THE USE OF AQUEOUS SMEARS IN THE STUDY AND IDENTIFICATION OF THE AMOEBAE OF MAN[1]

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This paper deals with observations of amoebae from the human stool when examined in direct smears in water and with the application of this procedure in the diagnosis of amoebiasis.

During the course of a survey for amoebic infections a stool was found to have trophozoites of *Endamoeba histolytica* which in the routine smear in normal saline solution had nuclei showing with unusual clearness. No explanation of this change could be found at that time. About a month later (August, 1934) the same phenomenon was seen in a stool from a patient with acute amoebic colitis. The collection of this stool had been observed and it had been noted that the bed pan into which the small amount of mucoid feces had been passed, contained some water. It was then decided to observe the effect of water on the trophozoites of *E. histolytica*. Direct smears were made using tap water instead of normal saline solution and as suspected the preparations revealed in the trophozoites the same clearly visible nuclei. Further observations on the trophozoites of various amoebae in aqueous smears elicited characteristic differences in the distensibility and fragility of the ectoplasm and appearance of the nucleus which proved of considerable importance in the differentiation of species.

TROPHOZOITES OF *Endamoeba histolytica* IN AQUEOUS SMEARS

In general the following changes were observed in the trophozoites of *E. histolytica* when examined in the aqueous smear. The

[1] The stools used in this study were obtained from patients in the Retiro Matias Hernandez, an institution for the insane of the Republic of Panamá. I am indebted to the superintendent of this institution, Dr. R. P. Hargreaves and his staff for their kind and ready coöperation in obtaining specimens.

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trophozoite quickly loses its motility, becomes spherical and distends. The endoplasm gradually becomes thin and watery and the nucleus stands out in bold relief showing clearly the nuclear membrane, the peripheral chromatin layer and the karyosome. These changes are illustrated in plate 1, the drawings of which show two trophozoites in the various stages of distention and disintegration, one from a stool of a patient with acute amoebic colitis (figures 1 to 9), the other, which happened to be bi-nucleate, from a stool of a carrier (figures 10 to 14) with very small trophozoites measuring only from 10 to 11 microns. As shown in the drawings even the small delicate trophozoites of this stage of amoebiasis allow a remarkable distention and exhibit their nuclei in a striking manner. More than 300 trophozoites from fifteen cases of amoebiasis have been observed as they passed through the various stages in the aqueous smear. A characteristic pattern can be seen in these changes the important features of which are: (1) the visibility of the nucleus, (2) the distensibility of the ectoplasm.

The visibility of the nucleus apparently is the result of two factors. A granulation and later clumping of the peripheral chromatin, and a thinning of the endoplasm allowing more penetration of light and better contrast between the dense chromatin granules and the lighter endoplasmic matter. In the fine gray annular line which first outlines the nucleus no granulations can be seen, probably because they are too minute or because of the obscuring effect of the still heavy endoplasm. Soon this outline of the nucleus darkens slightly and it then can be recognized as the nuclear membrane with minute, uniform, evenly distributed granules on its inner surface. At this time the karyosome usually becomes visible as a small gray central dot. In this stage the nuclear membrane and the chromatin granules can be seen only at the "equator" of the nucleus and it therefore appears as a beaded ring instead of a spheroid. Since the chromatin granules cannot be seen in all planes it is probable that each granule of the beaded ring represents several partially superimposed granules and that actually the peripheral chromatin granules are much more minute than even those first discerned.

As the trophozoite distends and the endoplasm becomes thinner, the fine beads of the chromatin layer gradually coalesce forming granules of increasing size and deepening shade. These larger peripheral chromatin granules usually can be seen in several planes and the spherical form of the nucleus now readily can be perceived. The karyosome changes from the rather inconspicuous gray dot to a clear black slightly irregular granule. Further coalesence of the chromatin leads to the formation of heavy black bars and masses which usually remain attached to the nuclear membrane. This disintegration of the nuclear chromatin usually takes place gradually with the increasing dilution of the endoplasm. Occasionally, however, a trophozoite will rupture early, while the nucleus is visible only as a gray beaded ring, and then suddenly, as soon as the water reaches the nucleus, the chromatin granules coalesce as if a chemical precipitation had taken place. At times this sudden change also results in the collapse and disintegration of the nucleus, but usually the nuclear membrane remains intact. As a matter of fact the nuclear membrane appears to be a remarkably strong structure. It frequently happens that when a trophozoite ruptures the nucleus pops out, very much like a seed squeezed from a cherry, and then lies free in the aqueous medium. In one instance a nucleus thusly isolated was seen to remain intact for fourteen minutes. In another aqueous smear it was observed as it was being carried away by a current under the coverglass revealing, as it rolled, very clearly its spherical form and peripheral layer of chromatin granules.

The remarkable tensile strength of the ectoplasm is an equally important feature. The average trophozoite in good condition will allow a distention which increases its diameter 60 per cent, and will withstand the increasing pressure for 30 minutes. With this distensibility there is associated a high degree of resilience. A distended trophozoite flattened out by pressure on the coverglass will resume its spherical form like a rubber ball when the pressure is released.

Deviations from the characteristic pattern have been observed. In order to interpret these correctly it is necessary to keep in

mind the well known fact that the trophozoites found in a stool vary in age and condition. Even in a stool examined within a few minutes after being passed they frequently can be seen in two or more of the following stages of viability: (1) Forms in rapid progressive motion with a freely flowing endoplasm in which no nuclei can be seen. (2) Forms similarly active but in which a faint gray or clear black annular outline of the peripheral chromatin of the nucleus is visible. (3) Forms in sluggish motion without progression, with the often vacuolated endoplasm in a central seemingly congealed mass within which not infrequently a nuclear ring of heavy chromatin granules can be seen. (4) Immotile forms, rounded up as if dead, having a central mass of granules and vacuoles and a clear peripheral layer of varying width. In these the nucleus when not concealed by the thick endoplasm shows advanced degeneration, appearing as a ring of large granules or clumps of chromatin. Not uncommonly the trophozoites in this stage will revive in normal saline smears and show sluggish or even active motility. (5) Forms obviously dead in which the clear ectoplasmic peripheral layer has disappeared leaving a round or oblong mass of grayish-yellow coarse granules in which remnants of the nucleus usually can be seen as large black granules or a few bars arranged in an incomplete circle or nondescript mass. In all but the last lifeless stage, the trophozoites distend in the aqueous smear, but the extent and duration decrease as they degenerate.

In the aqueous smear from the fresh exudate of acute amoebic colitis in which usually all the trophozoites are in an excellent condition, the degree and duration of the distention are quite uniform. There is an increase in the diameter of from 55 to 65 per cent. For example, two trophozoites increased from 20 and 24.5 microns to 31.5 and 39.5 microns, respectively. One trophozoite, the original size of which had not been determined measured 52.8 microns before it ruptured. A few of these healthy trophozoites will begin to rupture after fifteen minutes but the great majority will withstand the distention for thirty minutes. Some have been observed intact for one hour and a few for an hour and a half.

In the mushy stools of subacute cases and from carriers the trophozoites are less uniform in their physical condition. Some rupture after two or three minutes, but most of them take the distention and withstand it in the same manner as the vigorous trophozoites of acute amoebic colitis. As the stools become old and the trophozoites degenerate, they gradually lose their distensibility. The rapidity of this process depends to some extent upon the nature of the stool. With slight putrefaction as in the stools made up mostly of vegetable débris the trophozoites may exhibit a short but characteristic distention twenty-four hours after the stools were passed, while in the rapidly liquifying stools containing the muco-sanguinous exudate of acute amoebic colitis they degenerate and lose their distensibility in from five to nine hours.

The appearance of the nucleus in the aqueous smear also varies with the age and physical condition of the trophozoite. In smears from the fresh exudate of acute amoebic colitis almost all the trophozoites show their nuclei from the earliest phase, while in smears from stools of subacute cases or carriers the nuclei in some trophozoites will show, from their very first appearance, the beading of the peripheral chromatin or the large granules of further coalescence, even the large clumps and bars of complete disintegration of the peripheral chromatin layer. As a stool becomes old, an increasing number of nuclei with the chromatin layer in advanced stages of disintegration will be seen.

TROPHOZOITES OF *Endamoeba coli* IN AQUEOUS SMEARS

The trophozoites of *E. coli* in aqueous smears also exhibit distention and increased visibility of the nucleus, but unlike *E. histolytica* the ectoplasm appears to be very fragile and except in very fresh stools ruptures while the preparation is being made or within four to five minutes (plate 2, figures 1 to 8). During this short period of distention there is no thinning and spreading of the endoplasm as in *E. histolytica* but it remains as a central, almost undisturbed mass, while the ectoplasm distends into a wide clear peripheral layer. The distention terminates in rupture which frequently is explosive producing a wide tear in the

ectoplasm through which some of the endoplasm may protrude, but more often the trophozoites shrink without a visible rupture although careful observation will reveal in most instances a sudden coalescence and darkening of the nuclear chromatin and a sharpening of the contours of the endoplasmic granules and vacuoles just before the trophozoites begin to shrink, indicating that a rupture has occurred and water suffused into the trophozoite. The trophozoites which have suffered a gross tear in the ectoplasm are easily recognized as remnants, but those which have shrunken without any visible injury to the ectoplasm may appear as if they had suffered no change in the water except to round up and become immotile. They can be identified as remnants by a few minutes observation, since they will show no distention but further shrinking.

The nucleus of the trophozoites of *E. coli* in the aqueous smear is not as clearly seen as in the trophozoite of *E. histolytica*. This is apparently due to the fact that the endoplasm in which the nucleus is more or less imbedded remains almost unchanged in transparency. An early phase corresponding to the appearance of the fine, gray, annular line in the nucleus of *E. histolytica*, has not been observed. The nucleus of *E. coli* at its very first appearance is a circle of relatively large black granules. When the trophozoite ruptures, or sometimes a few minutes later, the chromatin layer coalesces into a few large granules and bars, sometimes into a crescent shaped or U-shaped mass, all of which are unimportant variations in the final disintegration of the chromatin layer. A karyosome which can be definitely identified as such is seldom seen. In 178 trophozoites of *E. coli* observed in the aqueous smear, a granule which unmistakably was the karyosome was visible in only eight nuclei. However, a granule which possibly was the karyosome was seen eccentrically placed close to the granules of the peripheral chromatin layer in many nuclei.

There are then some very definite differentiating features in the changes which the trophozoites of *E. histolytica* and *E. coli* suffer in the aqueous smear. In some stools however, there will be trophozoites in which these features have been lost. The

degenerate trophozoite of *E. histolytica* with shortened duration of distention and partial disintegration of the chromatin layer cannot be differentiated with certainty from *E. coli*. Since such degenerate trophozoites are present in almost all stools, except in some fresh exudates from acute amoebic colitis, it is obvious that not in all aqueous smears can all the trophozoites observed be identified.

In some fresh stools an occasional trophozoite of *E. coli* may allow a considerable distention which may last as long as ten minutes and which may be associated with thinning and dispersion of the endoplasm as in *E. histolytica*. Although these trophozoites present no positive points for differentiation from *E. histolytica* they usually can be identified on the shorter duration of distention, larger granules in the chromatin layer of the nucleus, and the absence of a central karyosome.

Endolimax nana IN AQUEOUS SMEARS

In the aqueous smear the trophozoite of *E. nana* appears to be very fragile. The cycle of distention, rupture and shrinking is completed within from one to four minutes and unless the smear is made quickly and examined immediately no trophozoites in distention may be seen. Since the small remnants are recognized with difficulty, it then may seem as if the trophozoites had disappeared. In the distended trophozoite (plate 2, figure 9) which measures from 12 to 14 microns, the endoplasmic granules are evenly distributed or more often in a cluster at the periphery and the nucleus which usually becomes visible when the maximum distention is reached appears as a gray or steel-blue disc of chromatin with a ring of minute granules at its periphery. After the short period of distention the trophozoites shrink rather quickly without visible rupture of the ectoplasm and become irregularly rounded masses of closely packed granules in which the steel-blue nuclear chromatin frequently remains visible (plate 2, figure 2). These changes have a practical value particularly in the differentiation of *E. nana* from the small trophozoites of *E. histolytica* sometimes seen in stools from carriers.

Dientamoeba fragilis IN AQUEOUS SMEARS

In the aqueous medium this supposedly fragile amoeba exhibits an ectoplasm of notable toughness and resilience. As in *E. nana* the distention is rapid, but it lasts from two to eight minutes thus affording ample time for observation. By the time the preparation has been made and is under the microscope all the amoebae have rounded up and become immotile. The endoplasm with uniformly distributed granules is thin and becomes visibly thinner as the distention progresses. When fully distended, amoebae which in the control smear in normal saline solution had a diameter of 10 to 13 microns, have measured from 16 to 20 microns. The distention terminates abruptly with an explosive rupture of the ectoplasm and a quick and complete expulsion of the endoplasmic mass, following which the ectoplasmic shell immediately regains its spherical form without trace of the tear. The nuclei usually become visible just before the amoeba ruptures. They appear as gray or steel-blue discs with a ring of very fine black granules at the periphery and resemble closely the nucleus of *E. nana*. When the amoebae ruptures the nuclei are either expelled with the endoplasm or they remain close to the periphery within the otherwise empty shell.

When expelled they may be seen sometimes at the edge of the endoplasmic mass or completely isolated lying free in the aqueous medium. When within the emptied shell they stand out very clearly, and these remnants of the amoebae present indeed a diagnostic picture, particularly in the bi-nucleate individuals (plate 2, figures 11 to 15).

It should be noted that all stools used for this study of *Dientamoeba fragilis* were obtained from one patient. It is possible that dientamoeba from other infections may show variations in the reaction above described.

ORGANISMS RESEMBLING AMOEBAE IN THE AQUEOUS SMEAR

The flagellates

The flagellates are subject to the same changes in the aqueous smears as the amoebae. They round up, become immotile, dis-

tend, shrink and disintegrate. And as in the case of amoebae, the duration of these events depends upon the physical condition of the animal. Vigorous flagellates from fresh stools may retain their motility for some minutes in a rather thick smear while the dying flagellates from old stools may disintegrate while the preparation is being made. During the distention they are quite characteristic with their thin cytoplasm in which only a few granules and slender rods are seen, and sometimes steel-blue granules of nuclear chromatin. While shrinking they become more granular and resemble the remnants of *E. nana*, particularly *Chilomastix mesnili*.

Blastocystis hominis

In the aqueous smear *Blastocystis hominis* also presents essentially the same changes as amoebae—distention, increased visibility of the nuclei and disintegration. The distention is always rapid and practically completed by the time the preparation is ready to be examined. It varies in duration, approximately from a minute to an hour. During the distention the peripheral cytoplasm becomes very clear and the nuclei within it show as prominent black or steel-blue granules. Small blastocysts may have a superficial resemblance to trophozoites of *E. nana* or *Dientamoeba fragilis* at some stages of their distention in the aqueous smear, but the clear or ground glass appearance of the central vacuole in the blastocysts differentiates them from the granular amoebae. The empty endoplasmic shell of ruptured dientamoebae from which the nuclei have been expelled with the endoplasm, may resemble a distended blastocyst, but these remnants always contain swarms of very minute granules in brownian movement.

In this connection it may be mentioned that for the purpose of finding protozoan cysts in feces with blastocysts, the aqueous smear is an excellent preparation. The difficulty of finding the cysts in smears crowded with blastocysts is well known. Frequently the protozoan cysts and the blastocysts cannot be distinguished under the 16 mm. objective; the smear then has to be examined with the 4 mm. objective and hundreds of blastocysts

may have to be scrutinized before it can be determined what cysts or that no cysts are present. No matter how patient the worker may be he probably will call the smear negative long before it has been thoroughly covered. In the aqueous smear the blastocysts have ruptured and disappeared or they have become so distended and watery that they can barely be seen and certainly not confused with the cysts.

TECHNIC

Tap water, which in this laboratory is a chlorinated water neutral to litmus, has been used for the routine aqueous smear. To determine whether or not some salt in this tap water influenced the process of distention and the nuclear changes, smears in distilled water have been made from many stools. Assuming that the distention of the trophozoites is the result of endosmosis, theoretically there should be more rapid distention in the distilled water, and this actually can be observed if the same amount of fecal material be used for making the smears. The rapidity of the distention and the nuclear changes can be regulated at will, however, in both tap water and distilled water by varying the amount of fecal material stirred in the drop of water. Presumably the osmotic pressure in the aqueous smear depends mainly upon the amount of salts dissolving out of the fecal material used. Thus a thick smear in distilled water will show the trophozoites distending less rapidly and lasting longer than in a thin smear in tap water. Successful aqueous smears can be made with wide variations in the dilution. A smear slightly thinner than the routine smear in normal saline solution is the most satisfactory. In this the distention and the nuclear changes progress slowly enough to allow careful observation and fecal débris seldom interfere with a clear view of the trophozoites. In very thin smears the trophozoites may rupture before the characteristic features develop or if they develop, before they can be observed.

In very thick smears the distention is unnecessarily slow and some trophozoites will be protected by fecal débris and escape the change. This is apt to occur particularly in smears from mucoid exudates of acute amoebic colitis in which lumps of mucus

may remain and within these, trophozoites well protected against the water. This condition may give rise to curious trophozoites, observed in several aqueous smears. Instead of rounding up, to distend and die, they seem to adjust themselves to the aqueous medium and keep moving. The endoplasm becomes thin and full of vacuoles and the nucleus disintegrates and disappears, but still they stay alive. In one smear they were seen in rapid flowing progressive movement five hours after the preparation was made. At this time the endoplasm appeared as thin as the surrounding water, there was no trace of the nucleus but numerous vacuoles clear as bubbles rolled around like marbles as the animal surged across the field.

SUMMARY

The trophozoites of *E. histolytica, E. coli* and *E. nana* and of *Dientamoebae fragilis* have been observed in aqueous smears and the essential findings recorded. Material for a similar study of the trophozoites of *Iodamoeba butschlii* has not been available. The observations indicate that certain features in the morphology of the amoebae can be demonstrated in the aqueous medium, such as (1) the distensibility of the ectoplasm of the *E. histolytica,* (2) the relative fragility of the ectoplasm of the *E. coli* and *E. nana,* (3) the resiliency of the ectoplasm of the *Dientamoeba fragilis,* (4) the remarkable strength of the nuclear membrane of the *E. histolytica* and *E. coli.*

Some of these characteristics together with the visibility of the nuclei help to differentiate the species of amoebae in the human stool.

It also has been recorded that the annoyance of *Blastocystis hominis* can be eliminated by the use of the aqueous smear.

I have been unable to find any reference in the literature to the use of aqueous smears in the study of the amoebae of man. However, it does not seem possible that such a simple procedure has not been used by some workers.

It is my pleasure to acknowledge the generous and valuable advice of Dr. C. M. Johnson, protozoölogist at the Gorgas Memorial Laboratory and the efficient assistance of J. F. Buckner and H. A. Down of the Hospital Corps, U. S. Navy.

PLATE 1

All drawings from aqueous smears. × 1,000. The time elapsed from the moment the fecal particle, used for the smear, first touched the water is noted under each figure.

FIGS. 1 TO 9. DRAWINGS OF A TROPHOZOITE OF E. HISTOLYTICA FROM A CASE OF ACUTE AMOEBIC COLITIS. ABSTRACTS OF NOTES MADE DURING THE OBSERVATION, AS FOLLOWS

FIG. 1. The trophozoite is immotile, rounded up. About one-fourth of its contents is free of granules, the rest is a finely granular mass in which are imbedded three partly digested erythrocytes. At the edge of the granular mass there appeared, while the trophozoite first was being observed, a faint gray annular ring, the earliest indication of the nucleus.

FIG. 2. The nucleus is clearer. A fine beading now can be seen in the annular line and a small central karyosome has appeared.

FIG. 3. Two of the erythrocytes have faded considerably. The peripheral chromatin granules of the nucleus have changed from gray to black and appear larger. The nuclear membrane is clearly visible. The karyosome has changed from gray to black and is strikingly prominent.

FIG. 4. The granular endoplasm has spread out to all parts. Two of the erythrocytes have faded out. Vacuoles forming.

FIG. 5. Distention continues, endoplasm thinner, more vacuoles, last erythrocyte has faded out. The chromatin granules of the nucleus are larger but still form an evenly beaded ring.

FIG. 6. The chromatin granules have coalesced into twelve larger ones. The granules of other planes of the chromatin layer are also visible and give the center of the nucleus a granular appearance.

FIG. 7. No further distention. Several clear spaces have formed. The nucleus, floating in one of these, is unchanged.

FIG. 8. The trophozoite ruptured and the contents which now lies partially expelled quickly changed to a mass of vacuoles separated by strands of granules. Nucleus unchanged.

FIG. 9. The nucleus crumbled into a mass of large granules.

FIGS. 10 TO 14. DRAWINGS OF A TROPHOZOITE OF E. HISTOLYTICA FROM A CARRIER

FIG. 10. The trophozoite is immotile and spherical, filled with finely and uniformly granular endoplasm. No nucleus can be seen.

FIG. 11. A very fine, gray, annular line has appeared in the endoplasm which now appears thinner.

FIG. 12. Endoplasm thinner. Minute granules of uniform size have appeared in the nuclear ring. The karyosome is not visible.

FIG. 13. The trophozoite was turned by a current in the preparation and a second nucleus revealed itself. The two nuclei are identical with finely beaded rings and small central karyosomes.

FIG. 14. No further distention. The small granules of the nuclear chromatin have begun to coalesce and have changed from gray to black. Observation of this trophozoite discountinued and the preparation searched for additional trophozoites. Nine more found intact and in the same stage of distention as in figure 14. One was bi-nucleate.

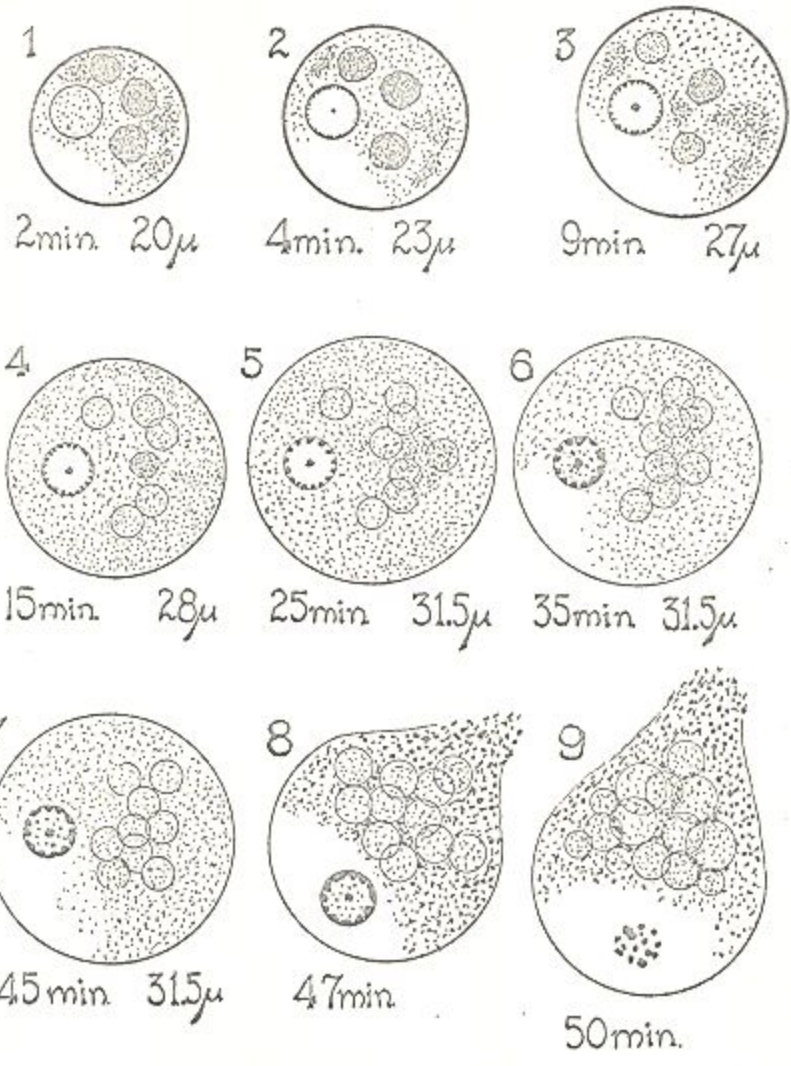
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PLATE 2

All drawings from aqueous smears. × 1,000. The minutes recorded under the figures is the time elapsed from the moment the fecal particle, used for the smear, first touched the water.

FIGS. 1 TO 4. DRAWINGS OF A TROPHOZOITE OF E. COLI IN WHICH THE DISTENTION IS FOLLOWED BY SHRINKING WITHOUT VISIBLE RUPTURE

FIG. 1. The trophozoite is rounded up, immotile. An annular line with irregular granules outlines the nucleus which appears to be imbedded in the granules and débris of the endoplasm.

FIG. 2. The clear peripheral ectoplasmic layer has widened; the endoplasm remains undisturbed as a rounded central mass. The chromatin of the nucleus has coalesced into a few large masses. No karyosome can be seen.

FIG. 3. The clear peripheral zone almost has disappeared. The chromatin masses of the nucleus have become very dense, but no karyosome is visible.

FIG. 4. The trophozoite has shrunken into a remnant of coarse granules and débris. The disintegrated nucleus appears as a U-shaped mass of chromatin.

FIGS. 5 TO 8. DRAWINGS OF ANOTHER TROPHOZOITE OF E. COLI IN WHICH THE DISTENTION TERMINATED BY VISIBLE RUPTURE

FIG. 5. The trophozoite is immotile but not yet rounded up. The nucleus is not visible.

FIG. 6. The clear ectoplasm now is evenly distributed in a peripheral layer, endoplasm coarsely granular, nucleus not visible.

FIG. 7. The peripheral ectoplasm has widened, increasing the diameter of the trophozoite from 24 to 31 microns. A few heavy masses of nuclear chromatin now can be seen, no karyosome.

FIG. 8. The trophozoite has ruptured and part of the endoplasm expelled through the wide tear of the ectoplasm. The nucleus is a mass of dense irregular granules.

FIGS. 9 TO 10. ENDOLIMAX NANA

FIG. 9. The distended trophozoite with thin, finely granular endoplasm and visible nucleus which appears as a gray or steel-blue disc.

FIG. 10. The trophozoite has shrunken without visible rupture and become a coarsely granular irregular mass with the nucleus still visible.

FIGS. 11 TO 15. DIENTAMOEBA FRAGILIS

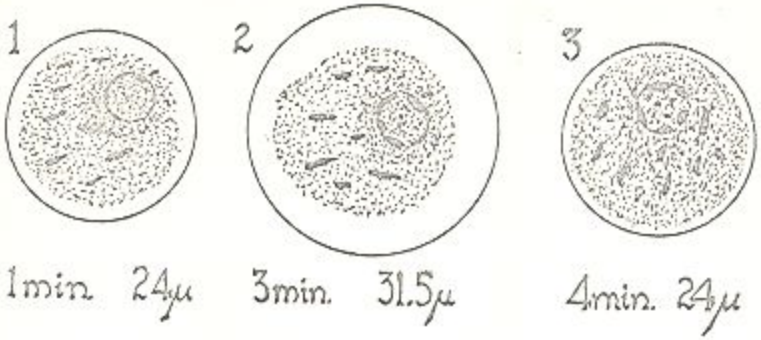
FIG. 11. Amoeba in early phase of distention. No nucleus visible.

FIG. 12. Distention has increased the diameter from 11 to 16 microns. Endoplasm very thin.

FIG. 13. Maximum distention, just before rupture. The two nuclei are seen as steel-blue discs.

FIG. 14. The amoeba has ruptured and after expulsion of the endoplasm, the ectoplasm has regained its spherical form and obliterated the tear. Nuclei remain within the ectoplasm.

FIG. 15. Another *Dientamoeba*, after rupture. One nucleus seen at the edge of the expelled endoplasmic mass. The ectoplasmic shell is empty except for some minute granules.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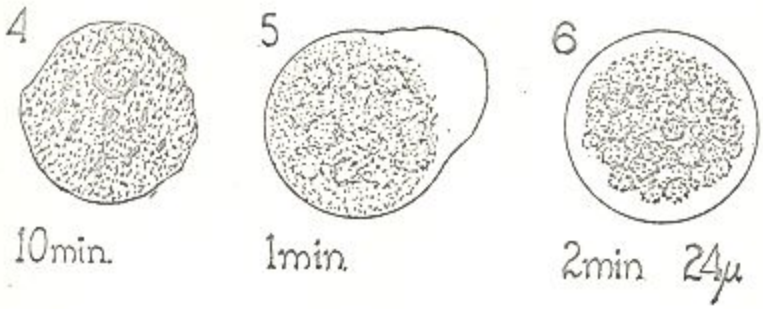
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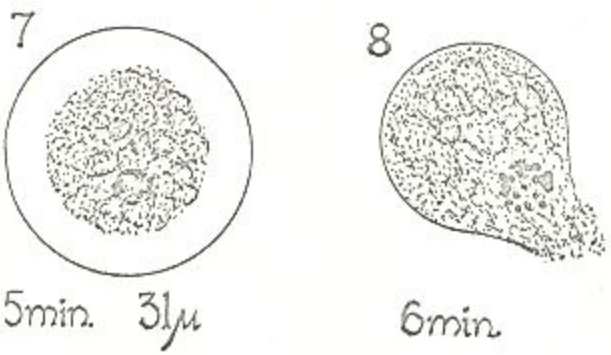
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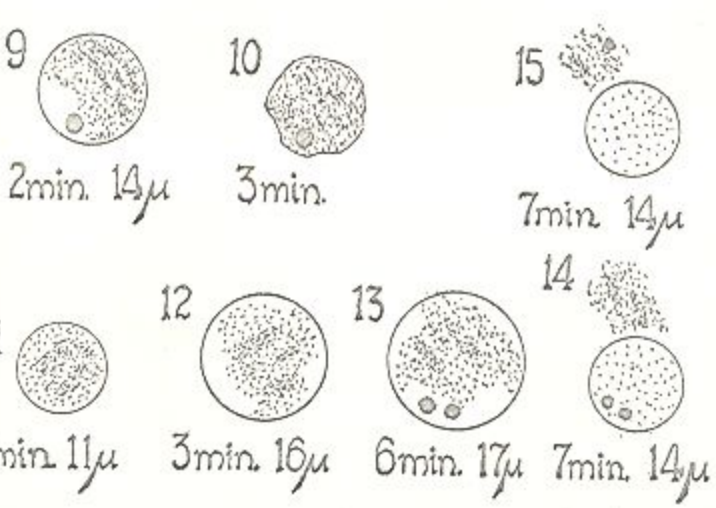
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