, 1957 (55)	E. histolytica, under bacteriostatic conditions, could not cause the dissolution of rat-tail tendon	E. histolytica does not elaborate a collagenase
Nakamura, 1957 (53)	preparations of E. histolytica were not capable of hydrolyzing milk-agar plates	E. histolytica does not produce a casease
Harinasuta and Maegraith, 1958 (35)	extracts of E. histolytica are capable of hydrolyzing the gelatin on exposed and developed photographic film	E. histolytica produces a gelatinase

TABLE I (continued)

Reference	Observation	Conclusion
Hilker and White, 1958 (39)	unreported	E. *histolytica* produces phosphoglucomutase, phosphohexokinase, aldolase, and glucose-6-phosphate dehydrogenase, but does not contain glyceraldehyde-3-phosphate dehydrogenase nor 6-phosphogluconate dehydrogenase

CHAPTER II

STATEMENT OF THE PROBLEM

Very little is known regarding the enzymes of
Entamoeba histolytica. Various workers have felt that
studies on the enzymes of the amoebae could lead to the
establishment of this protozoan parasite in pure culture.

The present investigation is a survey study of some
of the enzymes of E. histolytica.

CHAPTER III

METHODS AND MATERIALS

I. General Methods and Materials

(1) Strains of Entamoeba histolytica employed

Seven strains of E. histolytica (HUS-105, HUS-100, Washington, Griffith, ROK, Conrad and DKB) were used in these studies. The HUS-100 and HUS-105 strains, both large races, were obtained from Dr. Chia-tung Pan, Department of Tropical Public Health, Harvard School of Public Health, Boston. These strains were isolated from the stools of carriers during the South Bend, Indiana, (Singer Sewing Machine Co.) outbreak of amoebiasis in 1953 (59, 60). The DKB strain, a large race growing in association with Clostridium perfringens, was obtained from Dr. J. N. DeLamater, Department of Medical Microbiology, University of Southern California, Los Angeles. The DKB strain was isolated in England by Dr. J. Drbohlav in 1924, given to Professor C. Dobell in that year, and subsequently brought to the United States of America by Dr. H. E. Meleney in 1935 (8). The other strains, all small races, were obtained from Dr. R. M. McQuay, Jr., Mount Sinai Hospital, Chicago, Illinois. The Griffith strain was isolated by Dr. McQuay at the Mount Sinai Hospital from the stool of a patient with

symptomatic amoebiasis (70). The Washington strain was iso-
lated in December, 1956, and the ROK strain was isolated in
October, 1957, at the Mount Sinai Hospital, Chicago (46).
The Conrad strain of $\underline{E}$. $\underline{histolytica}$ was isolated in the
Parasitology Laboratory of the Hektoen Institute, Cook
County Hospital, Chicago, from a patient with chronic
symptomatic amoebiasis (70).

(2) Culture media and methods employed

Three different culture media were used in this
study. A modified Boeck-Drbohlav medium was used earlier
in the investigation, but as it was found possible to re-
place the serum overlay with liver extract (25), this
modification was employed. Later, the Cleveland-Collier
medium, overlayed with bovine serum, which produced better
growth, was employed.

The Boeck-Drbohlav medium (10) was prepared in the
following way: (1) One dozen fresh eggs and 120 ml of
Ringer's solution were beaten with an egg beater. (2) The
egg-Ringer suspension was dispersed into glass test tubes
(15 by 12.5 mm) in 4.0 ml amounts per tube. (3) The
tubes were plugged, slanted and insipissated in the Arnold
steam sterilizer at 75-80° C for 3 hours. This procedure
coagulated the egg suspension. (4) The tubes were then
autoclaved at 15 pounds pressure for 15-20 minutes. (5)
The egg slants were overlaid with 5 ml of horse serum

diluted 1/5 with Ringer solution (4). The serum was sterilized by filtration through a Selas filter (porosity #02). The horse serum was obtained from the Biologics Laboratory, Department of Public Health, Antitoxin and Vaccine Laboratory, Boston, Massachusetts. A modification of this medium in which horse serum was replaced by 0.5% Liver Extract "L" (Nutritional Biochemical Corp.) was also used.

The Cleveland-Collier medium was prepared in the following manner: (1) Thirty-three grams of Bacto End-amoeba Medium (Difco) was dissolved in 1000 ml of cold distilled water and heated to boiling. (2) This solution was dispersed into test tubes in 4 ml amounts and auto-claved at 15 pounds pressure for 15-20 minutes. (3) The tubes were allowed to cool in a slanted position in order to produce a solid slant (17). (4) The slants were over-laid with 4 ml of sterile bovine serum-Ringer (1/5). Bovine blood, from which the serum was separated, was generously supplied by J. R. Daily, Inc., Missoula, Mont.

Immediately prior to the transfer of the amoebae, approximately 10 mg of sterile Difco-Bacto Rice Powder was added to the culture medium. The rice powder was sterilized by autoclaving at 15 pounds pressure for 15 minutes, dried, and the tube vigorously agitated to obtain a fine powder. The amoebae cultures were transferred on Mondays, Wednes-days, and Fridays. The method of transfer consisted of

withdrawing approximately 0.5 ml of the sediment from the culture tube by means of a capillary pipette fitted with a rubber bulb. The cultures were incubated at 37° C. Every strain of amoebae utilized, except the DKB strain, had an unknown multiple bacterial associate.

(3) Cell preparations

Since the peak of the E. histolytica growth curve was found to be between 48-72 hours by Knight et al. (41), all assays were performed with cultures incubated for this length of time. Concentrated trophozoite suspensions were obtained by centrifuging the pooled amoebae at slow speeds (approximately 650xG for 5 minutes) and resuspending them each time in fresh physiological saline. As this process was repeated, the bacteria were diluted out and the amoebae were concentrated. The effectiveness of this method has been described by Seaman (68), and others (9, 31).

The amoeba preparations for these studies were washed 5 times. The supernatants from the second, third and fifth washings (containing residual bacteria) and the sediment from the fifth washing (containing amoebae) were assayed for activity. In addition, plate counts were made of the supernatant from each washing step in order to check the efficiency of the washing procedure in terms of elim- ination of the associated bacteria. Brain-heart infusion agar (Difco) was used in these procedures to determine

bacterial growth and brain-heart infusion broth (Difco) was used to obtain bacterial cells for assay controls; fluid thioglycollate medium (Difco) was used for the cultivation of Cl. perfringens.

In experiments where lysed amoebae were needed, cytolysis was accomplished by suspending washed amoebae in distilled water. A 15 minute treatment was usually sufficient to obtain 60-75% lysis.

(4) Counting method

The concentration of amoebae was determined with the aid of a hemocytometer. The method described by Hansen and Bennett (32) was used. The washed amoebae were suspended and placed in the chambers of the hemocytometer ("Bright Line," American Optical Co., with Neubauer-Levy ruling, 0.1 mm deep). The amoebae in four of the 1 mm^2 ruled areas were counted in each chamber and the number of amoebae per ml in the suspension was calculated.

(5) Preparation of buffers and salt solutions

Phosphate and acetate buffers of various pH values were used throughout this research. The compositions of the buffers utilized are listed in Table II.

Physiological saline was prepared by adding 8.5 g of sodium chloride (reagent grade) to a few hundred ml of distilled water and adjusting the volume to 1000 ml.

TABLE II

COMPOSITION OF BUFFER SOLUTIONS

(A)   Phosphate Buffer Solutions

M/15 Na_2HPO_4	M/15 KH_2PO_4	pH
2.5 ml	97.5 ml	5.28
10.0 ml	90.0 ml	5.90
30.0 ml	70.0 ml	6.46
61.1 ml	38.9 ml	7.00
95.0 ml	5.0 ml	8.04

(B)   Acetate Buffer Solutions

0.1 N sodium acetate	0.1 N acetic acid	pH
18.0 ml	82.0 ml	4.0
70.5 ml	29.5 ml	5.0
90.5 ml	9.5 ml	5.6

Buffered saline was prepared by adding 8.5 g of sodium chloride to a few hundred ml of M/15 phosphate buffer (pH 7) and bringing the volume up to 1000 ml with additional buffer.

The composition of Ringer solution is outlined below:

NaCl	6.5 g
$CaCl_2$	0.12 g
KCl	0.14 g
$NaH_2PO_4 \cdot H_2O$	0.01 g
NaH_2CO_3	0.20 g
Distilled water	1000 ml

II. Experimental Methods and Materials

(1) Catalase

The DKB strain of E. histolytica (growing in association with Cl. perfringens) was used in this study. Amoeba preparations were prepared from 48-hour cultures of E. histolytica grown on Cleveland-Collier media. In all trials of this assay, the amoeba count was at least 400,000 amoebae per ml. The supernatant fluid from each washing step was pooled and centrifuged to obtain packed bacterial cells. Purified catalase (General Biochemicals Inc.) and packed cells of Escherichia coli were used as positive controls.

A modification of the method described by Higgins and Plastridge (37) was used. The assay was carried out in calibrated Smith fermentation tubes completely filled with

3% H_2O_2 in buffered saline (pH 7.0). The material to be assayed was introduced into the Smith tubes in 1 ml amounts by means of a curved Pasteur pipette. The tubes were incubated at $4-5°$ C for 30 minutes; at the end of the incubation period the tubes were examined for the evolution of oxygen.

(2) Gelatinase

Six strains of E. histolytica were assayed for gelatin-hydrolyzing ability. The HUS-100, HUS-105, Washington, Griffith, ROK, and Conrad strains were tested. All strains were routinely grown in a modified Boeck-Drbohlav medium.

Two methods were used to investigate the gelatinase of E. histolytica: (1) a sensitive microtest developed by Thirst (75), and (2) an agar-gelatin plate test developed by Smith (72). The microtest consisted of using a thin film of gelatin (Difco) as substrate on a glass slide. The slides were prepared by dipping clean, dry microscope slides into melted aqueous gelatin solutions (5%) containing phenol (0.1%), and allowing them to drain and dry. The materials to be assayed were placed on the slide in large drops (0.3-0.4 ml) and incubated at $20°$ C for 10-12 hours in Petri dishes containing strips of moistened filter paper to minimize evaporation. The hydrolysis of gelatin was indicated by the appearance of clear areas on the slide when stained with dilute carbolfuchsin, a protein stain. The presence of

phenol prevented bacterial growth without affecting gelatin-
ase activity.

The second method consisted of preparing 1% agar
plates containing 0.4% gelatin. Large drops of the material
to be assayed were placed on the surface of the plates, and
incubated at 37° C for 10-12 hours. The plates were then
flooded with approximately 10 ml of mercuric chloride in
hydrochloric acid (15 g $HgCl_2$/120 ml of 0.37 N HCl), a
protein precipitant. A cloudy, white precipitate in the
agar indicated the presence of gelatin; clear areas indi-
cated zones of gelatin hydrolysis.

Assay materials consisted of: (a) cell preparations
of all strains were made in the manner previously described
(see page 15); (b) strain HUS-105 grown in the absence of
multiplying bacteria with the aid of growth factors, adeno-
sinetriphosphate, ribose-5-phosphate, and large concentra-
tions of antibiotics (52), was pooled and washed; (c) pre-
parations of pooled and washed HUS-105 grown in association
with Esch. coli. The HUS-105 strain growing in association
with Esch. coli was initiated for this experiment, but could
not be continued in culture for an extended period of time.
Yields of amoebae varied with the method of cultivation, but
at least 200,000 to 250,000 amoebae per ml were obtained.

Controls consisted of: (a) drops of sterile Ringer's
solution on slides and plates; (b) a 12 hour broth culture
of Proteus vulgaris and a 0.5% aqueous suspension of trypsin

(Fairchild Bros. & Foster Co.) as indicators of gelatin
hydrolysis; (c) inoculation of associated flora from the
amoeba cultures into conventional gelatin tubes to detect
liquefaction of gelatin by the bacterial flora; (d) de-
struction of amoebic gelatinase activity by autoclaving the
assay materials and also inhibition of the enzyme activity
with 20% copper sulfate; and (e) alteration of amoebic
enzyme activity by alternate freezing and thawing of the cell
suspensions.

An attempt was made to determine the optimum pH
range for amoebic gelatinase. The pH of the agar-gelatin
plates was adjusted with phosphate buffers. Finally the
effects of enzyme concentration were studied. The washed
amoebae suspensions were diluted with saline to obtain a
1/2, 1/4, 1/8, 1/16, and 1/32 dilution and tested for
gelatinase activity on gelatin agar plates.

(3) Lecithinase

A modification of the method by Sheldon et al. (71)
was used in this assay. The reaction is one in which the
enzyme lecithinase splits lecithovitellin to insoluble
fatty acids that appear as a cloudy precipitate (28).

The yolks of several fresh chicken eggs were stirred
with sufficient Ringer's solution to obtain a 20% suspension
(v/v). The suspension was filtered through Whatman #1
filter paper twice. Five ml of this crude lecithovitellin

was added to 95 ml of a buffered agar (1%) solution that had
been autoclaved and cooled to 50° C. The lecithovitellin-
agar mixture was poured into sterile Petri dishes in 15 ml
aliquots and allowed to harden. Antibiotic assay discs
(Schleicher and Schuell, #740-E) with a diameter of 12.7 mm,
were placed on the surface of the plates; the discs were
saturated with the materials to be assayed. The plates
were incubated at 20° C for 10-12 hours, and the presence
of precipitates recorded.

E. histolytica (strain HUS-105) was used in this
assay for lecithinase activity. The amoeba cells were pre-
pared as previously described (see page 15), except that the
amoeba were cultured on Cleveland-Collier medium. All cell
preparations contained at least 400,000 amoebae per ml.
Washed cell preparations of Bacillus subtilis and Cl. per-
fringens were used as positive controls.

(4) Desoxyribonuclease and ribonuclease

A modification of the method of Jeffries et al.
(40) was employed to study the nuclease activity of the
amoebae. Solutions of nucleic acids, containing 10 mg
per ml were prepared. The desoxyribonucleic acid (DNA)
(Nutritional Biochemicals Corp.) was dissolved in distilled
water, and the ribonucleic acid (RNA) (Nutritional Biochemi-
cal Corp.) was dissolved by the dropwise addition of 1 N
NaOH being careful not to allow the pH to rise above 5.
Fifty ml of the nucleic acid solutions were added to 50 ml

of the liquified agar (2% agar in water) and autoclaved.

The autoclaved solution was allowed to cool to 50° C, and

approximately 15 ml of the solution were poured into sterile

Petri dishes and allowed to harden. Experiments were per-

formed with plates containing either RNA or DNA as sub-

strates.

Six strains of E. histolytica (HUS-105, HUS-100,

Washington, Conrad, ROK and Griffith) were used in this

assay; all strains were grown in modified Boeck-Drbohlav

media. Cell suspensions were prepared in the usual manner.

Ribonuclease (Nutritional Biochemicals Corp.) (10 mg/ml in

water) and desoxyribonuclease (Nutritional Biochemicals

Corp.) (10 mg/ml in water) were utilized as positive con-

trols. Large drops of the materials to be assayed for

nuclease activity were placed on the nucleic acid-agar

plates and the plates were incubated at 25° C for 10-12

hours. After incubation the plates were flooded with

approximately 15 ml of 0.1 N HCl; the positive reaction

for nuclease activity was indicated by a clear zone on

the agar. Areas without nucleic acid hydrolysis became

cloudy. The mechanism is one in which the acid reacts with

the nucleate salts in the medium yielding free insoluble

nucleic acid and consequently a precipitate forms.

(5) Casease

The method utilized for the assay of the phospho-

protein-hydrolyzing enzyme, casease, was a modification of
the standard laboratory "milk plate" method as described
by Salle (67). Instead of using whole milk in the test
medium, purified casein (Merck) was used. The casein (final
concentration of 0.2% in 1% agar) was added to agar that
had been autoclaved and cooled to 50° C. Approximately
15 ml of the casein-agar mixture was poured in sterile
Petri dishes and allowed to harden. Large drops of the
materials to be assayed were placed on the surface of the
plates, and the plates were incubated at $20-25^\circ$ C for 12
hours. The test plates were then flooded with a solution
consisting of 15 g $HgCl_2$/120 ml of 0.37 N HCl. Clear areas
in the opaque casein-agar indicated zones of casein hydro-
lysis.

Two strains of E. histolytica (HUS-105 and Washington)
were assayed for casein-hydrolyzing ability. A modified
Boeck-Drbohlav medium was used for the cultivation of these
two strains. Amoeba cells were prepared as described —
earlier (page 15) and assayed; packed cells of the associ-
ated bacterial flora were also tested for activity. The
heat stability of the enzyme was studied by heating the cell
suspensions for 5 minutes at 45° C, 55° C and 65° C. To
determine the optimum pH for enzyme activity the pH of the
test plates was varied by the addition of phosphate buffers
of different pH values to the agar base. B. subtilis
was utilized as a casease-producing control; Esch. coli,

an organism unable to produce casease, was used as a control
to rule out non-specific casein hydrolysis.

(6) Urease

A modified Van Slyke and Cullen (36) method for the
determination of urinary ammonia was used to investigate
the urease activity of E. histolytica. An aeration train,
as shown in Figure 1, was set up in the following manner:
(1) 20 ml of concentrated sulfuric acid was added to tube A,
the air intake tube, in order to render the incoming air
ammonia-free; (2) tube B acted as a trap to prevent any
accidental carryover of the sulfuric acid during aeration;
(3) tube C was the reaction tube and contained 10 ml of
3% urea solution (Merck) (pH 7) and 2 ml of the material to
be assayed for urease activity; (4) tube D contained 25 ml
of 0.02 N sulfuric acid and served to trap the ammonia given
off during the reaction. Caprylic alcohol (2 drops) was
added to each tube to minimize frothing. Sintered glass
impingers were used to obtain good aeration of the solu-
tions. A water aspirator was used as a source of vacuum.

The reaction mixtures were incubated at 20-25° C for
varied periods of time. After the incubation period, the
tubes were aerated very slowly for 30 seconds, the aspira-
tor was turned off and 10 ml of a saturated potassium carbo-
nate solution added to tube D (reaction tube). The reaction
tube was resealed and aerated for 15 minutes. At the end of
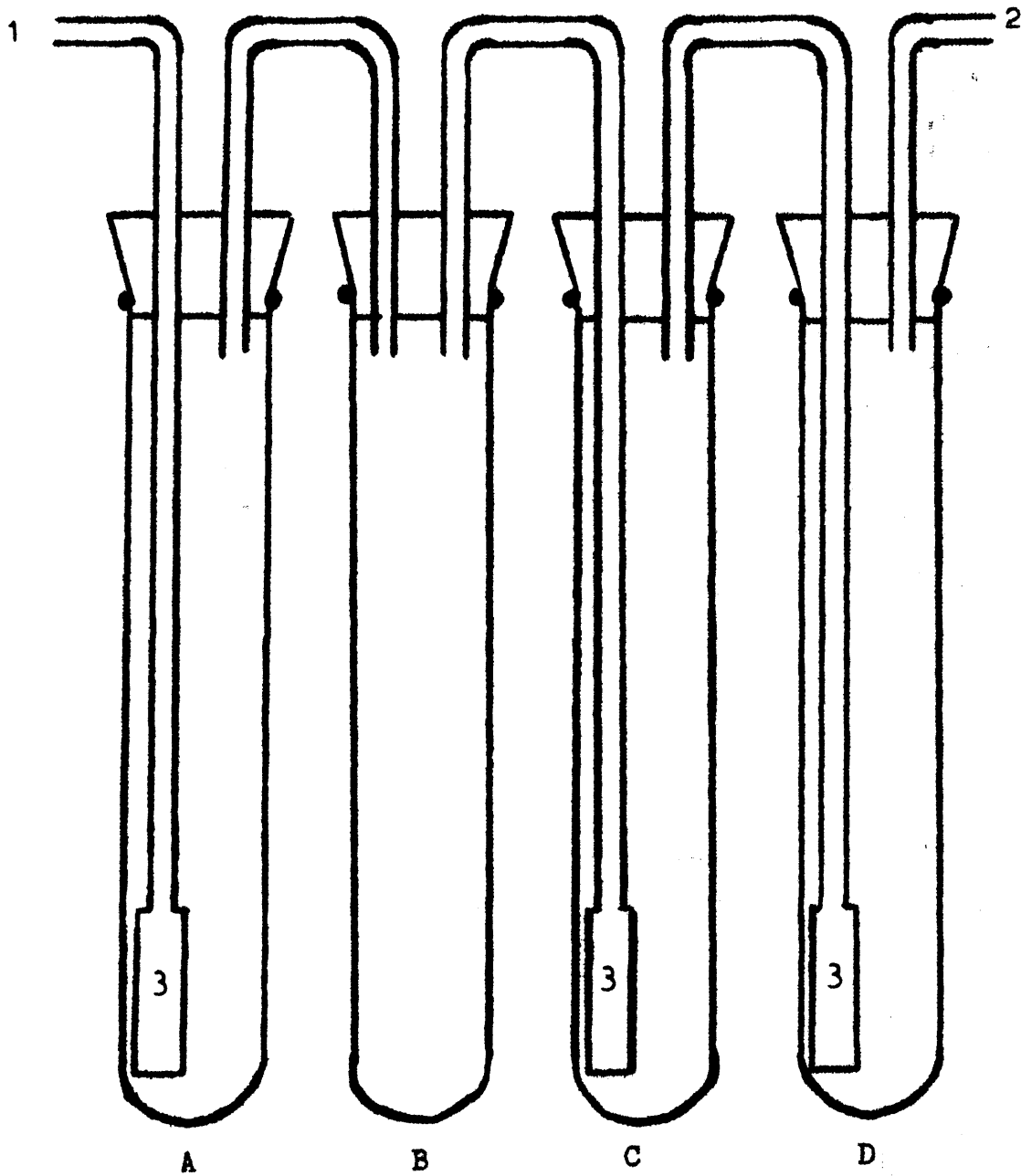
FIGURE 1

Aeration Train

(1) air intake
(2) to aspirator
(3) impingers

this period, tube D was disconnected and the contents transferred to a 100 ml volumetric flask to which 10 ml of Nessler's solution (Tenso-Lab. Inc., 6.8 g/100 ml of 8.5% NaOH), diluted to 30 ml with distilled ammonia-free water (boiled), was added and the volume brought up to 100 ml with ammonia-free water. The solution was mixed, allowed to stand for five minutes, and the optical density determined at 480 mu in a Spectronic-20 colorimeter (Bausch & Lomb) against a reagent blank. The reagent blank was prepared by carrying a solution of substrate through the complete analytical procedure (incubation, aeration, nesslerization) in exactly the same manner as the assay tubes. This was done in order to correct for ammonia in the reagents, decomposition of the substrates and errors in the analysis (36).

The DKB strain of E. histolytica, intact and lysed, was assayed for urease activity. This strain was cultured on Cleveland-Collier media. Washed amoebae were prepared as previously described. A determination of the ammonia content of the washed amoeba preparations was attempted by the same analytical procedure in the absence of substrate. The sensitivity of the method was determined by using dilutions of clinical urease tablets (Arlington Chemical Co.).

(7) Citrullinase

Citrullinase activity was determined using procedures

similar to those for the detection of urease. The only
modification was that a 2% solution of dl-citrulline (Nutri-
tional Biochemicals Corp.) (pH 7) was used as a substrate
instead of urea.

In this assay the DKB strain of E. histolytica
was employed. Positive controls consisted of packed cells
of Cl. perfringens. Both intact and cytolyzed amoebae were
studied. Amoebae were cultured on Cleveland-Collier medium.

(8) Asparaginase

The assay for this amidase was also carried out by
the Van Slyke-Cullen method (36). A 2% solution of dl-
asparagine (Nutritional Biochemicals Corp.) (pH 8) was
utilized as substrate. Both intact and lysed cells of
E. histolytica, strain DKB, grown on Cleveland-Collier
medium, were used.

(9) Glucuronidase

The glucuronidase activity of E. histolytica was
investigated using a modification of the method described
by Robinson et al. (65). Essentially, the method is based
upon the colorimetric measurement of the hydrolysis of a
chromogenic substrate, phenolphthalein glucuronidate.

The assay for glucuronidase activity was carried out
in 15 ml glass centrifuge tubes. Each tube received 0.4 ml
of 0.1 N acetate buffer of varied pH values, 0.4 ml of

0.001 M phenolphthalein glucuronidate (Sigma Chemical Co.),
and 0.4 ml of the material to be assayed for activity.
After a one hour incubation period at 37° C, 2.4 ml of 0.15 M
sodium carbonate were added and the tubes centrifuged for
30 minutes (3000xG). Concurrently, duplicate series of
tubes were treated in the same manner, except that one
series did not contain substrate, and the other contained
heat-inactivated (boiled) suspensions of the material
assayed.

After clearing by centrifugation, the supernatant
was decanted and the optical density determined at 540 mμ
in the Spectronic-20 colorimeter. The optical density was
set at zero with the supernatant from a tube from the series
lacking in substrate; the duplicate tubes (the same dilution
of the same material) from the heat-inactivated series and
from the enzyme series were compared with this tube, and the
optical density recorded. This method of negating changes
in optical density caused by bacterial cells and other non-
phenolphthalein opacities was carried out with each dilution
of each material assayed.

In order to become more familiar with the method and
to determine the sensitivity of the method, an assay of
various dilutions of a known glucuronidase-producing bac-
terium were deemed necessary. To obtain an organism that
produced glucuronidase it was found necessary to perform a
survey of several species of bacteria. Streptococcus

liquefaciens, Streptococcus pyogenes, Staphylococcus pyo-
genes, var. aureus, Escherichia coli, and an unidentified
Gram positive streptococcus, isolated from the HUS-105
flora, were assayed with a microtest for glucuronidase (65).

The microtest consisted of placing a small drop of
0.01 M phenolphthalein glucuronidate in a slight depression
in a sheet of aluminum foil. A loop of the bacterial
species to be tested was thoroughly mixed with the substrate,
and the entire suspension drawn up into a 1 mm glass capil-
lary tube. The suspension was incubated at 37° C for one
hour. White spot plates were prepared with 0.03 ml of
0.15 M sodium carbonate in each hollow. The capillary
tubes were removed from the incubator and the contents ex-
pelled into designated spots of the alkaline solution. The
prompt appearance of a distinct red color in the sodium
carbonate solution indicated the presence of glucuronidase.

Strain DKB of E. histolytica, cultured on Cleveland-
Collier medium, was used in this assay. The previous method
of cell preparation was utilized; whole and lysed amoebae
were assayed for activity.

(10) Beta-D-galactosidase

O-nitrophenyl-beta-d-galactopyranoside (Sigma Chemi-
cal Co.), a chromogenic substrate, was used in an assay for
galactosidase (lactase) activity in E. histolytica.
The method is based upon the colorimetric determination of

chromogen produced in the supernatant by the enzymatic hydrolysis of the substrate.

The method employed was a modification of a procedure described by Lederberg (43). Amoeba cells, lysed and whole, were resuspended in phosphate buffer (pH 7) and 4.5 ml of the suspension were pipetted into 15 ml centrifuge tubes containing 0.5 ml of M/200 substrate. The tubes were incubated at 37° C for 30 minutes, cleared by centrifugation (3000xG) and the optical density determined at 420 mμ in the Spectronic-20 colorimeter. The method of correcting for the opacities produced by factors other than the chromogen used in the assay for glucuronidase was also used in the assay for the galactosidase.

Strain DKB, cultured on Cleveland-Collier medium, was used in this study. In addition, the sensitivity of the method was assayed using Esch. coli as the test organism.

(11) Succinic dehydrogenase

The Thunberg technique was used for the assay of succinic dehydrogenase in E. histolytica (HUS-105). The method consists of determining the time necessary to decolorize methylene blue. Methylene blue acts as the final hydrogen acceptor in the oxidation (dehydrogenation) of succinic acid.

Conventional Thunberg tubes were used; 1 ml of the material to be assayed was placed in the side arms. Two

ml of phosphate buffer (pH 7), 1 ml of a 1:10,000 dilution
of aqueous methylene blue, and 2 ml of M/50 succinic acid
(Nutritional Biochemicals Corp.) were added to each tube.
Inhibitors of succinic dehydrogenase were assayed by placing
1 ml of inhibitor (M/50) into the Thunberg tubes in addition
to buffer, dye, and succinate (1 ml). The tubes were evacu-
ated for 3 minutes with the aid of a water aspirator and
then equillibrated for 10 minutes in a 57° water bath, and
the cells were tipped into the body of the tube from the
sidearm.

The tubes were incubated at 57° C and visually com-
pared with a control tube (containing methylene blue but no
substrate) at 10 minute intervals over a period of 90 min-
utes. The time required for the decolorization of the
methylene blue to a color intensity equivalent to the
control, which represented 90% reduction, was recorded. The
control tube contained 1 ml of heat-inactivated cells, 2 ml
of buffer, 2 ml of substrate and a 1/100,000 dilution of
methylene blue.

The amoebae, cultivated on egg slants overlayed with
0.5% liver extract, were prepared in the usual manner.
Bacterial and endogenous (preparations of amoeba in absence
of substrate) controls were also used in the prescribed
fashion.

Arsono-acetate obtained from Dr. G. R. Seaman,
University of Texas Medical Branch, Galveston, and malonic

acid (Nutritional Biochemicals Co.) were used as competitive inhibitors.

The assays were performed at 57° C to inhibit the rapid endogenous dehydrogenation of starch by E. histolytica. The use of 57° C to inhibit the endogenous metabolism was suggested by the work of Quastel and Wooldridge (63). This temperature apparently does not affect succinic dehydrogenase activity.

(12) Indole

The production of indole, a biochemical reaction dependent on an enzyme system, by E. histolytica was investigated.

For this assay, actively growing cells of the DKB strain in association with Cl. perfringens (a species unable to produce indole) were used. The Gnezda oxalic acid method and Kovac's method as described by Salle (67) were employed.

The procedure consisted of inoculating two tubes of Cleveland-Collier media with Cl. perfringens, two tubes with E. histolytica strain DKB-Cl. perfringens, and two tubes with strain DKB-Cl. perfringens plus Esch. coli. Strips of filter paper previously dipped in saturated oxalic acid solution and dried were then carefully placed in the necks of the tubes, avoiding contamination during the process. The tubes were then incubated at 37° C for 48 hours and the oxalic acid papers inspected for the presence of a pink

coloration that indicates production of indole.

In order to minimize the possibility that extremely small amounts of indole were produced that could not be detected by the Gnezda method, the tubes were centrifuged, the supernate separated from the sediment, and both tested for indole with Kovac's reagent. The appearance of a deep cherry-red color, when 5 ml of Kovac's reagent were introduced into the tube containing the material to be analyzed, was considered to be indicative of indole production.

The composition of Kovac's reagent is indicated below:

Para-dimethyl-amino-benzaldehyde	5 g
Butyl alcohol	75 ml
Hydrochloric acid (37%) c.p.	25 ml

CHAPTER IV

RESULTS

(1) Catalase

In no instance was the evolution of oxygen from
hydrogen peroxide observed when this substrate was incu-
bated with either whole or lysed cells of strain DKB,
Entamoeba histolytica. Clostridium perfringens, an obli-
gate anaerobe known not to possess catalase, was unable to
decompose the peroxide molecule. On the other hand, as
little as 7 gamma of purified catalase and as few as 300,000
cells of Escherichia coli produced detectable amounts of
oxygen from the substrate. When the assay vessels were
incubated at 4° C there was no spontaneous breakdown of the
hydrogen peroxide solution. Results are recorded in
Table III.

(2) Gelatinase

All six strains of E. histolytica hydrolyzed gelatin.
The mixed bacterial flora of all six strains were also
capable of splitting this protein. However, the supernatant
from the final washing did not exhibit gelatinolytic activi-
ty, indicating that the observed gelatinase was of amoebic
origin. The lytic activity of the supernatants progressive-
ly decreased and finally ceased after the third washing

TABLE III

RESULTS OF CATALASE ASSAY

Preparation	Oxygen Evolved (ml)	
	Series 1	Series 2
Amoebae (500,000/ml)	0	0
Amoebae, lysed (500,000/ml)	0	0
Cl. perfringens	0	0
Esch. coli (1.5×10^7 cells/ml)	0.40	0.50
Esch. coli (3×10^5 cells/ml)	trace	trace
Catalase (70 gamma)	2.80	2.60
Catalase (7 gamma)	0.2	0.25
Endogenous (no enzyme)	0	0

procedure. The amoebae were also able to hydrolyze gelatin when grown in a monoassociated culture with Esch. coli, a species known not to hydrolyze gelatin. The lytic activity of the amoebae was completely inhibited by auto-claving and copper sulfate and partially inhibited by freezing and thawing. The degree of hydrolysis of gelatin decreased as the amoeba preparations were diluted. A 1/16 dilution exhibited slight activity and a dilution of 1/32 had no activity. Amoebic gelatinase activity was not affected by pH changes from a pH of 6.5 to pH of 8. The activity was reduced at pH values of 6.0 and lower. Studies were not made at pH values of above 8 because of the de-gradation of gelatin at these values. Because of the limi-tations of the method of determining amoebic numbers, no comparison of activity between strains was attempted.

The results of the gelatinase assay (microtest) utilizing all strains is tabulated in Table IV. Figure 2 illustrates the areas of hydrolysis on the gelatin-coated slides. Table V records the results of the effects of various pH values on the amoebic gelatinase.

(3) Lecithinase

There was no lecithinase activity in the amoebae (HUS-105) even though as many as 950,000 cells/ml were used. Cell suspensions of Cl. perfringens and Bacillus subtilis produced large zones of flocculant, white

TABLE IV

GELATINASE ACTIVITY OF E. HISTOLYTICA

Preparation		Strains					
	HUS-105	HUS-100	Washington	Conrad	ROK	Griffith
Supernatant (2nd wash)	≠	≠	≠	≠	≠	≠
Supernatant (3rd wash)	≠	-	-	-	-	-
Supernatant (4th wash)	-	-	-	-	-	-
Amoebae (washed 5x)	≠	≠	≠	≠	≠	≠

≠ indicates clear areas on the slide

- indicates no clear areas on the slide

38
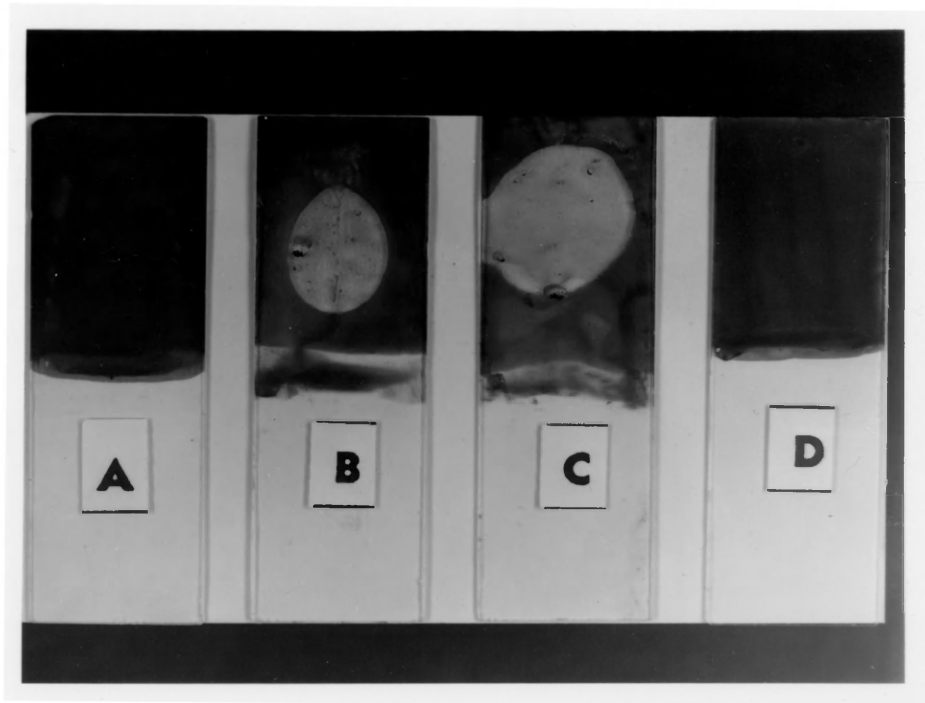
FIGURE 2

Gelatinase Microtest

(A) absence of gelatin hydrolysis by the super-
     nate fluid from final wash;

(B) gelatin hydrolysis by E. histolytica;

(C) gelatin hydrolysis by trypsin;

(D) absence of gelatin hydrolysis by Ringer's
     solution.

TABLE V

EFFECT OF pH ON THE GELATINASE ACTIVITY OF

E. HISTOLYTICA (HUS-105)

| pH | Degrees of gelatin hydrolysis | |
	Series 1	Series 2
5.0	+*	+
6.0	++	+
6.5	++++	++++
7.0	++++	+++
8.0	++++	++++

*   + indicates very slight area of hydrolysis
    ++ indicates slight area of hydrolysis
    +++ indicates good area of hydrolysis
    ++++ indicates maximum area of hydrolysis observed

precipitate, indicating strong lecithinase activity. The associated flora of the amoebae also produced lecithinase, but the activity was lost after the third washing procedure. A typical experiment is tabulated in Table VI.

(4) Desoxyribonuclease and ribonuclease

All six strains of E. histolytica surveyed possessed both ribonuclease and desoxyribonuclease. The associated flora of all strains also possessed the ability to depolymerize both substrates, but the washing procedures decreased the numbers of bacterial cells to such a level that hydrolysis of RNA or DNA was not observed after the third washing. Positive reactions were obtained with the purified nucleases; no hydrolysis or dissolution of the substrates occurred with sterile Ringer's solution. A plate from a typical experiment is shown in Figure 3; results are presented in Table VII.

(5) Casease

The associated bacterial flora from the two strains of E. histolytica utilized in this assay were not capable of hydrolyzing casein. Both strains of the amoebae hydrolyzed casein. Large clear areas of hydrolysis were produced on the plates by the amoebae. The supernatant fluid from all of the washings did not possess casease activity.

The amoebic casease was destroyed by heating at 65° C

TABLE VI

RESULTS OF LECITHINASE ASSAY

Preparation	Enzyme Activity	
	Series 1	Series 2
Supernatant from 2nd washing	++*	+
Supernatant from 3rd washing	-	-
Amoebae after 5th washing (950,000/ml)	-	-
Bacillus subtilis	+++++	++++
Clostridium perfringens	+++	++++

    * - indicates no detectable precipitate
      + indicates slight precipitate formation
      ++ indicates fair precipitate formation
      +++ indicates good precipitate formation
      ++++ indicates extensive precipitate formation
      +++++ indicates maximum precipitate formation
          observed

TABLE VII

NUCLEASE ACTIVITY OF E. HISTOLYTICA

Preparation	Strain											
	HUS-105 Hydrolysis of		HUS-100 Hydrolysis of		Washington Hydrolysis of		ROK Hydrolysis of		Griffith Hydrolysis of		Conrad Hydrolysis of	
	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA
Supernatant (2nd wash)	≠ *	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
Supernatant (3rd wash)	≠	≠	-	-	-	-	≠	-	-	-	-	-
Supernatant (4th wash)	-	-	-	-	-	-	-	-	-	-	-	-
Amoebae (5th wash)	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠

* ≠ indicates formation of a clear area

- indicates no detectable clear area formed
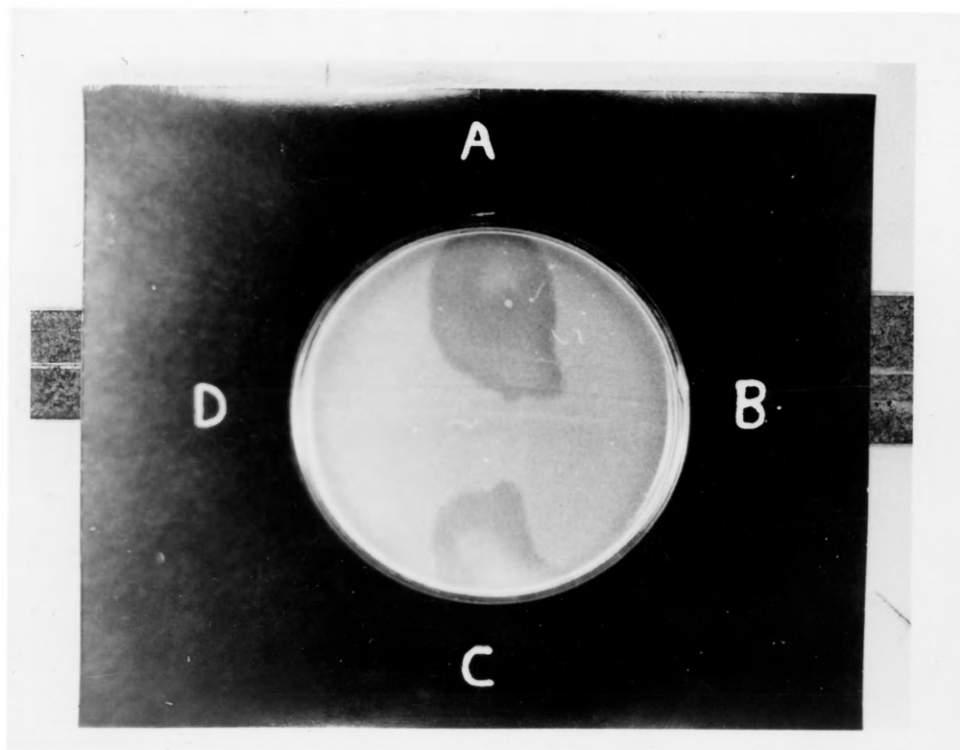
FIGURE 3

Nucleic Acid Test Plate

(A)   hydrolysis of ribonucleic acid (RNA) by
        ribonuclease;

(B)   absence of RNA hydrolysis by Ringer's solu-
        tion;

(C)   hydrolysis of RNA by washed E. histolytica
        in suspension;

(D)   absence of RNA hydrolysis by the supernatant
        fluid from final amoeba washing preparation.

for 5 minutes; the activity was not affected by heating the preparations for 5 minutes at 55° C. The experiments on the effects of pH on the amoebic casease showed that the enzyme was active over the whole pH range assayed (pH 5 to pH 8). It was not possible to quantitate any difference in hydrolytic activity over the pH range studied; all that could be determined in this case was that casein hydrolysis did occur at every pH value assayed over the mentioned range.

(6) Urease

It was not possible to detect urease activity in whole or lysed amoeba preparations. Although the preparations were incubated for as long as 12 hours, no detectable amounts of ammonia were produced from the action of the amoebae on urea solutions. The bacterial associates, however, produced increases in the optical densities of the reagent solutions in 15 minutes. Direct nesslerization of the amoeba reaction tubes did not result in the production of any detectable color. This confirmed the observation that E. histolytica does not produce urease.

(7) Citrullinase

The amoebae tested did not produce ammonia from citrulline. It was not possible to obtain increases in the optical density of the reagent solutions. Direct

TABLE VIII

SENSITIVITY OF THE UREASE METHOD USING

PURIFIED ENZYME PREPARATIONS

Urease Concentration	Optical Density	
	Series 1	Series 2
0.01 g/ml + urea	2.0	2.0
0.001 g/ml + urea	0.21	0.20
0.0001 g/ml + urea	0.025	0.035
0.01 g/ml (no substrate)	0.01	0.01

TABLE IX

RESULTS OF THE ASSAY FOR UREASE

IN E. HISTOLYTICA

Cell Preparations (containing urea as substrate)	Incubation Period	Optical Density	
		Series 1	Series 2
Amoebae (300,000/ml)	15 minutes	0	0
Amoebae (300,000/ml)	1 hour	0	0
Amoebae (300,000/ml)	12 hours	0	0
Amoebae (lysed)	15 minutes	0	0
Amoebae (lysed)	1 hour	0	0
Amoebae (lysed)	12 hours	0	0
Amoebae (no substrate)	1 hour	0	0
Cl. perfringens (2nd wash)	15 minutes	0.015	
Cl. perfringens (2nd wash)	1 hour	0.020	
Cl. perfringens (2nd wash)	12 hours	0.020	0.025

nesslerization of the reaction tubes indicated a lack of
citrulline breakdown.

The associated bacteria, Cl. perfringens, produced
citrullinase. Changes in the optical density of the reagent
solutions were observed after 30 minutes of incubation.
Large amounts of ammonia were produced from citrulline by
these bacteria after 12 hours.

The results of the citrullinase assay appear in
Table X.

(8) Asparaginase

There was an increase in the optical density of
the reagent solution when the amoebae were incubated with
dl-asparagine indicating that ammonia was being produced.
This is evidence for the production of asparaginase by
E. histolytica.

Cl. perfringens, the associated bacterium, also
attacked asparagine, but there was greater ammonia produc-
tion by the washed amoeba preparations than by the bacteria.
The ability of the amoeba preparations to produce ammonia
from asparagine was completely lost by boiling for 5
minutes. A 1/2 dilution of the amoeba suspension also
produced less ammonia from the substrate. The results
of this assay are presented in Table XI.

TABLE X

RESULTS OF THE CITRULLINASE ASSAY

Cell Preparation (containing citrulline as substrate)	Incubation Period	Optical Density	
		Series 1	Series 2
Amoebae (370,000/ml)	30 minutes	O	O
Amoebae (370,000/ml)	12 hours	O	O
Amoebae (cytolyzed)	30 minutes	O	O
Amoebae (cytolyzed)	12 hours	O	O
Cl. perfringens (2nd wash)	30 minutes	0.05	0.05
Cl. perfringens (2nd wash)	12 hours	0.12	0.10

TABLE XI

RESULTS OF THE ASPARAGINASE ASSAY

Cell Preparation (containing asparagine as substrate)	Incubation Period	Optical Density	
		Series 1	Series 2
Amoebae (200,000/ml) + substrate	15 minutes	0.12	0.18
Amoebae (lysed) + substrate	15 minutes	0.08	
Amoebae (boiled) + substrate	15 minutes	0	
Amoebae ($\frac{1}{2}$ dilution) + substrate	15 minutes	0.10	0.07
Amoebae no substrate	15 minutes	0	
Cl. perfringens (2nd wash) + substrate	15 minutes	0.045	0.025

(9) Glucuronidase

The results of the assay for a glucuronidase-produc-
ing bacteria are tabulated in Table XII. The results of
the tests on the sensitivity of the method are shown in
Table XIII and Figure 4.

Neither whole cells nor lysed amoebae were capable
of hydrolyzing the substrate at pH values of 4.0, 5.0 and
5.6; free phenolphthalein was not released during the incu-
bation periods employed. There was some activity exhibited
by Cl. perfringens. The results of the glucuronidase assay
are tabulated in Table XIV.

(10) Beta-D-Galactosidase

The data on the determination of the sensitivity of
the method are shown in Table XV and Figure 5.

The production of galactosidase by the amoebae could
not be detected using this sensitive method. There was no
change in the optical density of the incubation tubes over
the reagent blanks even after 12 hours of incubation. The
data are tabulated in Table XVI.

(11) Succinic dehydrogenase

Washed preparations of amoebae rapidly decolorized
methylene blue in the presence of the substrate, succinic
acid, at 57° C. The endogenous dehydrogenating ability of
the amoebae was almost completely inhibited at this

TABLE XII

A SURVEY OF BACTERIA FOR GLUCURONIDASE ACTIVITY

Organism	Comparative Activity	
	Series 1	Series 2
Streptococcus liquefaciens	-*	-
Streptococcus pyogenes	-	-
Staphylococcus pyogenes var. aureus	-	-
Escherichia coli	+	+
An unidentified Gram positive streptococcus	+++	+++

* - indicates no detectable color produced
+ indicates slight pink color produced
+++ indicates deep red color produced

TABLE XIII

SENSITIVITY OF THE GLUCURONIDASE METHOD

USING AN UNIDENTIFIED GRAM POSITIVE STREPTOCOCCUS

Number of cells/ml	Optical Density	
	Series 1	Series 2
2×10^8	0.27	0.29
1×10^8	0.14	0.15
5×10^7	0.06	0.08
3.3×10^7	0.05	0.06
2.5×10^7	0.03	0.03
2×10^7	0	0
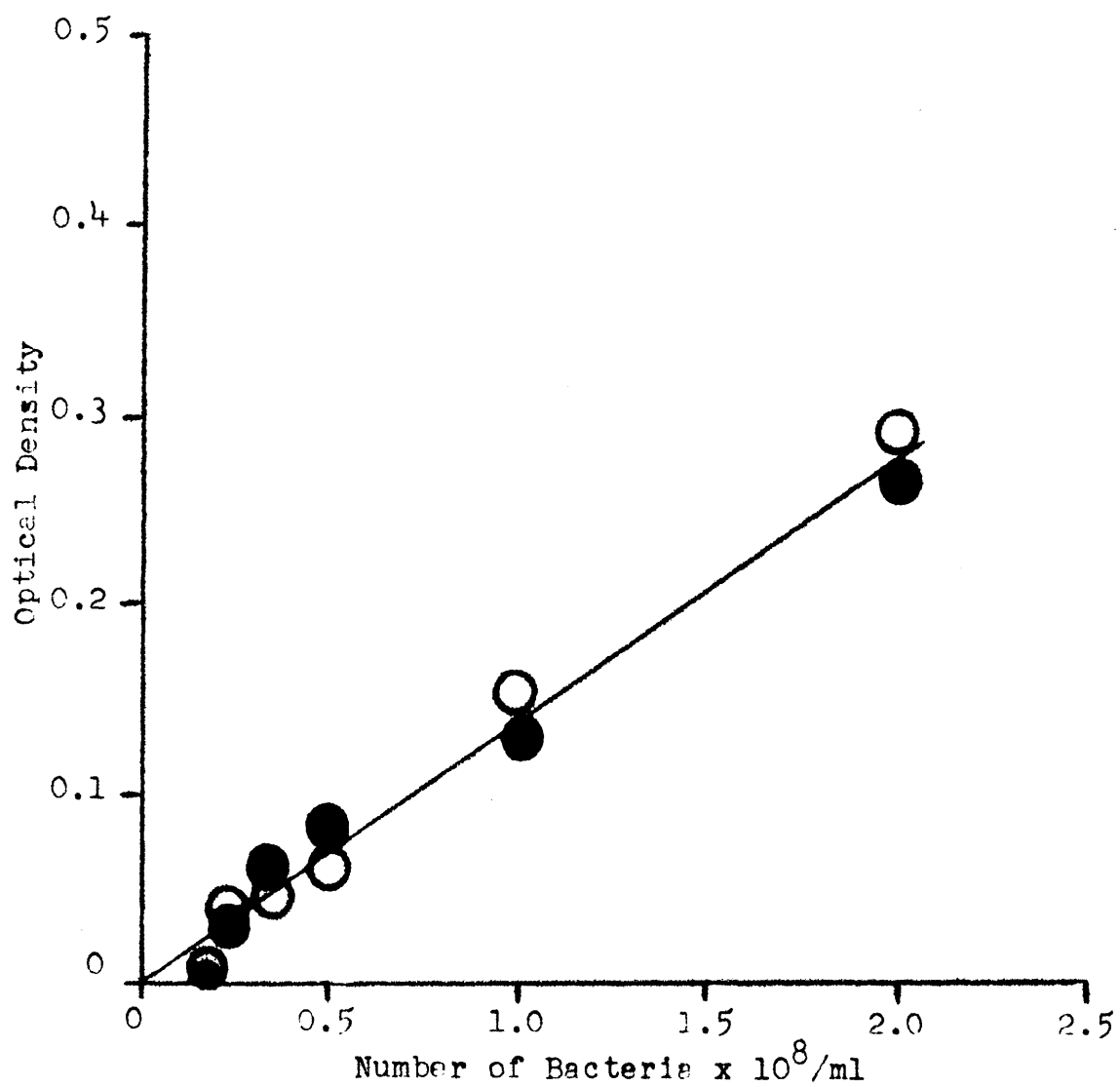
FIGURE 4

Sensitivity of the Glucuronidase Method

○ assay 1
● assay 2

TABLE XIV

ASSAY FOR GLUCURONIDASE IN E. HISTOLYTICA

Cell Preparation (containing phenolph- thalein glucuronidate as substrate)	Incubation Period	Optical Density	
		Series 1	Series 2
Amoebae (325,000/ml)	1 hour	0	0
Amoebae (325,000/ml)	12 hours	0	0
Amoebae (lysed)	1 hour	0	0
Amoebae (lysed)	12 hours	0	0
Cl. perfringens	1 hour	0.01	0.01
Cl. perfringens	12 hours	0.025	0.02

TABLE XV

SENSITIVITY OF THE GALACTOSIDASE METHOD

USING ESCHERICHIA COLI AS THE TEST ORGANISM

Number of cells/ml	Optical Density	
	Series 1	Series 2
5.4×10^8	0.32	0.28
2.7×10^8	0.15	0.17
1.35×10^8	0.06	0.08
9×10^7	0.05	0.07
6.75×10^7	0.04	0.04
5.4×10^7	0.02	0.02
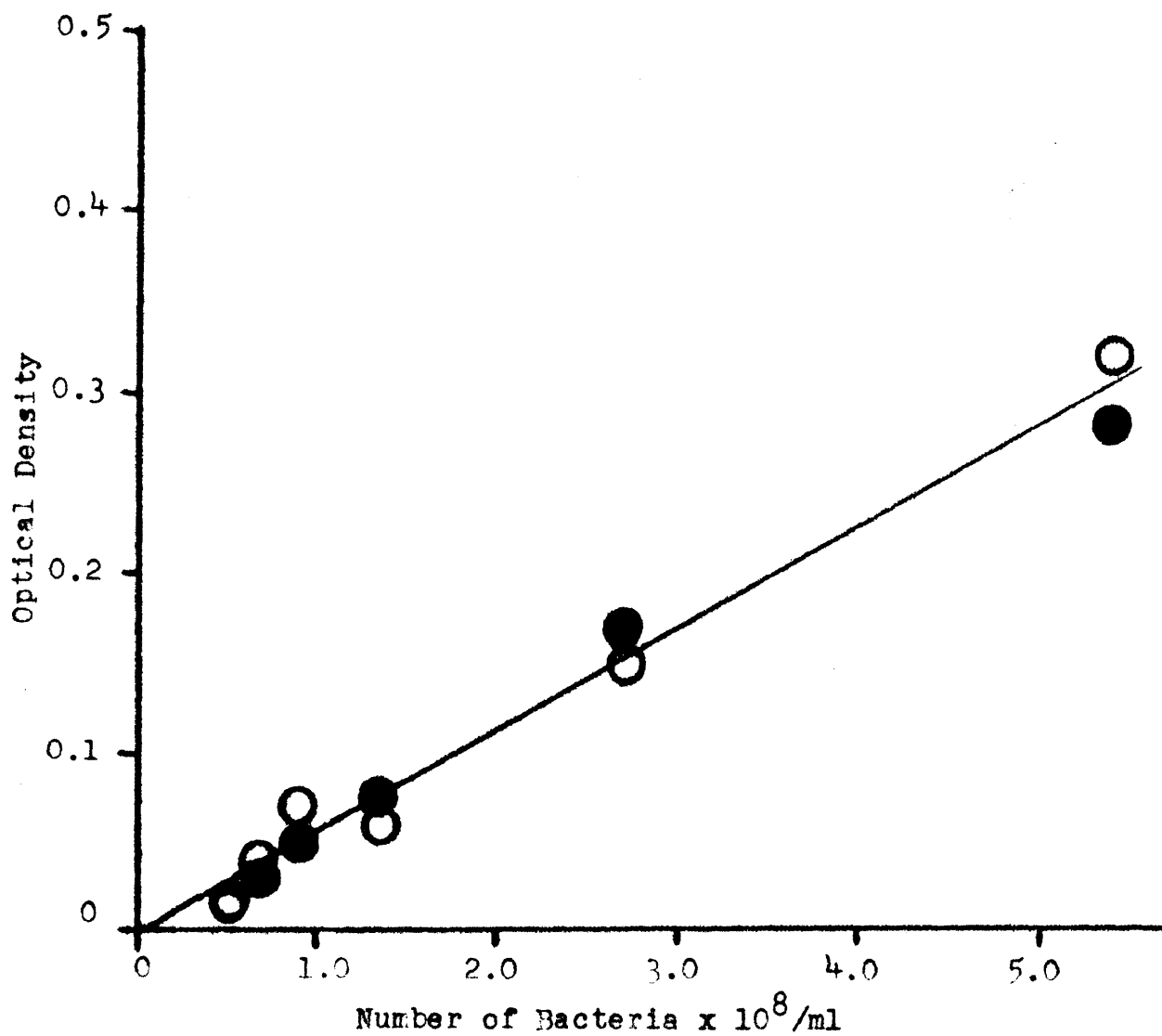
FIGURE 5

Sensitivity of the Galactosidase Method

○ assay 1.

● assay 2.

TABLE XVI

RESULTS OF THE GALACTOSIDASE ASSAY

Cell Preparation (containing O-nitrophenyl beta D galactoside as substrate)	Incubation Period	Optical Density	
		Series 1	Series 2
Amoebae (400,000/ml)	30 minutes	0	0
Amoebae (400,000/ml)	12 hours	0	0
Amoebae (lysed)	30 minutes	0	0
Amoebae (lysed)	12 hours	0	0
Cl. perfringens (2nd wash)	30 minutes	0.02	0.01
Cl. perfringens (2nd wash)	12 hours	0.06	0.06

temperature. After the second washing procedure, the super-
natant fluid, still containing residual bacteria, exhibited
no decolorizing activity. The succinic dehydrogenase of
E. histolytica was completely inactivated by boiling for
five minutes. Both arsono-acetate and malonic acid were
shown to be powerful inhibitors of the amoebic enzyme. A
typical experiment is tabulated in Table XVII.

(12) Indole

The production of indole by the amoebae plus Cl.
perfringens or by Cl. perfringens alone was not detectable
under the conditions of these experiments. Tubes containing
metabolizing cells of Esch. coli consistantly produced a
positive reaction. In all cases the results of the Gnezda
paper were confirmed by Kovac's test. The Gnezda oxalic
acid paper turned pink within the first 24 hours of incu-
bation when E. histolytica, in association with Esch. coli,
was the test organism. All tubes were inspected for amoe-
bic growth before results were recorded. If amoebic growth
was poor or absent, the tubes were discarded.

TABLE XVII

RESULTS OF THE SUCCINIC DEHYDROGENASE ASSAY

Enzyme Preparation	Substrate	Time for 90% Reduction Series 1	Series 2
Supernate (2nd wash)	2 ml M/50 succinic acid	in excess of 90 min.	in excess of 90 min.
Amoebae (300,000/ml)	2 ml M/50 succinic acid	25 min.	15 min.
Amoebae (300,000/ml)	2 ml saline	80 min.	in excess of 90 min.
Amoebae (boiled)	2 ml M/50 succinic acid	no reduction	no reduction
Amoebae (300,000/ml)	1 ml M/50 succinic acid 1 ml M/50 arsono acetate	in excess of 90 min.	in excess of 90 min.
Amoebae (300,000/ml)	1 ml M/50 succinic acid 1 ml M/50 malonic acid	80 min.	65 min.
Amoebae (300,000/ml)	1 ml M/50 succinic acid 1 ml saline	30 min.	40 min.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Two important factors involved in the enzyme assays
performed in this investigation were the efficiency of the
washing procedure and the numbers of amoebae utilized in
each experiment. The washing procedures were efficient as
indicated by the fact that plating of the fifth-wash sedi-
ments or supernatant fluids showed a great reduction in
numbers of bacteria as compared with the initial popula-
tion. The fact that it was not possible to eliminate com-
pletely all of the bacteria, however, does allow some
criticism of the work. Since adequate bacterial controls
were utilized, there is little doubt that positive reac-
tions for any given enzyme detected were due to amoebic
activity. Since a few bacteria were present, it was not
possible to eliminate completely the possibility of the
existence of synergistic actions. Furthermore, it was not
possible to rule out the remote possibility that the elim-
ination of so many of the associated bacterial cells re-
sulted in an alteration of the metabolism of Entamoeba
histolytica. Perhaps the amoebae function in a different
biochemical manner in the absence of supporting associated
cells. These are problems not answered by this research.
However, it is felt that in view of the meager knowledge

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available concerning the metabolism of E. histolytica, this investigation, and others in related areas, might offer clues which may lead to a better understanding of the biochemical reactions of this protozoan parasite.

The second factor, the numbers of amoebae utilized in the assays, becomes important in the experiments where enzyme activity could not be detected in the amoebae. The inability to detect activity might have been due to the comparative paucity of cells used. Most bacterial assays are carried out with many millions of cells, not a few hundred thousand as was the case in this work with E. histolytica. Theoretical calculations indicate that a little more than a million trophozoites would have a dry weight of one milligram (26). Since at least 200,000 amoebae were used, and the method of assay was highly sensitive, it is probable that sufficient quantities of cells were employed.

Thenard (73) first observed that certain tissues of plant and animal origin were capable of decomposing hydrogen peroxide. Later workers characterized the degradation as enzymatic, and Loew, in 1901, named this enzyme catalase (73). Catalase, an iron-containing enzyme, is concerned with the removal or utilization of hydrogen peroxide produced by biological oxidation reactions of the cell. It catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen. The enzyme can be found in all

aerobic and most facultatively anaerobic microorganisms, but has never been found in obligate anaerobes (62, 66, 74).

The question of anaerobiosis as a requirement for the growth of E. histolytica has received much attention (5, 50), but many conflicting opinions have been reported (50). As recently as 1958, Elsdon-Dew (20) posed the question as to whether or not E. histolytica is a strict anaerobe.

The results of this work indicate that the amoebae do not produce catalase (in amounts detectable by the methods utilized). As an indication of the sensitivity of the catalase detection methods, it has been calculated that a single molecule of catalase can catalyze the degradation of more than five million molecules of hydrogen peroxide per minute (61). The observation that the DKB strain of E. histolytica did not produce catalase supports the view that these amoebae are probably obligate anaerobes.

The existence of proteolytic enzymes in E. histolytica have been previously reported (2, 47, 64), but until the paper by Neal in 1956 (57) and the work of Harinasuta and Maegraith (35) in 1958, some doubt existed as to whether the previously observed proteolysis was due to amoebic or bacterial enzymes. Attempts were made to determine gelatinase activity in the amoebae using different assay methods and also to see if casease is produced by this organism. It was found that E. histolytica elaborated gelatinase and

casease. These observations confirmed the reports of Neal
and of Harinasuta and Maegraith. Although proteolysis by
the amoebae has been demonstrated, with our present know-
ledge about the organism it cannot be incriminated as a
mechanism of pathogenicity. However, as Meleney (48) points
out, research into the proteolytic enzymes of E. histolytica
may lead to the identification of the pathogenic component
of this organism.

Very little is known concerning the nucleic acid
metabolism of the amoebae. Pan and Geiman (59) recently
studied the distribution of ribonucleic acid and desoxy-
ribonucleic acid in this organism using cytochemical meth-
ods. Studies with antimetabolites have indicated the impor-
tance of nucleic acid metabolism in E. histolytica (54).
Inhibition of the amoebae by purine and pyrimidine analogs
and the subsequent reversal of the inhibitory effects by the
metabolites have established the roles of nucleic acid pre-
cursors as nutritional requirements for the amoebae.

The present work has shown that E. histolytica
produced both ribonuclease and desoxyribonuclease. These
enzymes split nucleic acids into oligonucleotides; pos-
sibly the amoebae possess enzymes for completing the degrad-
ation to the respective bases, pentoses, and phosphates.
Additional work will be necessary to determine this. Since
rapidly growing parasites synthesize large amounts of
nucleic acid (12), it can be imagined that nucleases capable

of degrading host nucleic acids to compounds readily utilizable by the parasite might be an important aspect of the mechanism of tissue invasion by the amoebae. Perhaps, as in the case of the bacterial enzyme, streptodornase, the nucleases of the amoebae are involved in pathogenicity.

The degradation of lecithin is accomplished by some snake venoms and toxins. The hemolysis of red blood cells by some bacterial toxins, such as the Cl. perfringens "theta toxin," is usually attributed to the action of lecithinase on the phospholipid of the erythrocyte stroma. Since E. histolytica engulfs and lyses red cells, it was thought that a study of lecithin hydrolysis by the amoebae would be interesting. E. histolytica, using this sensitive method (71, 77), was unable to hydrolyze lecithovitellin. It is possible that the dissolution of red blood cells by the amoebae is due to proteolysis and not lipolysis.

Since urease and citrullinase were not produced by the amoebae, the Krebs-Henseleit cycle probably does not operate in E. histolytica (58, 69). The preparations of E. histolytica utilized in this assay did not produce detectable amounts of ammonia from either substrate. The method utilized for the determination of ammonia-nitrogen is considered very sensitive. Increases in optical densities are reported to occur with less than ten micrograms of ammonia-nitrogen (36). Therefore the evidence is strong

that urease and citrullinase were not produced by the strain of the amoebae used in these experiments.

Glutamine, the amide of glutamic acid, and asparagine, the amide of aspartic acid, are generally considered to be readily available sources of ammonia for the organism possessing glutaminase or asparaginase. These enzymes catalyze the reversible hydrolysis of the amides to release ammonia and the free amino acids (19). Glutaminase production by E. histolytica has been previously reported by Nakamura and Goldstein (56). The present study on asparagine indicates the presence of this deaminase in the amoebae as well. Since approximately 70% of the dry protoplasmic constituents of microbial cells are nitrogenous compounds of varying complexity (62), it is not surprising that the amoebae produce deaminases which are means for obtaining utilizable sources of nitrogen for synthetic processes. These results indicate that glutaminase and asparaginase, but not urease nor citrullinase, may function in this capacity in E. histolytica.

Dolkart and Halpern (18) in 1958, reported excellent growth of the amoebae in a medium consisting entirely of gastric mucin in phosphate buffer. Mucins are enzymatically degraded to hexosamines and uronic acids. Greenberg et al. (27) found that glucosamine was an irreplacable component of their medium for the amoebae. Loran et al. (45a) also reported that the main action of the associated

bacteria on glucose was to convert it to glucosamine for use by the amoebae. In view of these observations it was deemed of interest to learn if the amoebae were capable of splitting glucuronic acid. There has also been a report that beta-glucuronidase was able to hydrolyze hyaluronic acid (24). It was thought that a study of beta-glucuronidase in the amoebae might be valuable.

The aglucuronometric method utilized in this assay is extremely sensitive (24, 65) and quantitative measurements can be made with only a few micrograms of phenolphthalein. On the basis of the results obtained, and on the basis of extreme sensitivity of the assay method, the conclusion reached is that E. histolytica did not produce beta-glucuronidase under these conditions.

Some progress is now being made in the study of the carbohydrate metabolism of the amoebae (23, 38, 39), but it is obvious that a great deal of knowledge is yet to be accumulated before the diverse pathways and enzymatic reactions of E. histolytica can be elucidated. A report by Hilker et al. (38) on the absence of lactase in the amoebae was considered important because of the widespread distribution of this disaccharide and also because of previous reports on the breakdown of glucose and galactose (hydrolytic products of lactose) by the amoebae (42).

The extremely sensitive method of Lederberg (43) was used for the analysis of the lactase activity of the

amoebae. At one time it was thought that lactase and beta-galactosidase were entirely different enzymes, but it has been shown that lactose and lactose derivatives are hydrolyzed by beta-galactosidase (76).

The fact that the amoebae were unable to hydrolyze the glycoside, o-nitrophenyl-beta-d-galactopyranoside, indicates that the amoebae did not produce lactase. The results of this work add support to the previous findings that E. histolytica did not possess a lactase.

In 1909, Thunberg, and later, Batelli and Stern, showed that the addition of succinic acid to minced animal tissues increased the uptake of oxygen (73). The mediator of this reaction was an enzyme, succinic dehydrogenase. This enzyme oxidizes succinic acid to fumaric acid by the removal of two hydrogen atoms. In aerobic microorganisms, the hydrogens are passed on to a cytochrome system (which act as hydrogen acceptors) until finally the hydrogens are transferred to oxygen, producing water. Oxygen is not the final hydrogen acceptor in anaerobic organisms. Instead of oxygen, various organic molecules and even portions of the substrate molecule can be utilized as the ultimate hydrogen acceptor (7, 15).

In the Thunberg technique, an aqueous solution of methylene blue is employed as the hydrogen acceptor. Methylene blue is a complex heterocyclic compound that is blue in its oxidized form and colorless in the reduced form

(leuco methylene blue). Thus, the dye is useful as an oxidation-reduction indicator for many biological systems.

Succinic dehydrogenase production by E. histolytica has been previously reported (68), but it seemed that the materials assayed were of questionable purity and it was probable that bacterial dehydrogenase activity was being studied. Furthermore, no bacterial controls were included in this previous work. Therefore, repetition of this phase of the work with adequate controls seemed justified. The present data indicate that succinic dehydrogenase was definitely present in E. histolytica. Since the supernatant fluids (controls) did not have any activity, the conclusion that the decolorization observed was due to amoebic enzymes seemed valid. Malonic acid, a specific competitive inhibitor of succinic dehydrogenase, inhibited the reduction of methylene blue. This is good evidence that the reduction of the dye observed in the Thunberg tubes was due to the action of succinic dehydrogenase on succinate.

Indole is a putrefactive compound produced by the action of some bacteria on tryptophane. The conversion of tryptophane to indole involves a system of enzymes rather than one specific enzyme. E. histolytica did not produce indole in Cleveland-Collier medium. A positive reaction was obtained with Esch. coli in this medium indicating that there was sufficient tryptophane present in the medium to yield positive reactions.

CHAPTER VI

SUMMARY

1. Methods for obtaining _Entamoeba_ _histolytica_ relatively free of associated bacterial cells are described.

2. Procedures for controlling the activity of the residual bacteria in the enzyme assays are discussed.

3. Methods are described for the assay of catalase, gelatinase, ribonuclease, desoxyribonuclease, lecithinase, casease, urease, citrullinase, asparaginase, glucuronidase, beta-galactosidase and succinic dehydrogenase, and for the detection of indole production.

4. The present study has shown that _E._ _histolytica_ produced gelatinase, casease, ribonuclease, desoxyribonuclease, asparaginase and succinic dehydrogenase.

5. _E._ _histolytica_ did not possess catalase, lecithinase, urease, citrullinase, glucuronidase and beta-galactosidase activity.

6. _E._ _histolytica_ did not produce indole.

7. The significance of the findings is discussed.

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