Morphological and Life History Studies on *Entamoeba terrapinae* and its Comparative Morphology with *E. histolytica* and *E. invadens*.

by

Arlene R. McMorran

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A C K N O W L E D G E M E N T S

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To Dr. M.J. Miller, of the Institute of Parasitology, for isolating the cultures used in the present studies and for his helpful direction of my work, I express my appreciation.

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INTRODUCTION

The genus Entamoeba, Casagrandi and Barbagallo 1895, includes a number of species parasitic on a wide range of animals from ciliates to man. Many of these are morphologically similar but differ in growth requirements and hosts. Entamoeba histolytica, Schaudinn 1903, in man, and Entamoeba invadens, Rodhain 1934, in snakes, are the only two species known to invade host tissue.

In 1930 Sanders and Cleveland described a new species, Entamoeba terrapinae, from the terrapin, Chrysemys elegans. Since then no further work has been published on this amoeba, and much information regarding it is still wanting. According to Sanders and Cleveland the life-cycle of E. terrapinae differs from any other life history of a parasitic amoeba heretofore described. As their observations have not been confirmed it was deemed advisable to reinvestigate the life-cycle of this parasite.

A number of species of parasitic amoebae have been described in turtles by various workers. In many of these the specific identity, which was established on inadequate morphological study, is still in doubt. In some cases size alone was the characteristic used for differentiating a species. In the present investigation a detailed study of the morphology of Entamoeba terrapinae has been made. In an attempt to evaluate morphological characteristics and size as taxonomic characters a comparison of the morphology of Entamoeba terrapinae with that of two morphologically similar species, Entamoeba invadens and Entamoeba histolytica, all growing under identical conditions, is presented.

The results of this study are presented in two parts, as follows:

Part I. Morphological and life history studies on *E. terrapinae* from the host and from culture.

Part II. A comparison of the morphology of *E. terrapinae*, *E. invadens*, and *E. histolytica*.

HISTORICAL REVIEW

Entamoeba terrapinae, Sanders and Cleveland 1930, was described from the turtle, Chrysemys elegans. According to their description it is characterized by its small size (trophozoites 10-15 u, nuclei 2-4 u, cysts 8-14 u, average 10 u), a typical "histolytica" nucleus, and cysts "indistinguishable from a small race of E. histolytica." Their description of the life-cycle shows it to differ from that described for any other Entamoeba: mature cysts have four nuclei and excyst as a four-nucleate trophozoite; this divides into two binucleate trophozoites, each of which, by a cytoplasmic division, gives rise to two unicleate trophozoites.

A number of other Entamoeba species have been described from reptiles. As reported by Wenyon (1926) and Geiman and Ratcliffe (1936), a large amoeba, Entamoeba testudinis, was described by Hartmann in 1910 from the turtle, Testudo graeca. Hartmann found that trophozoites ranged from 50-70 u, with a nucleus of 11.5-12.5 u; no cysts were described. Of E. testudinis Geiman and Ratcliffe (1936) state that "the size and nuclear structure serve to differentiate this species from other reptilian Entamoebae."

Entamoeba barreti, Taliaferro and Holmes (1924), was cultivated in 1923 by Barret and Smith from the snapping turtle, Chelydra serpentina. Trophozoites from the intestine measured 14.5-22.4 u, average 18.7 u, and from culture 13.8-22.9 u, average 17.5 u, nucleus 5-6 u; no cysts were described. As discovered by Geiman and Ratcliffe (1936) this amoeba produces eight-nucleate cysts in mucin - saline - rice starch medium.

Entamoeba varani, Lavier 1928, was described from the snake, Varanus niloticus. No cysts were described. Trophozoites ranged from 12-25 u, nucleus 5 u, and the nuclear structure was stated to be identical with that of E. dysenteriae (i.e., E. histolytica). The amoebae were found in the posterior part of the intestine and had produced no pathological effects. Geiman and Ratcliffe (1936) considered that E. varani is probably synonymous with E. invadens but state that "in the absence of disease this amoeba may be a distinct species."

In 1930 Knowles and Das Gupta described an Entamoeba species from culture material obtained from the rectal contents of a Gangetic turtle, Trionyx gangeticus. The trophozoites ranged from 10-60 u, average 30.9 u, nuclei 5-6 u, cysts 7.5-20.7 u, average 11.7 u. Mature cysts had four nuclei and were strikingly similar to those of E. histolytica.

In 1934 Rodhain described Entamoeba invadens (trophozoites from liver of snake host 17-30.6 u, average 22 u; from culture 21-25.5 u, nuclei 3.5-5 u; cysts 12.75-18 u) and noted that it was closely related morphologically to E. dysenteriae. In 1936 Geiman and Ratcliffe studied the morphology and life-cycle of an amoeba pathogenic to snakes and called it E. invadens, although they were dissatisfied with Rodhain's evidence for the pathogenicity of his species. They found E. invadens to be so strikingly similar to E. histolytica both in morphology and life-cycle as to necessitate their distinction on purely physiological grounds. Ratcliffe and Geiman (1938) found that a high percentage of water-snakes died from experimental infections of E. invadens.

In 1935 Rodhain and Van Hoof succeeded in experimentally infecting three turtles, Testudo tabulato, with E. invadens. Eighty-three days after

infection they sacrificed one of the turtles and found numerous motile amoebae and cysts in a small lesion in the large intestine. A culture from the liver was positive. Three snakes, Tropidonotus natrix, were placed in a cage with the other two infected turtles, which showed amoebae in their faeces three and one-half and four months after infection. All three snakes acquired infections and died after seventeen, sixty-one, and seventy-eight days, but the turtles continued to live in a healthy state. The authors concluded that turtles could act as carriers for amoebae pathogenic to snakes.

Geiman in 1947 carried out cross-infection experiments with E. invadens, E. terrapinae, and E. barreti with the following results: E. invadens was infective but non-pathogenic for frogs and turtles; E. terrapinae and E. barreti were infective but non-pathogenic for snakes.

Entamoeba ranarum (Grassi 1879) Dobell 1908 has been found in the intestine of frogs and tadpoles. Taliaferro and Fisher (1926) studied this amoeba from cultures originally isolated from tadpoles by Barret and Smith and found that trophozoites ranged from 12.0-38.5 u, average 22.8 u, cysts 9.6-20-6 u, average 14.8 u. Many workers have noted a remarkable similarity between this amoeba and E. histolytica. Dobell (1918) tried to establish its synonymity with E. histolytica by infecting tadpoles with cysts of E. histolytica, but failed and concluded that they are probably two distinct species.

According to Wenyon (1926) spontaneous amoebic abscess of the liver in frogs was described by Ilowaisky in 1922. Dobell (1908) stated that when the amoebae were numerous in the large intestine, they readily ingested blood-corpuscles and epithelium cells. However, this amoeba is usually regarded as a harmless commensal.

Wenyon (1926) reproduced two figures, one by Collin (1913) showing a multinucleated trophozoite of E. ranarum, and one by Mercier and Mathis (1918) showing a multinucleated encysted form. These forms, which had from sixteen to thirty nuclei, were described as schizonts. The evidence that schizogony does occur has never been accepted. In 1931 Cleveland and Sanders worked out the life history of E. ranarum and showed it to be identical with that of E. histolytica as described by Dobell in 1928.

Endamoeba insolita, Geiman and Wichterman 1937, was described from Testudo elephantina and two Testudo vicina. Stained trophozoites from faeces ranged from 12.8-33 u, average 21.1 u, in length, and 12-23 u, average 16.8 u, in width, nuclei 3.6-7 u, average 5.2 u. Cysts ranged from 12.8-19.5 u, average 15.7 u, and when mature had four nuclei. This is the first record of cysts from a turtle host. The distinctive characters of this amoeba were its eccentric karyosome and peculiar cyst formation. At the time of encystation "a thick irregular gelatinous, brownish, membranous matrix begins to envelope the organism."

Carini in 1944 described two new species, Entamoeba testudinea and Entamoeba jaboti, from the turtle, Testudo tabulata. I was unable to obtain his original paper, and the only data I have been able to find on these two species is from Carini's table showing the distinctive characters of the various Entamoeba species from turtles. This table was reproduced by Rodhain and Van Hoof in 1947 when they described Entamoeba knowlesi, new species. Trophozoites of E. testudinea ranged from 50-120 u, nucleus 10 u. Cysts averaged 25 u and had two to four nuclei. Trophozoites of E. jaboti ranged from 20-30 u, nucleus 6 u. Cysts had two nuclei and measured 16-18 u.

Entamoeba knowlesi, Rodhain and Van Hoof 1947, was described from

Terrapina cinosternoides and Platysternum megacephalum. The trophozoites, which resembled E. invadens, ranged from 20.5-29.5 u. The nucleus had an average diameter of 4.8 u and showed a small central karyosome. Cysts ranged from 7.2-13.8 u, average 10.78 u, and when mature had four nuclei. The authors considered their species very closely related to, if not synonymous with, the Entamoeba species of Knowles and Das Gupta.

MATERIALS AND METHODS

The three species of Entamoeba used in these studies were isolated at the Institute of Parasitology, Macdonald College, Quebec. E. histolytica was isolated from the stool of a human carrier in Montreal, Quebec, and had been cultivated for one year at a temperature of 30°C by Dr. M.J. Miller. E. invadens was isolated from a water-snake, Natrix toxospilota, obtained from Florida; E. terrapinae was isolated from a turtle, Chrysemys elegans, obtained from Lake Ontario.

The amoebae were maintained in Boeck and Drbohlav's culture medium consisting of whole egg slants with approximately ten milliliters of overlay to which a small amount of dry rice starch was added. Two types of overlay were used: five per cent human serum in Ringer's solution and Locke's solution.

The turtles used were Chrysemys elegans and Chrysemys picta obtained from Lake Ontario. The small turtles, Chrysemys elegans (length of shell 1 1/2 - 2 inches), were kept in glass aquarium tanks (12 inches x 8 inches x 8 inches) with sand, water, green reeds and snails. The larger turtles, Chrysemys elegans and Chrysemys picta (length of shell approximately five inches), were kept in a large sloping tank partly filled with water.

The snakes used for experimental infections were garter snakes, Tropidonotus, water-snakes Natrix, and whip snakes, Coluber. The garter snakes were caught in the vicinity of Ste. Anne de Bellevue, Quebec, and were fed earthworms. The water and whip snakes were obtained from Florida and were force fed ground-up beef heart. The snakes also were kept in glass aquarium tanks.

Snakes and turtles were infected orally, the snakes with both cysts and trophozoites, the turtles with cysts only, by means of a glass pipette. Faeces was obtained from the snakes by using gentle pressure on the ventral surface in the direction of the anus. Examinations for amoebae from the stomach, intestine, and liver were made by mixing the material in saline. In some cases the presence of amoebae had to be determined by inoculating some of the material into culture tubes.

The cultures of E. terrapinae on which the following studies were made consisted of:

(a) a mixed culture obtained from the large intestine of a turtle, Chrysemys elegans;

(b) a clone culture established from the above culture;

(c) a culture established with a single species of bacteria, Clostridium tertium, from the mixed culture (a).

Tissues were fixed in Zenker's fluid and stained with Mallory's ferric chloride, with and without Van Gieson's counterstain, Mallory's acid phosphotungstic haematoxylin, and Heidenhain's iron haematoxylin, with and without eosin as a counterstain. Permanent slide preparations from the host and from culture were fixed in Schaudinn's mercuric sublimate plus five per cent acetic acid, and were stained with Heidenhain's iron haematoxylin and Mallory's acid phosphotungstic haematoxylin. As it was found that amoebae from culture would not adhere to the slide unless the fluid was mixed with some adhesive substance, the following methods of obtaining culture preparations were used:

(1) the culture fluid was mixed with a mucus from the stomach of a hamster and spread out on a glass slide;

(2) Dobell's cover-slip method, in which sterile cover-slips are dropped into the culture tube and removed with the attached amoebae.

In studying excystation, the cysts were first freed of trophozoites and debris by means of the zinc sulphate flotation technique. To reduce the bacterial flora, a sterile technique was adopted throughout the procedure. The desired number of cysts were picked up with a micropipette and transferred to a slide containing a few drops of Ringer's serum with bacteria inside a ring of vaseline. A cover-slip was mounted and the ring was then completely sealed off with vaseline. It was later found that sterility was unnecessary and a drop of saline containing the cysts was placed directly on the slide. Large numbers of cysts for excystation studies were easily obtained by this method.

To determine the number of nuclei in a trophozoite or cyst at any given time, the following method for staining the fluid within the vaseline was devised by Dr. M.J. Miller: Two small openings are made in the vaseline at opposite ends of the cover-slip, so that the fluid communicates with the exterior. Two equal-sized pieces of blotting paper are placed one at each opening, but not in contact with the fluid. A drop of D'Antoni's iodine or of a one per cent solution of methyl green is added to one piece of blotting paper and the two pieces are simultaneously brought into contact with the fluid. This should be done slowly and with care if all the trophozoites and cysts are to remain in the same position. The staining requires about one minute and the whole process can be followed under the microscope. A 50x oil immersion was found most useful in studying these slides, as it showed up the nuclei clearly. In examining with a 97x oil immersion it was always necessary to flatten the cover-slip and most, if not all, the trophozoites

and cysts were lost.

For the studies on comparative morphology, the three species E. histolytica, E. invadens, and E. terrapinae, were each established in culture with a single species of bacteria, Clostridium tertium, by Dr. M.J. Miller. All three species were cultured at 30°C and at 35°C. The cultures were maintained by serial transfer every three days. To check for contamination of the monobacterial cultures with other species of bacteria, the cultures were stained with Gram's stain every three days. Doubtful cases were plated out on blood agar plates which were then incubated at 37°C both aerobically and anaerobically.

In making growth counts the liquid media was first thoroughly mixed by sucking in and blowing out fluid with a Pasteur pipette, to insure a uniform distribution of amoebae throughout the fluid media. A small quantity was then withdrawn and allowed to run beneath the cover-slip of a red blood cell counting chamber. The amoebae on both sides of two slides were counted, so that the number obtained represented the number of amoebae in 3.8 cubic millimetres of culture fluid. The growth curves were obtained by taking the average of the counts from four to eight different cultures. Cultures found to be contaminated at any stage of the counts were discarded and replaced by new ones.

All measurements were made on organisms from three-day old cultures. To enable removal of all three species from culture at the same time, only twenty-five of each species were measured at any one time. The measurements were therefore made from four different cultures. As all the cultures were not growing and encysting equally well, it was sometimes impossible to measure

twenty-five organisms, and the number of trophozoites or cysts measured for any one species therefore varied, depending on the media and the temperature. The amoebae were stained with D'Antoni's iodine solution.

Fixed and stained preparations were obtained by Dobell's cover-slip method with one modification. To eliminate unnecessary manipulation, and to cut down possible sources of contamination, the cover-slips were inserted into the culture tubes after they had been inspissated but before they were autoclaved. Three days after the amoebae were added to the cultures, the cover-slips were removed, fixed in Schaudinn's fluid plus five per cent acetic acid and stained with Heidenhain's iron haematoxylin. The same staining procedure was applied to each cover-slip.

Throughout this work care was taken to maintain exactly the same conditions for all three species.

Magnifications used were 100x, 430x, 500x, 970x, and 1,455x. All measurements were made at a magnification of 970x.

MORPHOLOGICAL and LIFE HISTORY STUDIES on ENTAMOEBA TERRAPINAE

I. GENERAL DESCRIPTION

A. Trophozoites

In fresh, active trophozoites, both from the host and from culture, the ectoplasm cannot be distinguished from the endoplasm. The trophozoites are very active, and when they are travelling rapidly in a straight line there is no observable formation of pseudopodia. Often a small piece of debris is seen clinging to the posterior end. The amoebae constantly change their direction by thrusting out a clear pseudopod into which the cytoplasm flows. When degenerating they remain in one position but send out clear pseudopodia in all directions.

The cytoplasm is granular and in fixed and stained specimens is seen to contain a number of vacuoles.

In culture the amoebae readily ingest bacteria and starch grains which are clearly visible in fresh preparation. Only rarely do trophozoites ingest cysts. Five trophozoites containing one cyst each, and one containing four cysts, have been seen.

In trophozoites examined fresh from culture or the host, the nucleus is invisible but becomes increasingly clear as the amoebae degenerate. In fixed and stained specimens the nucleus is usually spherical, but may be oval, pear-shaped, rectangular, or irregular in outline. Occasionally nuclei with a tail-like process are found, suggesting a recent division. The most typical nucleus, from the host and from culture has a small central karyosome surrounded by a clear halo and the nuclear membrane is lined with

fine evenly distributed chromatin granules (Figure 1). However the karyosome may contain from one to five distinct granules or may simply be elongated. When four or five granules are present the karyosome consists of a tiny centriole surrounded by a number of granules (Figure 2). When three granules are present they are either in a straight line or in the form of a triangle. Karyosomes, split into two granules, either in close contact or widely separated (Figure 4), are very common. Occasionally a thin line of chromatin connects the two dots (Figure 3). Around the karyosome is a clear zone outside of which is an achromatic ring. In forms from culture the space between the karyosome and the nuclear membrane is usually devoid of chromatin, but forms from the host frequently have, on the achromatic ring, a number of granules, from five to sixteen, which stain like chromatin (Figure 6). Occasionally "spoke radii" were observed running from the edge of the halo to the nuclear membrane. When these radii were present the area between the karyosome and the nuclear membrane was always free of chromatin.

In forms from the host the nuclear membrane is always uniformly beaded with a single layer of chromatin granules. This is usually true of forms from culture, but not infrequently the peripheral chromatin may be concentrated in a number of dots or bars. A fair number of nuclei have been found with all the peripheral chromatin contained in five large granules, spaced at regular intervals on the nuclear membrane. As all fixed and stained specimens were removed from culture on the third and fourth day after its inoculation, there is no reason to suppose that these forms were degenerate.

Two to five per cent of the trophozoites are binucleate, and in

these forms one nucleus is often larger than the other (Figure 6).

B. Cysts

The cysts are usually spherical but may be ovoid. In culture they adhere to one another often in such thick clumps that it is difficult to study any one cyst. When examined fresh in saline the cyst wall and the chromatoid bodies are seen as clear and highly refractile structures but the nuclei are invisible. The glycogen mass, incysts stained with D'Antoni's iodine, takes up a deep brown stain and is usually diffuse at its edges, but in fixed and stained specimens it appears as a clear vacuole with a very definite outline.

In young cysts the glycogen vacuole often fills the cyst, pressing the nucleus against the cyst wall. Very young cysts frequently have no chromatoid bodies, while others have many small ones arranged outside the glycogen vacuole. As the cyst matures the amount of glycogen decreases and the chromatoid bodies enlarge. Cysts stored in the refrigerator gradually lose their chromatoid bodies and it has been found that excystation never occurs until all of the chromatoid bodies have disappeared. The size, shape, and number of these bodies is of no diagnostic significance due to the great variation in all three of these characters. They may be rod-shaped, oval, rounded, or slender and filiform. The number has been found to vary from one to sixteen, and as the number increases, the size decreases. Cysts from experimental infections of snakes tend to have fewer and larger chromatoid bodies than cysts from culture.

Cysts have never been found in turtles. In experimental infections of snakes, most of the cysts have four nuclei. In culture fifty to eighty-five per cent of the cysts have only two nuclei, and it is unusual to

find a culture having more than twenty to thirty per cent of four-nucleate cysts.

The nuclei progressively decreases in size with further divisions. When only one nucleus is present it lies in close contact with the cyst wall and is usually elongated into a spindle shape (Figure 7). When two nuclei are present they may be spherical in shape or elongated with two widely separated karyosome granules (Figure 8). They do not always divide simultaneously as the trinucleate cyst is fairly common, one nucleus being much larger than the other two (Figure 11). In the quadrinucleate cyst the nuclei are commonly seen lying in pairs at opposite ends of the cyst (Figure 13), and frequently appear to be incompletely separated (Figure 12).

Occasionally supernucleate cysts, containing five or eight nuclei, are found. In the eight-nucleate cyst the nuclei are usually in pairs of two. In the five-nucleate cyst, two of the nuclei are small and spherical with a single karyosome granule; the other three are always larger and are usually in the process of dividing. Supernucleate cysts are usually larger than the normal mature cyst which contains a maximum of four nuclei, but there is no difference in the size of uninucleate, binucleate, trinucleate, and quadrinucleate cysts.

II. SIZE

The following measurements were made on fixed and stained specimens.

A. Trophozoites

The average diameter of 125 trophozoites from the large intestine of a turtle, Chrysemys elegans, was 14.40 u with a range in size of 9.88-20.52 u

and a standard deviation of 1.79. The average diameter of their nuclei was 4.63 u with a range of 3.19-6.38 u and a standard deviation of 2.04. The nucleo-cytoplasmic ratio was 0.32.

The average diameter of 41 trophozoites from the faeces of a garter snake was 20.32 u with a range of 12.76-30.52 u and a standard deviation of 1.21. The average diameter of their nuclei was 5.19 u with a range of 3.88-7.33 u and a standard deviation of 1.42. The nucleo-cytoplasmic ratio was 0.26.

The average diameter of 100 trophozoites from a culture at 30°C was 18.26 u with a range of 12.92-31.92 u. The average diameter of their nuclei was 5.34 u with a range of 3.34-6.99 u. The nucleo-cytoplasmic ratio was 0.29.

B. Cysts

The average diameter of 215 cysts from a clone culture of two months' duration was 9.69 u, with a range of 7.44-12.40 u, and a standard deviation of 1.60.

The average diameter of 114 cysts from the faeces of a garter snake was 10.36 u with a range of 7.55-15.10 u and a standard deviation of 1.53.

The average diameter of 215 cysts from the faeces of another garter snake was 10.63 u with a range of 8.21-16.87 u and a standard deviation of 2.31.

III. GROWTH REQUIREMENTS AND ENCYSTATION

Growth, with encystation and excystation, was obtained in cultures

at room temperature (20°-23°C), at 30°, and at 35°C.

In media without rice starch the amoebae grow poorly, do not encyst, and sometimes do not survive beyond a few days. Better encystation, but not better growth, was obtained in Locke's medium than in Ringer's serum.*

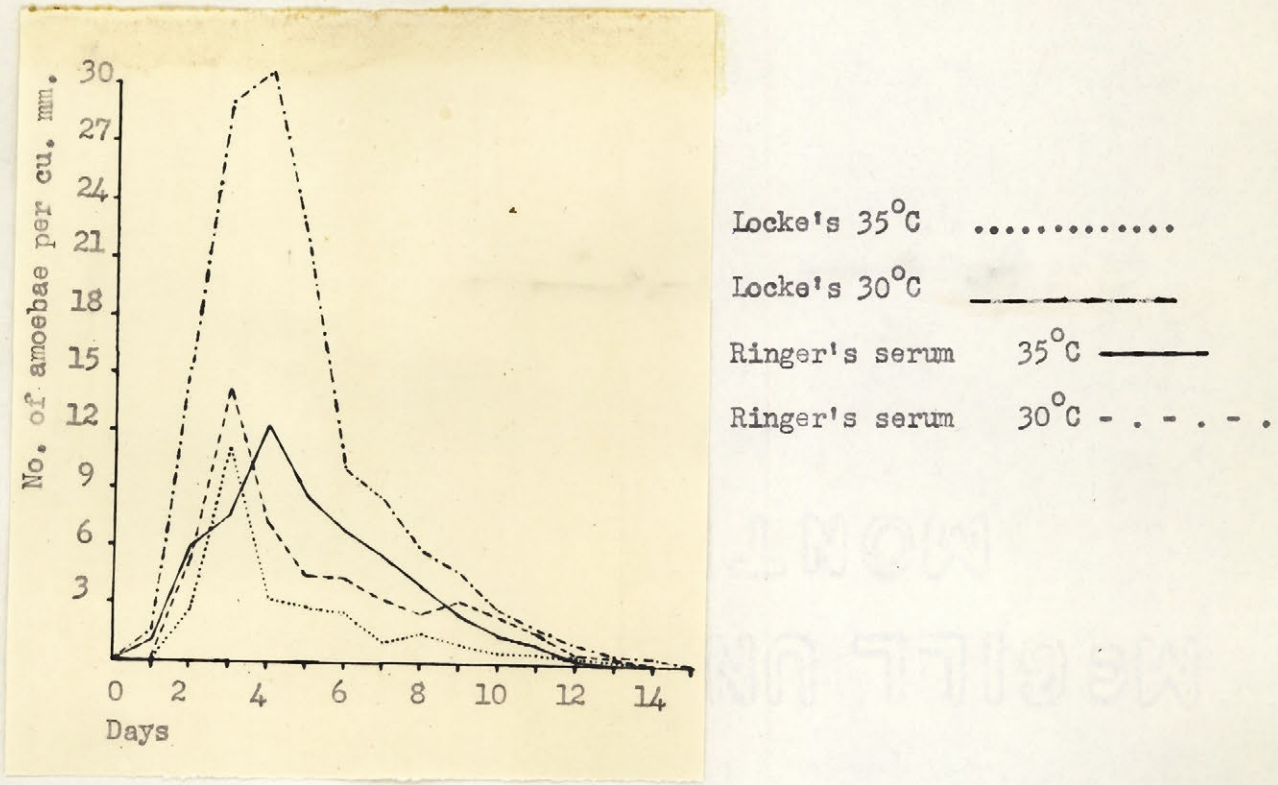
Cultures at room temperature showed good, even growth from the third to the twelfth day after inoculation and survived three weeks and sometimes longer. They did not consistently show a good yield of cysts, probably due to the variations in room temperature.

Cultures at 30°C always contained a large number of cysts on the third and fourth days after inoculation, the peak of cyst formation being on the fourth day. Occasionally good encystment was found two days after inoculation. Cultures at 35°C always showed a fair number of cysts three days after inoculation, but not to the extent found in cultures at 30°C.

Cultures at 30°C and at 35°C reached a growth peak on the third or fourth day after inoculation. Thereafter growth declined and the cultures did not survive beyond fourteen days.

Text Figure 1 gives a comparison of growth in Locke's solution and in Ringer's serum at 30°C and at 35°C. The amoebae were growing with a single species of bacteria, Clostridium tertium. The growth curves were obtained by the method described in the section on "Materials and Methods."

* All culture tubes contained whole egg slants. Locke's and Ringer's serum refer to the overlay used to cover the slants, and these terms will henceforth be used to distinguish the two types of media used.



Text Figure 1. Comparison of growth of E. terrapinae in Ringer's serum and in Locke's at 30°C and 35°C.

IV. STUDIES ON THE LIFE HISTORY

A. Trophozoites

Trophozoites in culture feed on starch grains and bacteria and the endoplasm frequently becomes packed with food material. These active feeding forms may attain a large size with nuclei measuring approximately 6 u. Most of the trophozoite nuclei have a single granule in the karyosome (as shown in Figure 1), but forms with four to six granules in the karyosome (Figure 2) are also found, and nuclei having two granules in the karyosome are common. Occasionally a thin line of chromatin may be seen connecting the two dots (Figure 3) but usually they are widely separated with no chromatin in the intervening space (Figure 4).

Hundreds of trophozoites have been studied but amoebae with the nucleus in the actual process of dividing have never been found. One trophozoite with two nuclei at opposite ends of the cytoplasm, which was constricted at its centre, indicates that the amoebae divide by simple binary fission (Figure 5).

B. Precystic Amoebae

Prior to encystation the amoebae lose their food inclusions, become less active and round up. Some precystic amoebae could easily be mistaken for uninucleate cysts were it not for the absence of a cyst wall. Cysts have never been found in turtles, but in culture and in experimental infections of snakes, they are seen regularly.

C. Cysts

In uninucleate cysts the nucleus is usually drawn out into a

spindle shape, tapers at both ends and usually stretches across the cyst (Figure 7). It usually contains a variable number of indefinite granules, and no distinct karyosome can be seen, but I have never been able to see these structures clearly enough to give an accurate description. At this stage the glycogen mass is large and sometimes fills the cyst. The chromatoid bodies are small and are arranged outside the glycogen mass; occasionally they are completely absent.

In the binucleate stage the first sign of division in the nuclei is seen in the karyosome which splits into two definite granules. The nucleus elongates but remains bluntly rounded at its ends, the two granules separate, and finally a faint constriction is seen at the centre of the nucleus (Figure 8). I have never been able to make out any chromosomes or spindle fibres. In a few cysts a single elongated halo may be seen surrounding the two karyosome granules, while in others a small complete halo surrounds each granule (Figure 9). In both uninucleate and binucleate cysts, the dividing elongated nucleus is frequently seen to have on either side of it, and diagonally situated to one another, a long slender bar of chromatin which stretches across at least one-half the width of the cyst (Figure 10).

In the binucleate cyst the nuclei do not always divide simultaneously. Trinucleate cysts are quite common. In these cysts division of one nucleus may be complete while that of the other has not started, so that two small nuclei and one larger nucleus are seen (Figure 11). In others one nucleus has completed division and the other is in the process of dividing. Chromatoid bodies are always present and are usually larger in binucleate and trinucleate cysts than in uninucleate cysts. As these bodies enlarge, the amount of glycogen decreases.

In the quadrinucleate cyst the nuclei usually lie in pairs of two at opposite ends of the cyst, but occasionally are found grouped together next to the cyst wall or even in a central position. Frequently they are not completely separated and there is no constriction at the point where the two nuclei are joined by a common nuclear membrane. This membrane, which may be curved or perfectly straight, is usually thinner and contains less chromatin than the rest of the nuclear membrane. At the ends of the line connecting the two nuclei a concentration of chromatin may be seen on the membrane of each nucleus, giving a "ring" appearance to both of them (Figure 12).

When they are completely separated the four nuclei are small and spherical with a single karyosome granule (Figure 13). They are smaller than the nuclei in any of the preceding stages as there is a marked decrease in the size of the nuclei with each division.

A small number of quadrinucleate cysts have been seen in some of which two nuclei were lighter stained, while in others two nuclei were smaller than the other two.

The glycogen mass in the four-nucleate cyst has either disappeared altogether or is present in a much smaller quantity than in the uninucleate cyst. The chromatoid bodies are usually larger but these too eventually disappear. Excystation never occurs until all of the glycogen and chromatoid bodies have been used.

D. Excystation

Excystation occurs readily at room temperature, at 30°C, and at 35°C.

Initial excystation is very rapid. They have been found to excyst in sterile saline while being picked up with a micropipette and are often found excysting on the slide immediately after it has been prepared. If excystation does not occur promptly at room temperature it may be speeded up by incubating the slides at 30°C. The process of excystation is usually very short requiring from five to thirty minutes, and in a few cases the amoebae have literally walked out of the cysts as though no wall existed. Immediately before excystation the cyst begins to revolve slowly and slight movement can be seen within the cyst. A small mass of cytoplasm is extruded through a pore in the cyst wall and then withdrawn. This may be repeated a number of times, and even when completely emerged, the amoeba frequently crawls back into its cyst. While the empty cyst wall usually appears to be intact, a small opening is occasionally seen.

Though the mature cyst has four nuclei, it always excysts as a two-nucleated amoeba. Trophozoites followed from the time of excystation and stained at varying intervals of from thirty minutes to four hours after excystation were found to have only two nuclei.

By staining a number of slides when movement was first observed within the cyst, when the amoeba had partly emerged, and when it had just completed excystation, it was discovered that only two nuclei were present prior to excystation. The following observations bear directly on this point.

(a) The most conclusive evidence that four-nucleate cysts revert to the two-nucleate stage prior to excystation was obtained from a culture with a single species of bacteria, Clostridium tertium. A count on the number of cyst nuclei was made immediately after their removal from culture. Two slides

were prepared, incubated at 30°C, and stained fourteen and twenty-five hours after preparation. The results are given in the following table in per cent:

Time	No. of cysts counted	Uni-nucleate	Bi-nucleate	Tri-nucleate	Quadri-nucleate
At time of removal from culture	30	3.3	36.6	13.3	43.3
14 hours later	74	2.7	64.7	9.5	23.0
25 hours later	56	1.8	64.3	16.1	17.8

No excystation had occurred on either slide. The increase in the percentage of two-nucleate cysts can only be explained by the corresponding drop in the percentage of four-nucleates. The number of uninucleate cysts was too low to account for the marked increase in the number of binucleate cysts.

(b) From a culture containing ninety-two per cent quadrinucleate cysts (thirty-two cysts were counted) four slides were prepared. At the end of one hour no excystation had occurred and there was no change in the percentage of four-nucleate cysts. A slide stained two hours later gave the same results. The third slide was stained five hours after preparation and resembled the first two with the exception that in most of the cysts the nuclei were lying in pairs. The fourth slide was stained fourteen hours after preparation and contained fifteen binucleate trophozoites, and four two-nucleate cysts. Because of the difficulty in counting nuclei on vaseline-sealed slides I was unable to discover the number of nuclei in the remaining cysts on this slide.

(c) A cyst revolving slowly and showing slight movement within the

cyst possessed only two nuclei.

(d) A trophozoite stained thirty minutes after excystation had two nuclei. It was followed from the time of excystation and had not divided.

(e) Movement was observed within a cyst ten minutes after a slide was prepared. Excystation was completed thirty minutes later when the trophozoite was stained. It contained only two nuclei, one of which had two chromatin granules in the karyosome (Figure 14).

(f) One slide containing two cysts was stained twenty-four hours after its preparation and showed one cyst and two uninucleate trophozoites.

(g) Figures 15 (a), (b), and (c) were drawn from a slide stained five minutes after its preparation and show three partly emerged trophozoites, each containing only two nuclei. A count made on the number of nuclei in fifty cysts on this slide showed that ninety-two per cent were binucleate; two per cent, trinucleate; and six per cent, quadrinucleate.

(h) The cysts on which the above experiment (exp. g) was performed were refrigerated and on the following day another slide was prepared and a count made on the number of nuclei in the cysts and excysted trophozoites. Of eleven cysts, nine had two nuclei; one had two elongated nuclei, each having two granules in the karyosome; the eleventh had four nuclei. Of twenty trophozoites, two were uninucleate; and eighteen were binucleate. Two of the binucleate trophozoites had just started to excyst, two were still clinging to their cyst walls, and one had just completed excystation. It is not known how long the other had been excysted, but certainly not longer than three hours.

(i) From a culture of cysts containing twenty per cent uninucleates,

twenty per cent binucleates, twenty per cent trinucleates, and forty per cent quadrinucleates a slide was prepared and stained seventy minutes later. One uninucleate and approximately fifty binucleate trophozoites were found. Six cysts examined from this slide were two-nucleated.

V. TURTLE INFECTIONS WITH ENTAMOEBA TERRAPINAE

A. NATURAL INFECTIONS

Thirty-four turtles were examined for natural infections with E. terrapinae. Of nine small Chrysemys elegans, only one was found infected. Twenty-five larger turtles (length of shell approximately five inches) were examined; two of twelve Chrysemys picta and two of thirteen Chrysemys elegans were infected with E. terrapinae.

(1) One small Chrysemys elegans had a few trophozoites in the large intestine. No cysts were found.

(2) The second Chrysemys elegans had few amoebae in the stomach and first part of the small intestine. A culture from the large intestine was negative and no cysts were found.

(3) The third Chrysemys elegans had numerous motile amoebae throughout the stomach, small, and large intestine. The morphology and size of trophozoites from the large intestine of this turtle have already been given. No cysts were found.

(4) One Chrysemys picta had nine small areas of necrotic tissue in the stomach, with numerous amoebae not only in the lesions but throughout the stomach and small intestine. A few trophozoites were found in the large intestine. Stained sections of necrotic tissue from the stomach showed amoebae lying in the mucus at the base of the stomach wall. As no amoebae had invaded the tissues it was impossible to attribute the lesions to the presence of the amoebae, and their cause is not known.

(5) The other Chrysemys picta had two areas of necrotic tissue in the large intestine. Amoebae, which were found only in the large intestine,

were especially numerous in the lesions. However, a study of sectioned and stained tissue failed to show invasion of the tissue by the amoebae. No cysts were found although a zinc sulphate flotation was done on material from the large intestine.

B. EXPERIMENTAL INFECTION OF TURTLES.

Three small Chrysemys elegans were experimentally infected with cysts of E. terrapinae. One turtle killed one hour after infection had a few cysts in the stomach. The other two turtles, killed two hours and three hours after infection, were both negative.

VI. EXPERIMENTAL INFECTIONS OF SNAKES

Two whip snakes (Coluber), five water-snakes (Natrix), and two garter snakes (Tropidonotus) were experimentally infected with both cysts and trophozoites of E. terrapinae.

EXPERIMENT I

One whip snakes, Coluber, was given four milliliters of a clone culture growing with mixed bacteria. The snake died six days later. Amoebae were present throughout the stomach and intestinal tract. A microscopic examination of amoebae from a culture from the stonach indicated that they were E. terrapinae. Moreover, examination of the faeces of this snake on five out of eight days previous to its infection showed it to be negative for amoebae. There was congestion of the blood vessels in the walls of the small and large intestine, but neither this nor the death of the snake could be attributed to the presence of amoebae. as all of these snakes died shortly after they were obtained from Florida.

EXPERIMENT II

Another whip snake was given five milliliters of a clone culture. I was unable to obtain faeces from this snake previous and subsequent to infection. The snake died five days after its infection. No active amoebae were found. A culture from the stomach was negative but one from the large intestine was positive.

EXPERIMENT III

A water-snake, Natrix, was given five milliliters of a clone culture. The snake died the following day and was found to be negative.

EXPERIMENT IV

A snake, Natrix toxopilota, was given six milliliters of a clone culture. It died fifty-three days after infection and amoebae were present in the large intestine.

EXPERIMENT V

Three snakes, Natrix, were given four to eight milliliters of a clone culture. The snakes died sixty, sixty-seven, and seventy-one days after infection, All three were negative.

EXPERIMENT VI

Two small garter snakes, Tropidonotus, were each given four milliliters of a culture of E, terrapinae growing with a single species of bacteria, Clostridium tertium. Both snakes passed trophozoites and cysts in their faeces on the day following their infection. They were killed five days later. One was negative; the other had numerous cysts, but no trophozoites, in its large intestine.

COMPARATIVE STUDIES on ENTAMOEBA TERRAPINAE, ENTAMOEBA INVADENS, and ENTAMOEBA HISTOLYTICA

I. SIZE

A comparison of the size of E. terrapinae, E. invadens, and E. histolytica is given in Tables I, II, III and IV.

Species and Temperature	RINGER'S SERUM No. measured	Range	Average Diameter	Standard Deviation	LOCKE'S No. measured	Range	Average Diameter	Standard Deviation
E. histolytica 30°C	50	15.20 - 27.06	19.38	1.11		No Growth		
35°C	80	13.68 - 29.74	19.78	1.19	50	13.68 - 30.52	19.82	1.60
E. invadens 30°C	75	15.20 - 38.00	27.78	1.64	110	16.72 - 44.08	31.25	1.88
35°C	106	12.77 - 56.24	29.04	2.41	85	17.02 - 46.48	27.84	2.05
E. terrapinae 30°C	100	15.20 - 38.00	26.63	1.77	100	16.72 - 35.72	24.78	1.70
35°C	118	18.24 - 57.76	28.49	2.16	100	15.96 - 45.60	28.32	1.90

Table I. Size comparison of trophozoites of E. histolytica, E. invadens, and E. terrapinae in Ringer's serum and Locke's at 30°C and 35°C. All organisms were stained with D'Antoni's iodine. Measurements are given in microns.

Species and Temperature	RINGER'S SERUM No. measured	Range	Average Diameter	Standard Deviation	LOCKE'S No. measured	Range	Average Diameter	Standard Deviation
E. invadens								
30°C	100	11.25 - 22.19	15.02	1.71	100	13.38 - 20.82	17.13	0.90
35°C	85	12.62 - 23.41	16.04	1.88	75	11.85 - 23.40	16.67	1.62
E. terrapinae								
30°C	100	11.86 - 19.15	14.77	1.20	105	10.64 - 20.06	15.55	1.40
35°C	60	11.25 - 21.28	16.22	1.10	75	12.92 - 24.32	18.33	1.18

Table II. Size comparison of cysts of E. invadens and E. terrapinae in Ringer's serum and Locke's at 30°C and 35°C. E. histolytica did not encyst in any of the cultures. The cysts were stained with D'Antoni's iodine solution and measurements are given in microns.

Species, Media and Temperature	TROPHOZOITES Number Measured	Range	Average Diameter	Standard Deviation	NUCLEUS Range	Average Diameter	Standard Deviation	Nucleo Cytoplasmic Ratio
E. invadens								
Ringer's serum 30°C	75	10.26-34.20	18.94	2.25	3.04-7.00	4.84	1.71	0.255
Locke's 30°C	75	16.72-39.90	24.54	1.71	4.10-7.60	5.90	1.49	0.240
E. terrapinae								
Ringer's serum 30°C	50	13.53-22.80	18.07	0.94	3.80-6.84	5.17	1.35	0.286
Locke's 30°C	50	12.92-31.92	18.45	1.38	3.34-6.99	5.50	1.50	0.297

Table III. Size comparison of *E. invadens* and *E. terrapinae* from cultures at 30°C. Specimens were stained with Heidenhain's iron haematoxylin.

Species and Host	TROPHOZOITES				NUCLEUS			
	Number Measured	Range	Average Diameter	Standard Deviation	Range	Average Diameter	Standard Deviation	Nucleo-Cytoplasmic Ratio
E. invadens								
Faeces of garter snake	90	9;58-21.28	14.76	1.88	3.04-6.23	4.68	1.77	0.32
E. terrapinae								
large intestine of Chrysemys elegans	125	9.88-20.52	14.40	1.79	3.19-6.38	4.63	2.04	0.32
E. terrapinae								
Faeces of garter snake	41	12.76-30.52	20.32	1.21	3.88-7.33	5.19	1.42	0.26

Table IV. Size comparison of E. invadens and E. terrapinae from the hosts. Measurements were made on fixed and stained organisms.

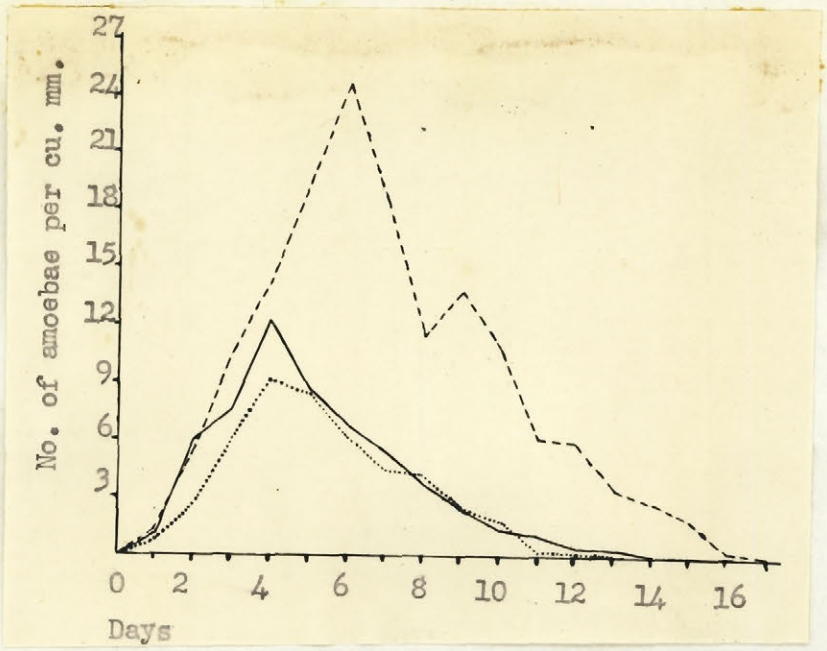
II. GROWTH IN CULTURE

E. histolytica showed best growth in Ringer's serum at 35°C. This species did not grow as well in LOCKE'S and it was usually necessary to add amoebae on the second day. In Locke's at 30°C the amoebae did not even survive despite heavy inoculations of amoebae on the second and third days. Very poor growth was obtained in Ringer's serum at 30°C, and to maintain the amoebae at this temperature it was always necessary to add amoebae on the second day and usually on the third day also. The amoebae were most abundant from the third to the seventh day, and cultures never lived more than twelve or thirteen days.

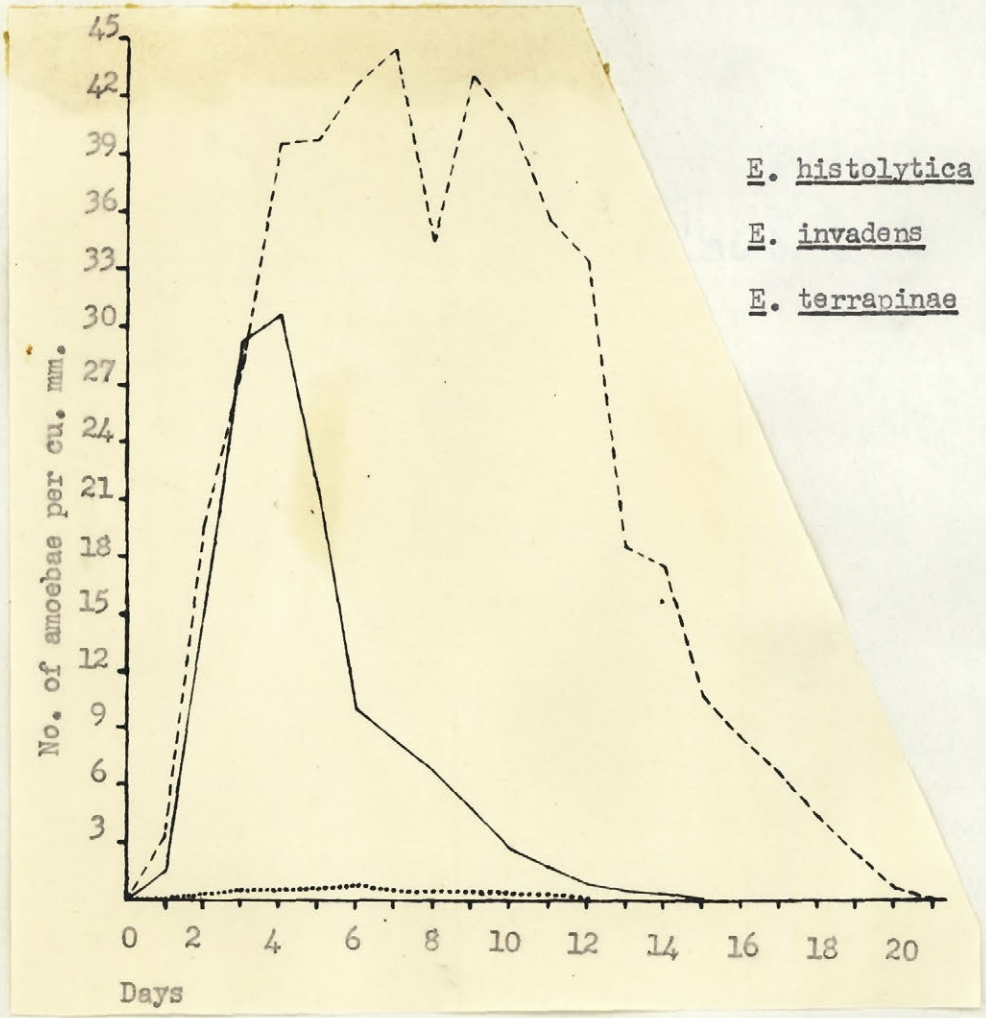
E. invadens showed best growth in Ringer's serum at 30°C, The amoebae grew well in Ringer's serum at 35°C and in Locke's at 30°C, but in Locke's at 35°C E. invadens showed poor growth and often the cultures did not survive unless amoebae were added on the second day. This species usually showed two or three growth peaks in culture, the best growth being obtained from the third to the tenth or eleventh day. Cultures survived three weeks in Ringer's serum at 30°C, sixteen to eighteen days in Ringer's serum at 35°C and in Locke's at 30 C. but only eleven or twelve days in Locke's at 30°C.

E. terrapinae showed best growth in Ringer's serum at 30°C. Good growth was obtained in Locke's at 30°C and in Ringer's serum and Locke's at 35°C. Growth of this species was not as affected by the temperature and media as was the growth of E. invadens and E. histolytica, and no extreme variation was found in cultures growing under different conditions. E. terrapinae reached a growth peak on the third or fourth day and thereafter growth declined, the cultures surviving thirteen or fourteen days.

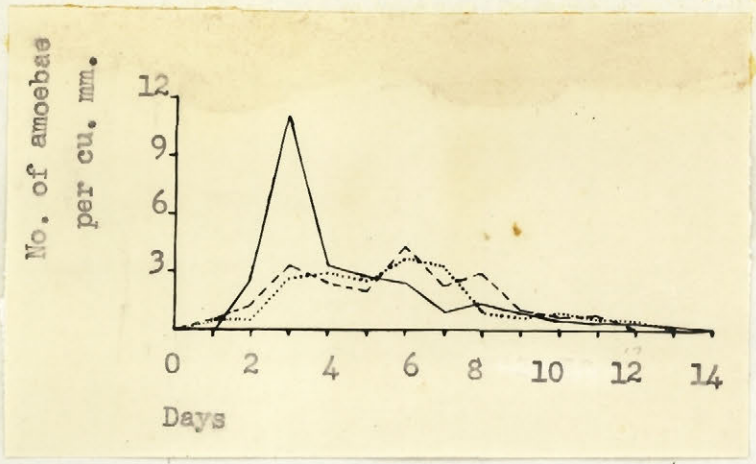
A comparison of the growth of E. histolytica, E. invadens, and E. terrapinae in Ringer's serum and in Locke's at 30°C and at 35°C is given in Text - Figures II, III, IV, and V.



Text Figure 2. Comparison of growth of E. histolytica, E. invadens and E. terrapinae in Ringer's serum at 35°C.



Text Figure 3. Comparison of growth of E. histolytica, E. invadens, and E. terrapinae in Ringer's serum at 30°C.

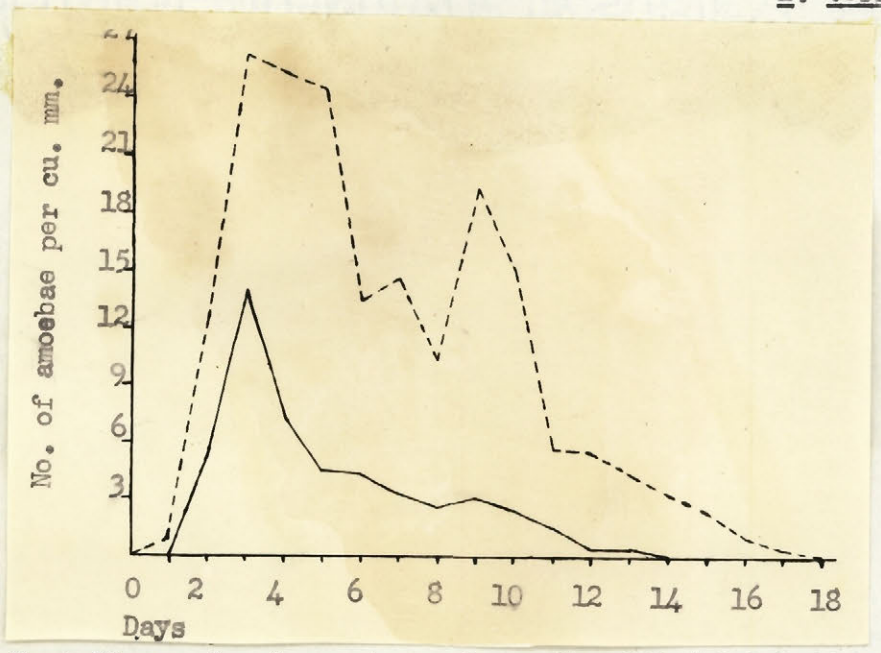


Text Figure 4. Comparison of growth of E. histolytica, E. invadens, and E. terrapinae in Locke's at 35°C.

E. histolytica .....

E. invadens - - - - - -

E. terrapinae _____



Text Figure 5. Comparison of growth of E. histolytica, E. invadens, and E. terrapinae in Locke's at 30°C.

III. ENCYSTATION IN CULTURE

E. histolytica did not encyst in either media at 30°C or at 35°C.

E. invadens showed a remarkable degree of encystation in both Ringer's serum and Locke's at 30°C and in Ringer's serum at 35°C. Cysts were usually present in twenty-four hour cultures and thereafter they increased in number to such an extent that they usually far outnumbered the trophozoites. As the cultures aged the increased proportion of cysts to trophozoites became more apparent. Examinations of cultures in which growth had almost died out revealed large numbers of degenerating cysts. Cysts of E. invadens from culture were always massed together in large clumps, some of which contained as many as eighty cysts.

E. terrapinae showed good encystment in all cultures on the third day and cysts were found until the eighth and ninth day. Cysts of E. terrapinae, like those of E. invadens, tended to clump together in cultures.

IV. MORPHOLOGY

A. UNSTAINED

Trophozoites of E. histolytica from culture were sluggish in comparison with those of E. invadens and E. terrapinae which showed great activity. The cytoplasm in the latter two species had a granular appearance and was more dense than that in E. histolytica. In active trophozoites of E. invadens and E. terrapinae, the streaming movement of the cytoplasm was clearly observable. In trophozoites of E. histolytica the cytoplasm was clear and homogenous, and when no definite pseudopodia were formed, the flowing of the cytoplasm was best seen by the movement of the starch grains within the trophozoites. All three species readily ingested starch grains in culture.

E. histolytica did not encyst in any of the cultures. Cysts of E. terrapinae and E. invadens, examined fresh from culture, were indistinguishable, both having the same appearance as that already described for E. terrapinae.

B. STAINED WITH D'ANTONI'S IODINE

In iodine-stained preparations trophozoites of all three species appeared morphologically identical. In some trophozoites the endoplasm was so packed with food material, starch grains and bacteria, that the nucleus was obscured, and often distorted. Usually, in all three species, it appeared as a spherical body with a central karyosome containing one to two granules.

Cysts of E. terrapinae and E. invadens, stained with iodine, were indistinguishable.

C. FIXED AND STAINED

Before permanent preparations of all three species were obtained, the cultures of E. histolytica growing with Clostridium tertium were lost, and the following studies from fixed and stained preparations were made on E. terrapinae and E. invadens.

(a) Trophozoites

(1) Trophozoites from cultures maintained at 30°C.

Trophozoites of both species from Locke's were more extended and the cytoplasm was therefore less dense than in trophozoites from Ringer's serum. This was especially true of E. invadens, the trophozoites from Locke's often being greatly elongated so that the cytoplasm stained lightly whereas trophozoites from Ringer's serum were more rounded, usually with no extended pseudopodia, and the cytoplasm stained more deeply.

In young trophozoites of both species the nucleus was always spherical with a small central karyosome. In older specimens it was usually spherical but sometimes assumed a number of shapes, the most frequent being an oval form. In these larger forms the nucleus usually maintained its shape better in trophozoites of E. invadens than in those of E. terrapinae. In most nuclei the karyosome was concentric, but nuclei with eccentric karyosome did occur and were more frequently seen in E. invadens than in E. terrapinae. Forms having two granules in the karysome were very common in both species. All the variations from the typical nucleus already described for E. terrapinae were found in E. invadens also. The only difference found between these two species was in their nucleo-cytoplasmic ratios, that of E. terrapinae being higher than that of E. invadens. In Ringer's serum the nucleo-cytoplasmic ratio of E. terrapinae was 0.286; in Locke's, 0.297; that of E. invadens in Ringer's serum was 0.255; in Locke's 0.240.

(2) Trophozoites from the hosts, snakes and turtles.

Trophozoites of E. terrapinae from the turtle and of E. invadens from the snake were identical in size, in their nucleo-cytoplasmic ratios, and in the structure of their nuclei. Amoebae from the hosts had more chromatin on the nuclear membrane and in the karyosome than was found in amoebae from culture. In organisms from culture the karyosome invariably showed up as one or more discrete granules. In trophozoites from the host it frequently gave the appearance of a blob-like structure, and while definite granules could not be counted, it is certain that it contained more than one. In trophozoites of E. terrapinae from the turtle, a variable number of chromatin granules were sometimes seen on the achromatic membrane surrounding the karyosome (Figure 6). The same appearance was occasionally seen in trophozoite

nuclei of E. invadens from the snake, with the exception that the granules were always more indefinite.

Both in size and in nucleo-cytoplasmic ratio, E. terrapinae trophozoites from a garter snake resembled those from culture more closely than those from the turtle or those of E. invadens from garter snakes. Moreover the structure of the nucleus may take on all the characteristics seen in trophozoites from culture but not in those from the natural host; i.e., the peripheral chromatin may be concentrated in a number of dots or bars, the shape of the nucleus may be as variable as in culture organisms, and the whole nucleus often has a granular appearance.

In trophozoites of E. invadens from liver smears of experimentally infected garter snakes, the nuclei sometimes contained many small granules scattered throughout the nucleo plasm.

(b) Cysts

Most of the cysts of E. invadens examined from culture were four-nucleate, whereas the greatest percentage of E. terrapinae cysts were two-nucleate. Cysts of both species recovered from experimental infections of snakes were usually four-nucleate.

In quadrinucleate cysts, the nuclei in E. invadens were often seen arranged in a row on one side of the glycogen vacuole, whereas in E. terrapinae they were usually found in pairs of two at opposite ends of the cyst. However, in cysts of either species the nuclei may occupy any position, and for all practical purposes, E. terrapinae and E. invadens were indistinguishable from the appearance of their cysts.

DISCUSSION

Morphology

My studies on the structure of trophozoites and cysts of E. terrapinae are in close agreement with those of Sanders and Cleveland (1930). The typical nucleus has a small central karyosome surrounded by a halo and the nuclear membrane is evenly beaded with chromatin granules. Sanders and Cleveland did not describe the karyosome beyond saying that it was central. I found that while it usually contains only a single granule it may have several to as many as five or six granules, sometimes appearing as a tiny "centriole" surrounded by a number of granules as described for E. histolytica by Dobell (1928). Karyosomes with two granules were very common and resembled the "prophase" stage of division described by Dobell (1938) for E. coli.

The variations which I found from this typical "histolytica" - like nucleus, if observed by Sanders and Cleveland, were not recorded. I found that in trophozoites from the turtle host a variable number of chromatin granules are sometimes present on the achromatic membrane surrounding the karyosome. In his studies on the nuclear structure of E. histolytica in 1919 Dobell states that in the normal nucleus no chromatin is present between the karyosome and the nuclear membrane and that all descriptions which state otherwise are made from degenerate or badly-fixed specimens. However, in 1928 he modified this statement by saying that the space between the karyosome and the nuclear membrane is usually free of chromatin "but sometimes shows a variable number of somewhat indefinite granules." In his studies on E. coli in 1938 Dobell states that "chromatin grains of variable size and number, are typically present, in E. coli, in the zone between the karyosome and the

membrane : in E. histolytica they are absent." In any case I do not believe the chromatin grains seen in nuclei of E. terrapinae were evidence of degeneration on the part of the amoebae as they were very active when examined fresh from culture and showed no tendency to round up. In nuclei of trophozoites from cultures and from snakes the peripheral chromatin was sometimes concentrated in a number of dots or bars on the nuclear membrane, instead of being evenly arranged as in organisms from the natural host. A number of workers described this appearance in nuclei of E. histolytica but Dobell (1919) stated that it is seen only in degenerate specimens. Geiman and Ratcliffe (1936) found that in trophozoites of E. invadens from old cultures the chromatin "tends to degenerate and clump in masses." I have observed the same appearance in nuclei of E.invadens from culture. My cover-slip preparations of both E. terrapinae and E. invadens were removed from three-day old cultures. In view of this and the fact that the amoebae were extended on the cover-slip in positions of activity, there is no reason to believe that they were degenerate. This unusual appearance of the nuclear membrane found in snake and culture organisms of E. terrapinae and culture organisms of E. invadens may, however, be due to the abnormal environment.

Sanders and Cleveland (1930) state that the chromatoid bodies in cysts of E. terrapinae may vary in size and number but are usually in the form of heavy blunt rods and "always have rounded ends." Of E. terrapinae cysts Geiman and Ratcliffe (1936) state that "the size and shape of the chromatoids are diagnostic." I found that the chromatoid bodies were too variable in size, shape, and number to be useful as a diagnostic character. Apart from this my observations on the cystic structure of E. terrapinae are in agreement with those of Sanders and Cleveland. (loc. cit)

Size

Snaders and Cleveland (1930) stated that trophozoites of E. terrapinae ranged from 10-15 u, nuclei 2-4 u, and cysts 8-14 u with an average of 10 u.

The result of the present studies on size show that it is a factor which is not determined by the species as much as by its environment. E. terrapinae varied in size depending on the source, turtles, snakes, or cultures. Again in culture, size was affected by the length of time the amoebae had been maintained in culture, by the temperature at which they were cultured, and by the type of overlay used, Ringer's serum or Locke's. Thus trophozoites from a turtle averaged 14.40 u, nuclei 4.63 u. Trophozoites from a snake averaged 20.32 u, nuclei 5.19 u. In Locke's at 35°C trophozoites averaged 28.32 u; in Locke's at 30°C they only averaged 24.78 u. The average diameter of cysts from an experimental infection of a garter snake was 10.63 u; from a clone culture of two months' duration, the average diameter was 9.69 u. After the amoebae had been maintained ten months in culture, cysts averaged 18.33 u in Locke's at 35°C, and 15.55 u in Locke's at 30°C.

The size of E. terrapinae as found for trophozoites from the turtle host and for cysts from a garter snake and an early culture agrees closely with that given by Sanders and Cleveland. However there is a marked difference in the size of the nuclei. Approximately ninety per cent of the nuclei I have measured exceeded the range given by Sanders and Cleveland and I have never found a trophozoite nucleus less than 3 u in diameter. Yet whether in trophozoites from the host or from the culture the size range and average diameter of the nucleus remains fairly constant. Trophozoites from snakes and cultures show a definite increase in size over those from the turtle host, but it is not accompanied by a corresponding increase in the size of the nucleus, which results in a lowered nucleo-cytoplasmic ratio.

Life-Cycle

Sanders and Cleveland found that "under laboratory conditions it was impossible for the complete life-cycle of Entamoeba terrapinae to take place at one temperature." Their amoebae grew well at 20-23°C but would not excyst at this temperature. The optimum temperature for excystation was 27°C at which temperature the amoebae would not grow. As opposed to this I found that E. terrapinae showed good temperature adaptability, growing well, with encystation and excystation, at any temperature between 20°C and 35°C.

Sanders and Cleveland found large numbers of four-nucleate cysts in six-day old cultures. In my cultures the amoebae reached a growth peak on the third or fourth day, also the height of encystation, and binucleate cysts usually outnumbered quadrinucleate cysts.

Sanders and Cleveland noted a rapid initial excystation of one and one-half to seven hours for cysts of E. terrapinae. My studies also showed that this species has a rapid initial excystation, many of the cysts excysting immediately after their removal from culture.

As described by Sanders and Cleveland, each cyst liberates a four-nucleate amoeba, which divides into two binucleate trophozoites, each of which by a cytoplasmic division, gives rise to two uninucleate amoebae. This description is based more on assumptions than on actual observations. They only saw one quadrinucleate amoeba dividing. Because they saw many binucleate trophozoites immediately after cysts had been planted in cultures free of amoebae, they assumed that they were the result of division of a four-nucleate trophozoite. My studies show that E. terrapinae always excysts as a two-nucleate amoeba and that they are not the result of a division. Moreover, it was found that the binucleate trophozoite comes from a quadrinucleate cyst

which had reverted to the binucleate stage prior to excystation.

One of the following two explanations may account for the loss of two nuclei in cysts of E. terrapinae prior to its excystation: (1) degeneration of two nuclei; and (2) autogamy or fusion of the nuclei.

Schaudinn in 1903 reported autogamy in cysts of E. coli. The following account of his description is from Wenyon (1926): "The single nucleus of the encysted form divides to give rise to two nuclei. Each of these gives off two reduction bodies, after which they divide to form four nuclei, which are arranged in pairs at opposite sides of the cyst. One of each pair is a stationary nucleus and one a migratory nucleus. The migratory nuclei move to opposite sides of the cyst, where they unite with the stationary nuclei. The cyst again has two nuclei, which proceed to divide till the characteristic eight nuclear stage is reached." This account was shown to be incorrect by Dobell in 1938. A number of other workers have described autogamy for E. coli, E. histolytica, E. ranarum, and E. muris but their evidence has never been accepted, and therefore a review of their work is not given here.

Dobell in his work on the life-cycle of E. coli in 1938 found that "in many cysts - indeed, in the majority - some of the nuclei degenerate and disappear before excystation; and consequently their amoebae, at the moment of hatching, contain not 8 nuclei but 7, 6, 5 or even only 4." These cysts he called "infranucleate" cysts as distinguished from "subnucleate" or immature cysts.

A small number of cysts have been seen in some of which two nuclei were smaller than the other two, while in others two nuclei were lighter stained. However, these observations have been made on so few cysts that no definite conclusions can be drawn.

With regard to the evidence for fusion of the nuclei in the quadrinucleate cyst prior to its excystation attention must be drawn to the appearance of the nuclei in Figure 12. This figure shows a cyst with four nuclei in pairs of two, each pair incompletely separated. Sanders and Cleveland (1930) described this and thought it might be due to pressure of the nuclei against one another and also suggested that it might be "a primitive form of division characteristic of this amoeba." Their first explanation implies an outside force and as the glycogen mass and the chromatoid bodies have usually decreased in size at this stage, it is extremely difficult to visualize a pressure on the nuclei which has not existed in the preceding stages, when the cysts were uninucleate, binucleate, and trinucleate. Furthermore, while my description of the division of cyst nuclei is far from being complete, there is nothing to indicate that they do not divide as a typical Entamoeba. Yet this stage if not caused by an outside pressure disagrees with the fact that a dividing Entamoeba nucleus elongates and constricts into two at its centre. In view of the fact that a four-nucleate cyst loses two of its nuclei, this stage might well be a fusion of the nuclei rather than "a primitive form of division," peculiar to this amoeba.

In his studies on the degeneration of nuclei in cysts of E. coli Dobell (1938) states that "infranucleate cysts are easily distinguishable from immature (subnucleate) cysts, containing the same number of nuclei, by the size of their nuclei." In cysts of E. terrapinae the nuclei in binucleate cysts are always larger than those in quadrinucleate cysts. If two of the nuclei degenerated, then the nuclei in a binucleate cyst, prior to its excystation, should be no longer than those in a quadrinucleate. Dobell also states that "the irregularity with which infranucleate cysts are formed, and

the insconstancy in the number of nuclei which degenerate, are inconsistent with any ordinary theory of gamete-formation." But while E. coli may excyst as an eight-, seven-, six-, five-, or four-nucleate amoeba, E. terrapinae always excysts as a two-nucleate amoeba. This again would suggest that there is fusion rather than degeneration of the nuclei.

COMPARISON of E. histolytica, E. invadens, and E. terrapinae

Geiman and Ratcliffe (1936) found that E. invadens, both in morphology and in life-cycle, was similar to E. histolytica, and noted the following differences between these two species:

(1) trophozoites of E. invadens have a greater density of cytoplasm than those of E. histolytica;

(2) thicker cysts wall in E. invadens;

(3) a lower nucleo-cytoplasmic ratio for E. histolytica, the average ratio for the latter species being 0.246, whereas the average ratio for E. invadens is 0.295;

(4) E. invadens grows well over a wide range of temperature; E. histolytica grows well between 35°C and 38°C;

(5) E. histolytica requires three to seven hours for excystation, whereas E. invadens requires five to fourteen hours;

(6) metacystic development in E. histolytica requires about seven hours; in E. invadens, twelve to twenty-four hours.

In the following table a comparison is given of the size and temperature requirements of E. histolytica as found by Dobell (1919, 1928), of E. invadens as found by Geiman and Ratcliffe (1936), and of E. terrapinae as found by Sanders and Cleveland (193 0).

SPECIES	NUCLEUS	TROPHOZOITES	CYSTS	TEMPERATURE REQUIREMENTS
E. histolytica	4-7 u	78 - 40 u; 20-30 u, when rounded	5 - 20 u average of small race, 6.6 u average of larger race, 15 u	35° - 38°C
E. invadens	3.5-7.3 u average 4.78 u	9 - 38.6 u average 15.9 u	11 - 20.2 u average 13.88 u	range of 10° - 33°C maximum for growth 20° - 30°C
E. terrapinae	2 - 4 u	10 - 15 u	8 - 14 u average 10 u	optimum growth 20° - 23°C no excystation; optimum temperature for excystation 27°C, no growth at this temperature

Table V. Size and temperature requirements of E. histolytica as described by Dobell (1919, 1928), of E. invadens as described by Geiman and Ratcliffe (1936), and of E. terrapinae as described by Sanders and Cleveland, 1930.

In the present comparative studies on E. histolytica, E. terrapinae, and E. invadens, all three species were cultured with a single species of bacteria, Clostridium tertium, in Locke's and in Ringer's serum at 30°C and 35°C.

Size

E. histolytica was constant in size at both temperatures (average diameter of trophozoites was 19.38 u from Ringer's serum at 30°C, 19.78 u from Ringer's serum at 35°C, and 19.82 u from Locke's at 35°C). This species was consistently smaller than E. invadens and E. terrapinae.

Both from their respective hosts and from cultures, E. invadens and E. terrapinae were remarkably similar in size. They approximated each other more closely at 35°C than at 30°C. At 35°C trophozoites of E. terrapinae averaged 28.49 u in Ringer's serum and 28.32 u in Locke's; those of E. invadens averaged 29.04 u in Ringer's serum and 28.74 u in Locke's. In Ringer's serum at 30°C E. terrapinae averaged 26.63 u and E. invadens 31.25 u. The above measurements were made on organisms stained with D'Antoni's iodine. In fixed and stained specimens from cultures at 30°C, E. terrapinae averaged 18.45 u 18.07 u in Ringer's serum and Locke's respectively. At 30°C E. invadens was consistently larger in Locke's than in Ringer's serum. On cover-slip preparations from Locke's the amoebae were more extended and the cytoplasm less deeply stained than on those from Ringer's serum. The greater size in Locke's, therefore, may have been due to greater activity on the part of the amoebae and not to greater volume of cytoplasm. In culture the nucleo-cytoplasmic ratio of E. invadens was 0.252; that of E. terrapinae was 0.292.

In culture cysts of E. terrapinae and E. invadens were almost

identical in size. In Ringer's serum at 30°C the average diameter of cysts of E. terrapinae and E. invadens was 14.77 u and 15.02 u, respectively. In Ringer's serum at 35 C cysts of E. terrapinae had an average diameter of 16.22 u; those of E. invadens had an average diameter of 16.04 u.

Trophozoites of E. invadens from the snake had an average diameter of 14.76 u, and their nuclei averaged 4.68 u. Trophozoites of E. terrapinae from the turtle had an average diameter of 14.40 u and their nuclei averaged 4.63 u. The nucleo-cytoplasmic ratio of each species from its respective host was 0.32.

Trophozoites of E. terrapinae from the snake had an average diameter of 20.32 u and their nuclei averaged 5.19 u, the nucleo-cytoplasmic ratio being 0.26. Cysts of E. terrapinae from the snake had an average diameter of 10.63 u. According to Geiman and Ratcliffe (1936) the average diameter of cysts of E. invadens from the snake was 13.88 u. It would thus appear that in snakes trophozoites of E. terrapinae are larger and the cysts smaller than those of E. invadens.

Growth

E. histolytica did not encyst in any of the cultures. The failure of this species to encyst in a monobacterial culture was also noted by Reardon and Bartgis in 1949. E. terrapinae showed good encystation in all cultures on the third and fourth days. E. invadens encysted remarkably well in all cultures except for Locke's at 35°C. Cysts were present in twenty-four hour cultures and thereafter they increased until they outnumbered the trophozoites. Cultures of these three species of Entamoeba were easily distinguishable by the degree of encystation, E. invadens showing the highest degree of encystation.

All three species showed best growth in Ringer's serum, E. histolytica at 35°C and E. invadens and E. terrapinae at 30°C. E. histolytica did not even survive in Locke's at 30°C and E. invadens grew poorly in Locke's at 35°C. The presence of serum in culture was a definite factor in obtaining good growth, especially in cultures of E. invadens and E. histolytica at an abnormal temperature. While it never grew as well or survived as long, E. terrapinae showed better adaptability to temperature and media than E. invadens, good growth being obtained in all of the cultures. Due to its non-pathogenicity, serum may not be an essential factor in the growth of E. terrapinae as it is in that of E. histolytica and E. invadens.

E. histolytica showed very poor growth in Ringer's serum at 30°C probably due to the fact that it was cultured with a single species of bacteria, as the same strain grew very well at 30°C with mixed bacteria.

Morphology

The cytoplasm in E. histolytica was clear and homogeneous; that in E. invadens and E. terrapinae was granular.

E. histolytica was sluggish; E. terrapinae and E. invadens were very active.

Stained in iodine, all three species were morphologically identical.

From their natural hosts and from identical cultures, E. terrapinae and E. invadens were indistinguishable in size and morphology.

In experimental infections of snakes, E. terrapinae was larger than E. invadens and had a lower nucleo-cytoplasmic ratio. Moreover snake infections

with E. terrapinae did not persist more than four to eight weeks and with no invasion of the tissue, whereas infections with E. invadens always end fatally.

Relationship of E. terrapinae with other species of Entamoeba.

The present studies have shown that, within narrow limits, size and morphology are poor criteria for differentiating species of Entamoeba, and indicate that, in addition, they should be distinguished on the basis of growth requirements and life history.

In view of the fact that E. terrapinae is infective but non-pathogenic for snakes, the possibility that it is synonymous with E. varani, Lavier 1928, must be considered. E. varani, which resembled E. histolytica in size and morphology, was found in a snake but had produced no disease.

In 1930 Knowles and Das Gupta described an Entamoeba species from culture material obtained from the turtle, Trionyx gangeticus. Trophozoites ranged from 10-60 u with an average of 30.9 u, nuclei 5-6 u, cysts 7.5-20.7 u with an average of 11.7 u. In 1947 Rodhain and Van Hoof cultivated an Entamoeba from Terrapina cinosternoides and Plastysternum megacephalum and described it as a new species, Entamoeba knowlesi. They found that trophozoites ranged from 20.5-29.5 u, nucleus 4-8 u, cysts 7.2-13.8 u with an average of 10.78 u. They considered that it might be synonymous with the Entamoeba species of Knowles and Das Gupta, and distinguished it from E. terrapinae because of the small size of the latter. In the present work the size of E. terrapinae from the host agreed with that given by Sanders and Cleveland (1930), whereas the size in cultures agreed with that given for E. knowlesi, Rodhain and Van Hoof 1947, and for the Entamoeba species of Knowles and Das Gupta 1930, both of

which were measured from cultures. These three species are also morphologically identical and as they have not yet been shown to differ in growth requirements or life history, they may be synonymous.

SUMMARY and CONCLUSIONS

Morphological studies were made on Entamoeba terrapinae, Sanders and Cleveland 1930, from the turtle, Chrysemys elegans, from an experimental infection of a garter snake, and from cultures. Trophozoites had a typical "histolytica" nucleus, consisting of a central karyosome containing from one to six granules, surrounded by a clear halo, and the nuclear membrane was beaded with a single layer of chromatin granules. Cysts when mature had four nuclei and contained varying amounts of glycogen and chromatoid bodies, the size, shape and number of the latter being too variable to be of any diagnostic significance.

The size of both trophozoites and cysts was affected by varying environmental conditions. Thus, trophozoites from a turtle, Chrysemys elegans, had an average diameter of 14.40 u, nuclei 4.63 u; trophozoites from a garter snake had an average diameter of 20.32 u, nuclei 5.19 u; trophozoites from Ringer's serum at 35°C had an average diameter of 28.49 u. Cysts from a garter snake had an average diameter of 10.63 u; cysts from a clone culture of two months' duration averaged 9.69 u. After ten months' growth in culture cysts averaged 14.77 u in Ringer's serum at 30°C and 16.22 u in Ringer's serum at 35°C.

In cultures E. terrapinae reached a growth peak on the third or fourth day. Good growth, with encystation and excystation, was obtained at any temperature between 20°C and 35°C. This differs from the findings of Sanders and Cleveland (1930) who reported that this species grew well at 20-23°C but did not excyst at this temperature, the optimum for excystation being 27°C at which temperature the amoebae would not grow.

The life-cycle of E. terrapinae was found to differ from that described for this species by Sanders and Cleveland (1930) and also from that described for any other Entamoeba species. According to Sanders and Cleveland, E. terrapinae excysts as a four-nucleate trophozoite, which divides into two binucleate amoebae, each of which, by a cytoplasmic division, gives rise to two uninucleate trophozoites. In the present studies it was found that prior to excystation the quadrinucleate cyst undergoes a reduction in the number of its nuclei, and always excysts as a binucleate trophozoite.

Studies on natural infections of turtles, Chrysemys elegans and Chrysemys picta, showed that E. terrapinae lives as a harmless commensal throughout the stomach, and the small and large intestines. Experimental infections of garter snakes, water snakes, and whip snakes with E. terrapinae showed that this amoeba is infective but non-pathogenic for snakes and that the infection dies out after a period of from four to eight weeks.

Comparative studies were made on E. histolytica, E. invadens, and E. terrapinae, all three species being maintained with a single species of bacteria, Clostridium tertium in Locke's and in Ringer's serum at 30°C and 35°C. In addition, a comparison of E. terrapinae and E. invadens from the hosts, turtles and snakes, was made. All three species were morphologically identical with the exception of a few minor differences listed below:

(1) the cytoplasm in E. histolytica was clear and homogenous; in E. terrapinae and E. invadens it had a granular appearance;

(2) E. histolytica was sluggish whereas E. invadens and E. terrapinae were very active;

(3) E. histolytica was more constant in size and was consistently smaller than E. invadens and E. terrapinae; from their natural hosts and

from identical cultures, E. invadens and E. terrapinae were strikingly similar in size and morphology, the one exception being that in Locke's at 30°C E. invadens was consistently larger than E. terrapinae;

(4) in culture the nucleo-cytoplasmic ratio of E. invadens was 0.252; that of E. terrapinae was 0.292;

(5) in experimental infections of snakes trophozoites of E. terrapinae had an average diameter of 20.32 u with a nucleo-cytoplasmic ratio of 0.26; trophozoites of E. invadens averaged 14.76 u with a nucleo-cytoplasmic ratio of 0.32.

These results indicate that E. histolytica, E. invadens, and E. terrapinae cannot be distinguished by such characters as size and morphology. Significant differences were found in the growth of these three species in vitro as follows:

(1) cultures could be distinguished by the degree of encystation, E. histolytica showing no encystation, E. terrapinae showing good encystation on the third and fourth days, and E. invadens showing a phenomenal degree of encystation, cysts increasing in number as the culture aged until they usually outnumbered trophozoites;

(2) all three species grew better in Ringer's serum, E. histolytica at 35°C and E. terrapinae and E. invadens at 30°C;

(3) at 30°C E. histolytica showed poor growth in Ringer's serum and did not even survive in Locke's at this temperature; E. invadens grew poorly in Locke's at 35°C; E. terrapinae showed good growth in both media at 30°C and at 35°C.

(4) E. histolytica did not consistently reach a growth peak; E. terrapinae reached a growth peak on the third or fourth day, and E. invadens showed two or three growth peaks in any one culture;

(5) cultures of E. invadens survived three weeks, those of E. terrapinae and E. histolytica survived two weeks.

These results indicate that E. terrapinae, E. invadens, and E. histolytica are more easily distinguished by their physiological requirements than by their morphological characters.

The possible synonymy of E. terrapinae with E. varani, Lavier 1928, should be considered. E. varani was found in a snake but had produced no disease. E. terrapinae is infective but non-pathogenic for snakes. Moreover, the two species resemble each other closely in size and morphology.

It was found that the size of E. terrapinae in the host agreed with that given by Sanders and Cleveland (1930) and the size from cultures agreed with that of E. knowlesi, Rodhain and Van Hoof 1947, and with that of Entamoeba sp., Knowles and Das Gupta 1930, both of which were measured from cultures. As these species have been distinguished on the basis of size alone, their possible synonymy should be considered.

BIBLIOGRAPHY

1. Dobell, C. (1909). Researches on the intestinal protozoa of frogs and toads. J. Micro. Sci. 53: 201.

2. Dobell, C. (1919). The Amoebae Living in Man: a zoological monograph. John Bale, Sons & Danielsson, Ltd., London.

3. Dobell, C. (1928). Researches on the intestinal protozoa of monkeys and man.
I. General introduction. II Description of the whole life-history of Entamoeba histolytica in cultures. Parasitology 20: 357.

4. Dobell, C. (1938). Researches on the intestinal protozoa of monkeys and man. IX. The life-history of Entamoeba coli, with special reference to metacystic development. Parasitology 30: 195.

5. Dobell, C. (1942). Some new methods for studying intestinal amoebae and other protozoa. Parasitology 34: 101.

6. Geiman, Q.M. (1937). Cross-infection experiments with three species of amoebae from reptiles. J. Parasit. 23: 557.

7. Geiman, Q.M. and Ratcliffe, H.L. (1936). Morphology and life-cycle of an amoeba producing amoebiasis in reptiles. J. Parasit. 28: 208.

8. Geiman, Q.M. and Wichterman, R. (1937). Intestinal protozoa from Galapagos tortoises (with descriptions of three new species). J. Parasit. 23: 331.

9. Knowles, R. and Das Gupta, B.M. (1930). On two intestinal Protozoa of an Indian turtle. Ind. J. Med. Res. 18: 97.

10. Lavier, G. (1928). Entamoeba varani n. sp. amibe parasite de reptile. Ann. Parasit. 6: 152.

11. Ratcliffe, H.L. and Geiman, Q.M. (1938). Spontaneous and experimental amoebic infection in reptiles. Arch. Path. 25: 160.

12. Reardon, L.V. and Bartgis, I.L. (1949). The cultivation of the small race of Endamoeba histolytica with a single species of bacteria. J. Parasit. 35: 218.

13. Rodhain, J. (1934). Entamoeba invadens n. sp. parasite de serpents. C.R. Soc. Biol., Paris. 117: 1195.

14. Rodhain, J. and Van Hoof, M. Th. (1935). Les Chéloniens porteurs d'Entamoeba invadens. C.R. Soc. Biol., Paris. 121; 156.

15. Rodhain, V. and Van Hoof, M. Th. (1947). Entamoeba knowlesi n. sp. parasite de deux tortues: Terrapinae cinostermoides et Platysternum megacephalum. Ann. Parasit. 22: 127.

16. Sanders, E.P. (1931). The life-cycle of Entamoeba ranarum, Grassi (1879). Arch. of. Protistenk. 74: 365.

17. Sanders, E.P. and Cleveland, L.R. (1930). The morphology and life-cycle of Entamoeba terrapinae, spec. nov. from the terrapin, Chrysemys elegans. Arch. f. Protistenk. 70: 267.

18. Taliaferro, W.H. and Fisher, A.B. (1926). The morphology of motile and encysted Entamoeba ranarum in culture. Ann. Trop. Med. and Parasit. 20: 89.

19. Taliaferro, W.H. and Holmes, F.O. (1924). Entamoeba barreti n. sp. from the turtle, Chelydra serpentina; a description of the amoeba from the vertebrate host and from Barret and Smith's cultures. Amer. J. Hyg. 4: 160.

20. Wenyon, C.M. (1926). Protozoology: A manual for medical men, veterinarians and zoologists. Vols. I and II, Baillière, Tindall & Cox, London.

EXPLANATION OF FIGURES

Figures 1-4. Trophozoites from cultures - drawn to show the structure of the nucleus and the division of the karyosome into two granules. Food inclusions are not shown. They were fixed in Schaudinn's fluid and stained with Heidenhain's iron haematoxylin.

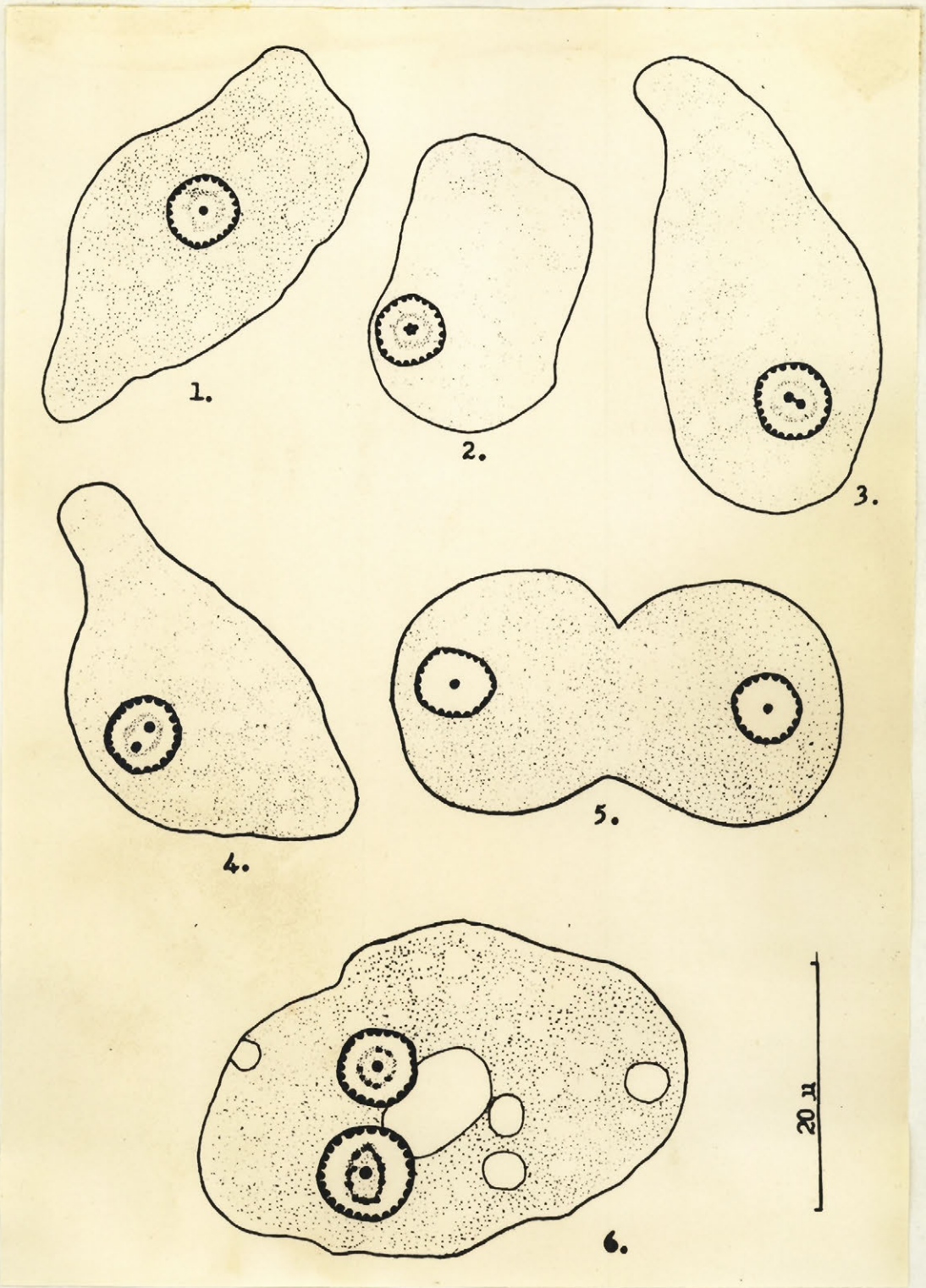
Figure 5. A binucleate trophozoite undergoing a cytoplasmic division. (Stained in D'Antoni's iodine solution).

Figure 6. A binucleate trophozoite from the large intestine of the turtle, Chrysemys elegans. The nuclei show a number of chromatin granules on the achromatic membrane surrounding the karyosome. (Stained with Heidenhain's iron haematoxylin).

Figures 7-13. Cysts from culture showing the progressive development from the uninucleate to the quadrinucleate stage. (Figures 7 and 8 were stained with Mallory's acid phosphotungstic haematoxylin; figures 9-13, with Heidenhain's iron haematoxylin).

Figure 14. A binucleate trophozoite which has just completed excystation. One nucleus shows two chromatin granules in the karyosome.

Figures 15 (a), (b), and (c), are diagrammatic drawings of three trophozoites just excysting. The slides were stained with D'Antoni's iodine solution.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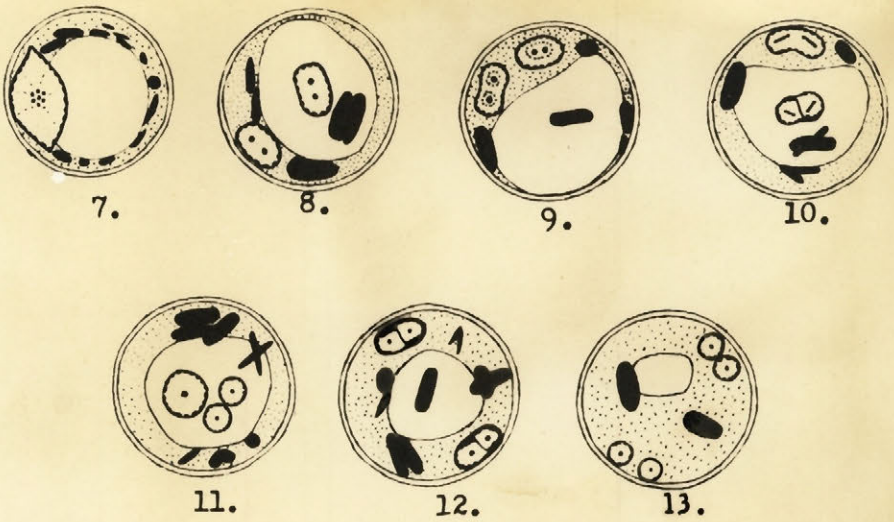
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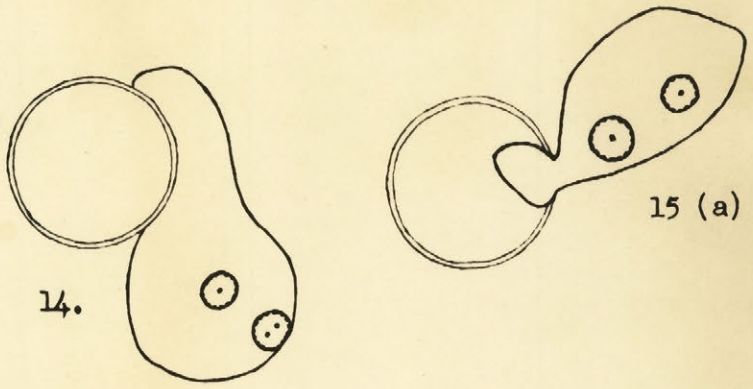
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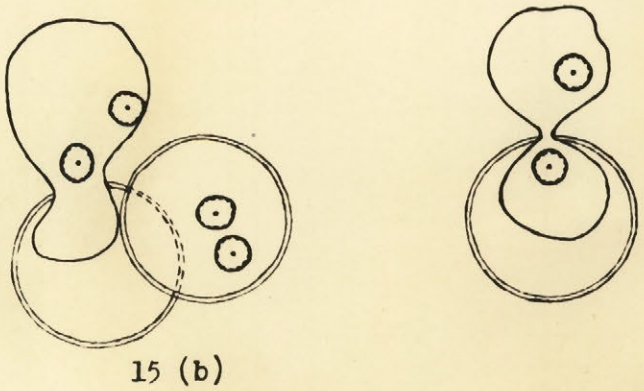
15 (b)
15 (c)