MICRURGICAL* STUDIES IN CELL PHYSIOLOGY.

I. The Action of the Chlorides of Na, K, Ca, and Mg on the Protoplasm of Amœba proteus.

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Plates 3 and 4.

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The rôle of electrolytes in the maintenance of protoplasmic structure and function is one of the most important aspects of cell physiology. The methods previously employed in studying this question have involved chiefly the immersion of cell and tissues in solutions of various electrolytes. By these methods much has been learned, and it is now well recognized that the presence of the cations Na, K, Ca, and Mg, and their maintenance in definite proportions is essential to the cell for its specific activities.

Little or nothing, however, is known of the direct action of electrolytes, or of any other substance, upon the physical state of living protoplasm. This is due to the fact that by the immersion method we have no means of knowing whether the changes observed within the cell are caused by the actual entrance of the substances in question, by their effect on the surface or by their action in causing the diffusion of substances from the cell. An analysis of this problem is possible by means of the micrurgical apparatus which permits the manipulation and dissection of the living cell and the introduction of substances directly into the internal protoplasm (2).

The experiments described in this paper had for their object an investigation of the action of varying concentrations of Na, K, Ca, and Mg chlorides separately, and in some cases in binary combinations, on the protoplasm of *Amœba*. This was ascertained by observing the

* Micrurgy (*micros*, small; *ergon*, work) is a term introduced by Péterfi (1) to denote manipulative technique in the field of the microscope.

effect of (*a*) immersing and of (*b*) tearing amebæ in the different solutions, and of (*c*) injecting the solutions directly into the interior of the cell.

From the results obtained conclusions are reached regarding:

1. The relative toxicity of the salts individually and in various combinations, the site of their toxic action, and their specific action upon the physical state of the protoplasm;
2. The relative penetrating ability of the salts;
3. The nature and site of the antagonistic action of the cations;
4. The nature of the reparation of the protoplasmic surface when mechanically injured in the different solutions.

Amœba proteus was selected because it reacts rapidly and in a characteristic manner to the various solutions and because it is not irreversibly injured by the micro-manipulations, so far as could be determined from its appearance and viability. Another important advantage of using fresh water amebæ is their relative indifference to marked variations in the osmotic pressure of the surrounding medium (3).

Through the courtesy of Dr. J. A. Dawson of Harvard University an abundant supply of carefully cultured material was available for a thoroughly systematic study. Repeated experiments with cultures obtained at different times of the year gave similar quantitative results.

Certain expressions used in this paper should be explained at the outset. By *internal protoplasm* is meant the hyaline material within the cell in which crystalloid and other visible granules and vacuoles are suspended. By *plasmalemma* (4) is meant the somewhat tenacious but delicate surface membrane which can be pulled out and torn with needles. By *liquefaction* is meant an increase in fluidity and not necessarily a solution of the plasmalemma or of the visible granules within the protoplasm. By *solidification* is meant a conversion of the cytoplasm into a non-flowing, jellied, inert mass which may or may not revert to its original, normal flowing state. Whenever a mixture of salts is used, the stated concentration of each salt is that actually present in the mixture. Thus, the expression M/4 NaCl + M/52 $CaCl_2$ indicates that equal volumes of M/2 NaCl and M/26 $CaCl_2$ were mixed so that the resulting solution contains salts in the con-

centrations indicated. Unless otherwise mentioned all solutions were maintained at about pH 7.

1.

Immersion Experiments.

It was first ascertained that the amebæ will live and remain in good condition for at least 5 days in distilled water. The toxicity of the salt solutions was then determined by observing the length of time that amebæ live in different concentrations of each solution.

Amebæ from a healthy stock culture were transferred with a minimum amount of fluid into Syracuse watch-glasses, each of which contained 5 cc. of the solution to be tested. For a given experiment the dishes were arranged in a series of diminishing concentrations of the salt. The successive dilutions were always carried well beyond the point where no toxic effects occurred. From five to ten amebæ were then placed simultaneously in each of the dishes of a series and examined at intervals for changes in motility, shape, internal appearance, and physical consistency, and for longevity.

For each concentration several experiments were performed with amebæ taken at different times. In a given concentration of a salt at least twenty-five amebæ were immersed and for critical values from 100 to 150 were used.

a. Immersion in Solutions of Single Salts.—In NaCl and KCl solutions the amebæ react quickly. They become increasingly sluggish and their pseudopodia are withdrawn until they assume a rounded shape. A period of quiescence follows, during which the larger cytoplasmic granules gradually sink. Upon manipulation with the needle the interior of the quiescent, rounded ameba is found to be liquid. All the granules finally fall and accumulate in a clump at the bottom. If the rounded ameba is rolled over with a needle the clump of granules remains adherent to the originally dependent surface. In NaCl the plasmalemma becomes extremely delicate, disrupts in places, and some of the contents of the interior escape. Slight agitation completes the disruption, so that the entire substance of the ameba tends to scatter in the surrounding medium. The higher the concentration of the surrounding solution the more rapid and complete is the disruption.

Even if the dead ameba is not disturbed the plasmalemma ultimately disappears, while a part of the interior persists as a loose coagulum. This coagulum is due possibly to a precipitation by long continued action of the salt. In KCl the surface becomes very viscid and is never as delicate and friable as in NaCl. In $CaCl_2$ and in $MgCl_2$ the amebæ are active and appear normal as long as they live.

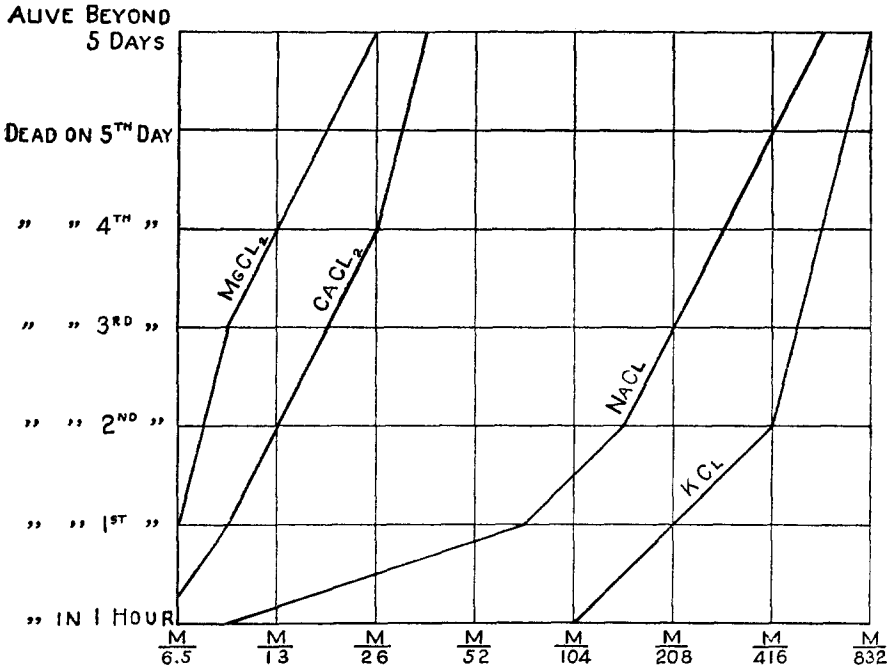

FIG. 1. Viability of amebæ in decreasing concentrations of $MgCl_2$, $CaCl_2$, NaCl, and KCl solutions.

Fig. 1 illustrates the relative toxic effects of each of the four salts in a given period of time. It will be noted at once that the monovalent cations are decidedly more toxic than the divalent. The toxicity effect for a given concentration is in the order KCl > NaCl > $CaCl_2$ > $MgCl_2$.

b. Immersion in Solutions of Two Salts.—The antagonistic action of $CaCl_2$ toward NaCl and toward KCl was determined by measuring the length of time that the amebæ remain alive in the various com-

binations. Concentrations of $CaCl_2$ which are non-lethal within 3 days were mixed with concentrations of NaCl and of KCl which are lethal within that time. It was found (Figs. 2 and 3) that $CaCl_2$ in a considerable range of concentrations above a definite minimum antagonizes the lethal action of the monovalent salts.

In Figs. 2 and 3 the abscissæ represent the different toxic concentra-

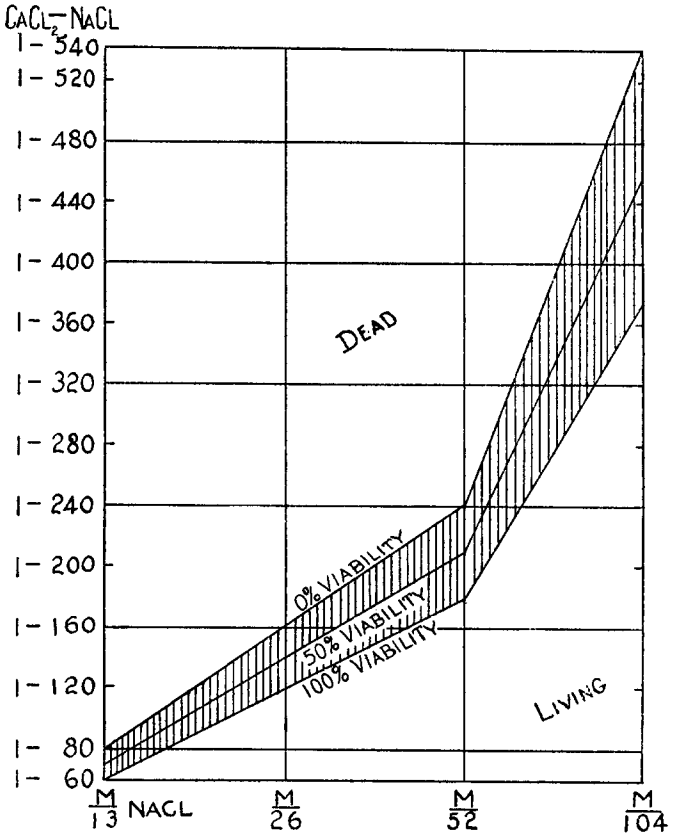

FIG. 2. Antagonism of $CaCl_2$ toward decreasing concentrations of lethal doses of NaCl, judged by survival of amebæ immersed in mixtures of these salts. The ordinates represent the ratios of NaCl to $CaCl_2$ for the different concentrations of NaCl represented by the abscissæ. The shaded area indicates the zone of gradation between viability and non-viability in the different concentrations. Above this the $CaCl_2$ is not sufficient to antagonize the lethal action of the NaCl. Below it, the lethal action is completely antagonized by the $CaCl_2$.

tions of NaCl and of KCl which were used in combination with $CaCl_2$. The ordinates represent the ratio of the concentrations of $CaCl_2$ to that of NaCl or of KCl. The shaded band represents the zone of the different combined concentrations which lie just between the lethal and the non-lethal doses. In the strengths which lie above the upper border, the amebæ die within the time limit of 3 days.[1] Below the lower border they live, and the line along the middle of the zone joins the points at which approximately 50 per cent of the amebæ can live.

Two further facts are brought out by these results. In the first place, as the toxic dose of the monovalent ion is increased, the antago-

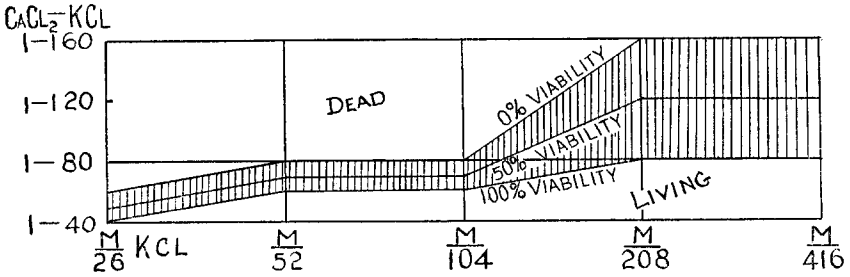

FIG. 3. Antagonism of $CaCl_2$ to decreasing concentrations of lethal doses of KCl, judged by survival of amebæ immersed in mixtures of these salts. The ordinates represent the ratios of KCl to $CaCl_2$ for different concentrations of KCl represented by the abscissæ. The shaded area indicates the zone of gradation between viability and non-viability in the different concentrations. Above this the $CaCl_2$ is not sufficient to antagonize the lethal action of the KCl. Below it, the lethal action of the KCl is completely antagonized by the $CaCl_2$.

nizing divalent ion must be increased beyond a proportional increment. In the second place, the zone of toxicity narrows as the toxic concentration of the monovalent salt increases; or, as the solution becomes less toxic, the amebæ show a greater variation in their ability to withstand the abnormal environment.

A striking feature of the immersion of amebæ in these combinations of NaCl and $CaCl_2$ is that the characteristic internal NaCl effect (see page 376) still occurs. If the concentration of the NaCl in the

[1] The time limit in the case of the combinations of $CaCl_2$ with M/13 NaCl was only 2 days as no strength of $CaCl_2$ was found which could antagonize this concentration of NaCl sufficiently to keep the amebæ alive for a longer time.

mixture is not too high recovery takes place with a redistribution of the granules.

c. Effect of Variations in Hydrogen Ion Concentration.—It was found that amebæ die within 24 hours in solutions of HCl more acid than M/24,000 (pH 5.6). The presence of none of the four salts used counteracts this toxicity. It was also found that increasing the alkalinity of water or solutions of any of the four salts from pH 7 to 9.8 with NaOH produces no change in the duration of life of the amebae immersed in them.

II.

Injection Experiments.

The procedure for injecting solutions into amebæ is as follows: Several amebæ are placed in a drop on a cover-slip. After a few seconds they fall and adhere to the glass. A drop of the solution to be injected is then placed on another part of the cover-slip and this is inverted over the moist chamber on the microscope stage. While under observation in the microscopic field, the tip of the micro-pipette is raised into the hanging drop of the solution and filled. The pipette is now lowered out of the drop and the moist chamber moved by the mechanical stage until an ameba is brought into view. The pipette is then raised and, when it punctures the ameba, the injection is made. In this way the entire procedure and the resulting effects can be continuously observed. The injection has to be made rather quickly after the puncture, because of the readiness with which a surface membrane may form around and over the tip of the pipette.

The aperture of the pipette in these experiments averaged 1 to 2 micra in diameter and the injections could easily be made under a Leitz No. 5 (5. 4 mm.) objective. The amount injected was estimated by comparing it to the volume of the nucleus or of the ameba. In this paper the terms small, moderate, and large are frequently used when referring to the amounts injected. A small amount equals approximately the volume of the nucleus; a moderate amount, a quarter to a third, and a large amount, one-half the volume or more of the entire ameba. On the average from twenty to thirty amebæ were injected with each concentration of salt studied.

a. Injection of Distilled Water.—As a control for the reaction to salt solutions the effect of injecting distilled water was first studied. A small or moderate amount immediately diffuses throughout the ameba. The cytoplasmic granules are driven away from the site of the injection, giving the effect of a temporary dilution. The diffusion of the water through the cytoplasm is followed, within a few seconds, by rapid streaming currents and the extension of pseudopodia. Gradually, the ameba quiets down and, within a minute, resumes its normal

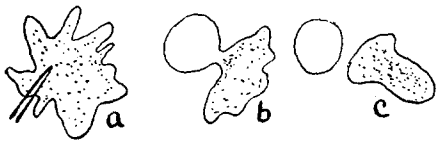

FIG. 4. Injection of a large amount of distilled water with pinching off: *a*, before injection; *b*, blister following injection; *c*, blister pinched off with return of ameba to normal.

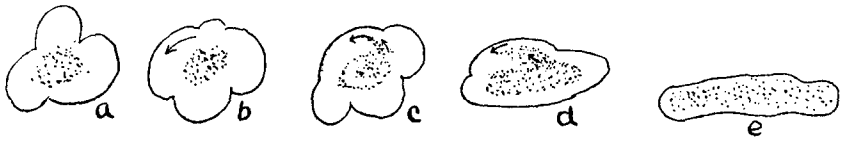

FIG. 5. Injection of a large amount of distilled water and subsequent recovery without pinching off. *a*, plasmalemma lifted in several places and granuloplasm clumped in center; *b*, sudden bulging of plasmalemma in direction of arrow; and, *c*, pouring of granules into hyaline region; *d*, continued scattering of granules throughout ameba and cessation of "rushing effect"; *e*, return to normal.

aspect. If a quantity equal to about half the volume or more of the ameba is injected, a clear fluid region quickly forms in the periphery and raises the plasmalemma locally in the form of a blister. Subsequently this blister is either resorbed or is pinched off by the healthy portion of the ameba (Fig. 4, *a* to *c*). Another phenomenon which occurs when distilled water in large amounts is injected rapidly is the so called "rushing action" of the ameba (Fig. 5). The outer portion becomes hyaline, the granules tend to accumulate in the center, *a*,

and then the plasmalemma is lifted by a sudden rush of clear fluid, *b*, *c*, *d*. This "rushing action" may last for several minutes before the ameba takes on a normal appearance again, *e*.

In general we may conclude that a moderate amount of water when injected diffuses through the cytoplasm and the ameba shows accelerated streaming movements. A larger amount injected causes a "rushing effect" or may accumulate under the plasmalemma in the form of a blister which is ultimately either pinched off or resorbed.

b. Injection of NaCl and KCl.—The injection of NaCl or of KCl produces a momentary dilution at the site of the injection followed by

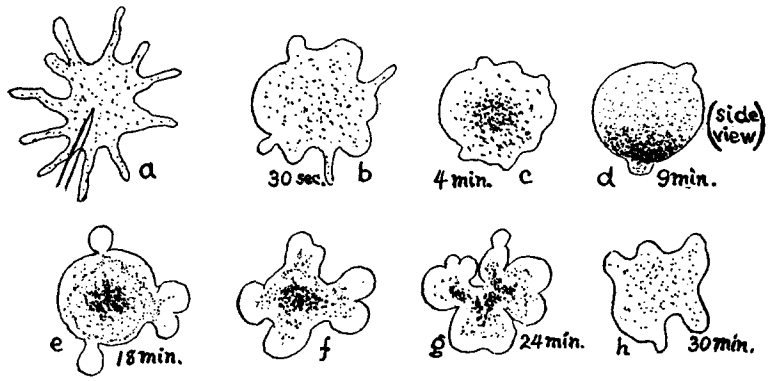

FIG. 6. Injection of a small amount of 2 M NaCl with recovery; *a*, before injection; *b*, pseudopodia being withdrawn; *c*, *d*, assumption of rounded shape and quiescence with sinking of granules; *e*, reformation of pseudopodia after period of quiescence; *f*, *g*, *h*, gradual resumption of normal appearance.

a slow retraction of the pseudopodia and a quiescence of the entire ameba. When very low concentrations or small quantities of the higher concentrations (M/4 to M/104) are injected the quiet phase is soon followed by a resumption of protoplasmic streaming and the extension of pseudopodia.

If higher concentrations are used (Fig. 6, *a* to *h*) the period of rounding and quiescence lasts a long time and a pronounced sedimentation of the cytoplasmic granules takes place, *b*, *c*, *d*. The larger and presumably heavier granules sink first and clump on the dependent surface of the rounded ameba. The smaller granules sink more slowly and accumulate in a middle zone, above which is left a granule-free

region. If recovery takes place, protoplasmic currents start up, pseudopodia are protruded from anywhere on the surface, but usually from the dependent part, and the granules are redistributed until the ameba appears normal again, *e*, *f*, *g*, *h*; (*cf.* Fig. 1, Plate 3). The largest granules, which are the first to sink and clump at the bottom, tend to adhere to the surface of the ameba for a long time (1 hour or more). These, however, also finally become completely redistributed. Complete recovery may require from a few hours to almost a day, according to the concentration of the salt.

If recovery does not take place all the visible granules finally collect in a clump at the bottom of the rounded body (Fig. 2, Plate 3). The plasmalemma becomes very delicate and ruptures easily. When this occurs the entire plasmalemma disintegrates, the clear liquid substance of the interior merges with the surrounding water, and the clump of granules tends to scatter.

In brief, the injection of NaCl or of KCl into amebae causes (*a*) a retraction of the pseudopodia followed by cessation of all movement, and (*b*) a liquefaction of the hyaloplasm and a sedimentation of the suspended granules. Death results with the internal protoplasm in a liquid state. It is significant that the amebæ have the same appearance and physical state when they are immersed in solutions of NaCl or of KCl as when these salts are injected into them.

By comparing the injection with the immersion experiments it will be seen that NaCl and KCl at a given concentration are less toxic when injected than when the amebæ are immersed in them. It might be suggested that this is due to the outward diffusion of the injected salt. The possibility of such a diffusion would probably be prevented by immersing the amebæ in the same concentration of the salt with which they are injected. Such an experiment is very difficult to perform because of the toxicity of the medium. Amebæ immersed in M/104 NaCl recovered completely from injections of M/4 NaCl. The result of this experiment is inconclusive because of the low concentration of NaCl in the immersion fluid. However, more concentrated solutions could be used for immersion by antagonizing the NaCl in the surrounding fluid with $CaCl_2$. With this in view a volume of M/4 NaCl equal to about one-third of the ameba was injected into an ameba immersed in a balanced combination of M/13 NaCl + M/256 $CaCl_2$.

The immediate reaction to the injection was the characteristic NaCl effect of rounding, quiescence, and sinking of the cytoplasmic granules. This was followed by a gradual resumption of movement, a redistribution of the granules and a return of the ameba to its normal state. Because the concentration of the NaCl in the immersion fluid used in this experiment closely approximates that of the salt in the ameba the inference is justified that the recovery of the internal protoplasm is not necessarily due to the outward diffusion of the injected salt.

c. Injection of $MgCl_2$.—When $MgCl_2$ is injected in concentrations of M/52 and stronger the pseudopodia withdraw and the granular cytoplasm of the affected region "sets" and contracts. During this process the plasmalemma may rise in one or several places so that blisters are formed into which the set, granular cytoplasm spasmodically breaks with a liberation of its granules. In sublethal amounts (*e.g.* small amounts of M/13 to large amounts of M/104) the formation of pseudopodia is resumed and the ameba gradually recovers. In lethal amounts (M/6.5 and stronger) the solidifying action spreads until the entire ameba is set into a mass of putty-like consistency. When large amounts are injected the entire ameba is immediately solidified, often with extended pseudopodia.

d. Injection of $CaCl_2$.—The effect of injecting $CaCl_2$ resembles that of $MgCl_2$ in its solidifying action. $CaCl_2$, however, differs from all the salts hitherto mentioned in causing the ameba to cast off the area affected by the injection. The pronounced solidification of the area injected and the pinching-off process are the two striking results when $CaCl_2$ is introduced into the interior.

All strengths of $CaCl_2$ from 2 M to M/208 immediately solidify the internal protoplasm. If the amount injected is large the entire ameba with its extended pseudopodia sets into a rigid mass. With smaller amounts the solidifying action is localized to the immediate region of the injection. The part solidified tends to be relegated to the posterior end of the ameba as the streaming movements carry the ameba forward (5). With concentrations of M/104 and stronger (Fig. 7; *cf.* Fig. 3, Plate 3), the ameba immediately starts flowing away from the region involved, which assumes the form of a welt or blister covered by an intact pellicle, *b*, *c*. The base of the blister progressively constricts and hyaline pseudopodia appear on either side of

the constricting stalk against which they bulge, *d*, *e*. By enlarging, first on one side and then on the other, the pseudopodia push on the inert blister until the stalk becomes attenuated and breaks, *f*, *g*, so that the blister is completely pinched off and discarded as a sphere. The granular content of this sphere is a solid mass and its pellicle is rigid, *h* to *k*. Occasionally, when a solid body such as the nucleus, an ingested rotifer, or a diatom is caught in the stalk, the pinching-off process is delayed. In such cases the pseudopodia at the base of the stalk may push the dead portion from side to side until the obstructing

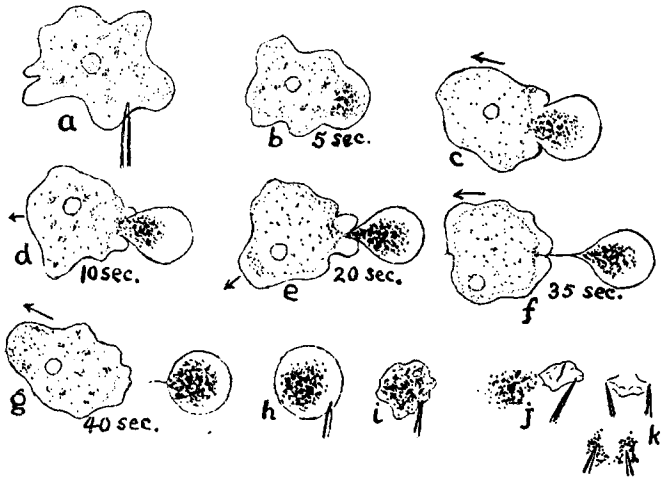

FIG. 7. Injection of M/13 $CaCl_2$ with pinching off; *a*, before injection; *b*, after injection, solidified material relegated to posterior of ameba; *c*, *d*, *e*, *f*, and *g*, successive stages in the pinching-off process; *h*, *i*, *j*, *k*, pinched-off body consisting of a coagulated granular mass surrounded by hyaline liquid and a hard, friable pellicle; *k*, granular mass being torn with needles.

body either moves into the dead blister or is drawn back into the body of the ameba. The stalk then rapidly attenuates and finally breaks. Occasionally, the front end of the ameba turns around and pushes against the blister. With each successive injection the pinching-off process is repeated until only a diminutive living portion remains. Fig. 8, *a*, *b*, exhibits an interesting case of an ameba with extended pseudopodia at the moment when 1 M $CaCl_2$ was injected into its center. The greater part of the ameba immediately became solidified while the tips of the pseudopodia farthest from the site of the

injection pinched themselves off and moved away. With M/208 there is a long delay and, occasionally, after a marked constriction of the stalk, a flow of cytoplasm from the main body of the ameba into the stalk gradually widens it and the blister becomes completely incorporated (Fig. 9, *a* to *f*).

An extraordinary feature of the pinching-off phenomenon is the marked effort of the ameba to retain its nucleus. If the nucleus is caught in the constricting stalk the force of the constriction is great enough to distort it. At the same time there is a decided and usually

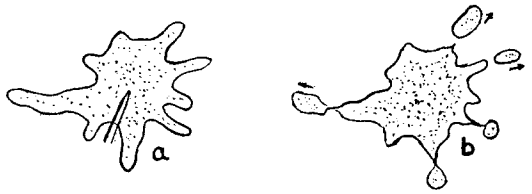

FIG. 8. Injection of 1 M $CaCl_2$: *a*, before injection; *b*, 10 seconds after injection, ameba solidified except for living tips of several pseudopodia which are pinching themselves off and moving away.

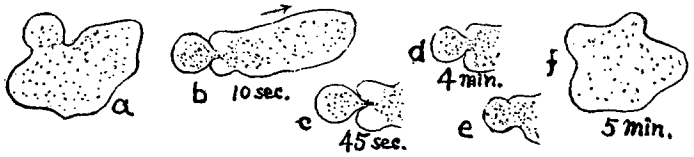

FIG. 9. Injection of M/208 $CaCl_2$ with attempted pinching off and resorption: *a*, 5 seconds after injection; *b* and *c*, continued constriction of base of affected region; *d*, *e*, and *f*, successive steps in broadening of base and final resorption of affected region.

successful attempt on the part of the living portion of the ameba to recover the nucleus before the pinching-off process is completed. The nucleus, which may have been compressed into the shape of a pear or of a dumb-bell, then gradually resumes its original contour.

In brief, both $CaCl_2$ and $MgCl_2$ produce a solidification of the internal protoplasm of the ameba. However, when $CaCl_2$ is injected the ameba reacts vigorously and usually localizes the injury to a region which is quickly thrown off. With $MgCl_2$, on the other hand, all movement ceases and the entire body is affected by the deleterious action of the salt.

e. Quantitative Comparison of the Injection Experiments of the Four Salt Solutions.—Fig. 10 illustrates the ability of the internal protoplasm to recover after it has been injected with different concentrations of each of the four salt solutions. $CaCl_2$ in concentrations of M/104 and stronger immediately solidifies the area injected and, if the amount injected is not excessive, the ameba pinches off the affected region. With M/208 solidification also occurs but is followed by either a pinching off or a resorption of the affected region. When the pinching-off reaction takes place the ameba survives minus the portion injected. As the curves on the chart represent the recovery of the protoplasm which has actually come into contact with the salt, $CaCl_2$ may be considered the most toxic of the salts. However, this conclusion must be qualified because it is impossible to determine whether the affected region might not have recovered if it were not pinched off. $MgCl_2$ is decidedly more toxic than NaCl and KCl.

f. The Rate of the Pinching-Off Process When $CaCl_2$ Is Injected.—The constancy in the rate of the pinching-off process for a given concentration of $CaCl_2$ permits the construction of a curve in which the time taken for pinching off is plotted against the concentration of the solution injected (Fig. 11).

When the concentration is M/13 or stronger the pinching-off process occurs in 30 to 45 seconds; with M/26 it takes 50 to 60 seconds; with M/52, 55 to 75 seconds; with M/104, 60 to 90 seconds. The dilution of M/208 appears to be the critical strength at which the pinching off is either delayed from 2 to 10 minutes or is never completed. In the latter case, the involved region is ultimately resorbed. With the dilution of M/416 no pinching off is even attempted and the ameba reacts as if it had been injected with water alone.

g. The Antagonistic Action of the Salts When Combinations Are Injected.—The rate of the pinching-off process when $CaCl_2$ is injected affords an excellent means for studying the antagonistic action between $CaCl_2$ and the monovalent cations on the interior of the cell. Fig. 12 shows the delay in the pinching-off process which occurs when NaCl is combined with $CaCl_2$. The curves marked CaCl + 1 M, +M/2, and +M/4 NaCl illustrate the reaction of the ameba when different concentrations of $CaCl_2$ are combined with concentrations of 1 M, M/2, and M/4 NaCl. The chart shows that the concentration of NaCl

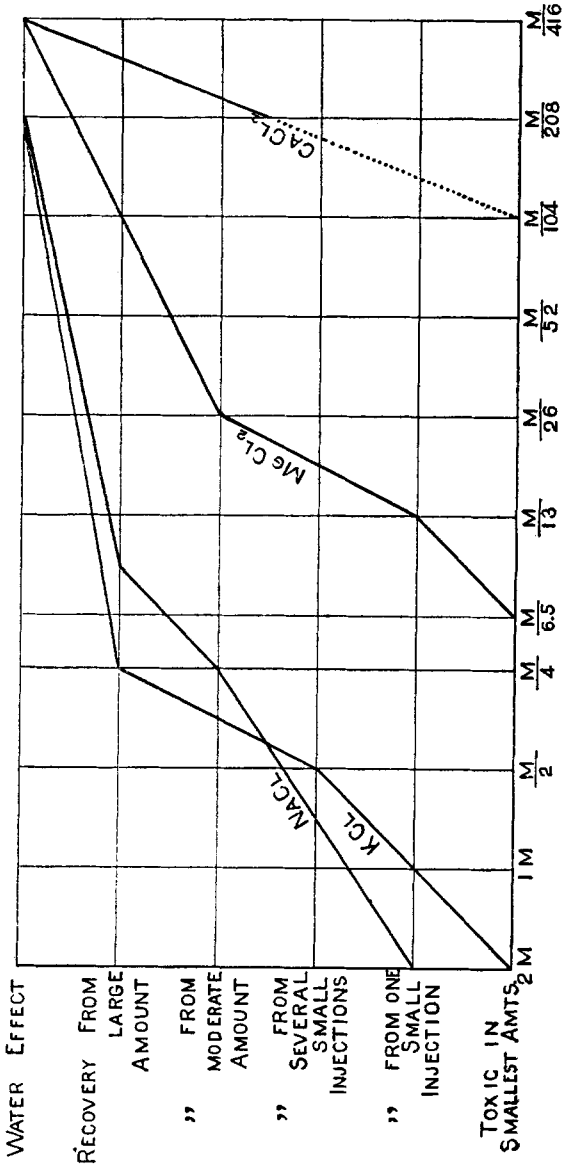

FIG. 10. Recovery of protoplasm of amebæ from injection of decreasing concentrations of NaCl, KCl, $MgCl_2$, and $CaCl_2$ solutions. The broken portion of the $CaCl_2$ curve represents conditions in which injected region is pinched off so that possibility of recovery of the affected part cannot be determined.

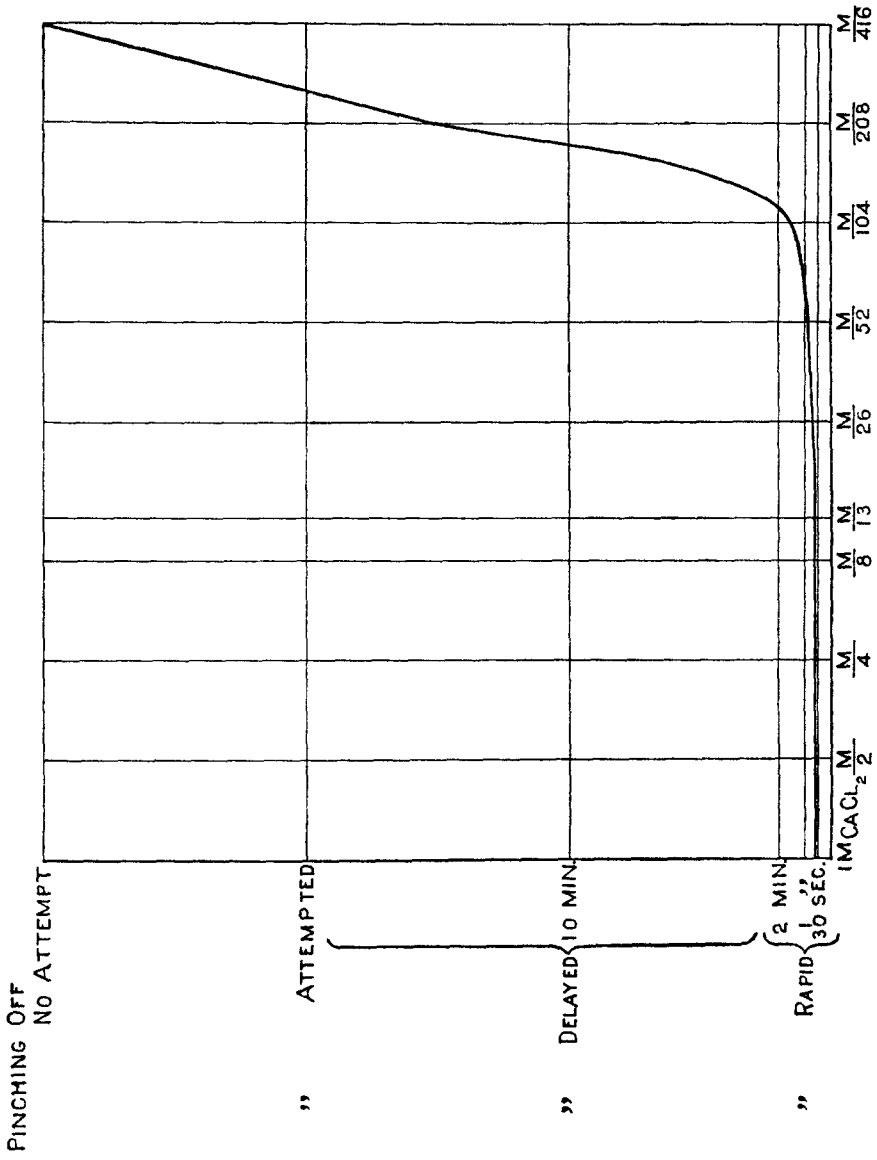

FIG. 11. Pinching-off effect produced by injection of different concentrations of $CaCl_2$ into the ameba.

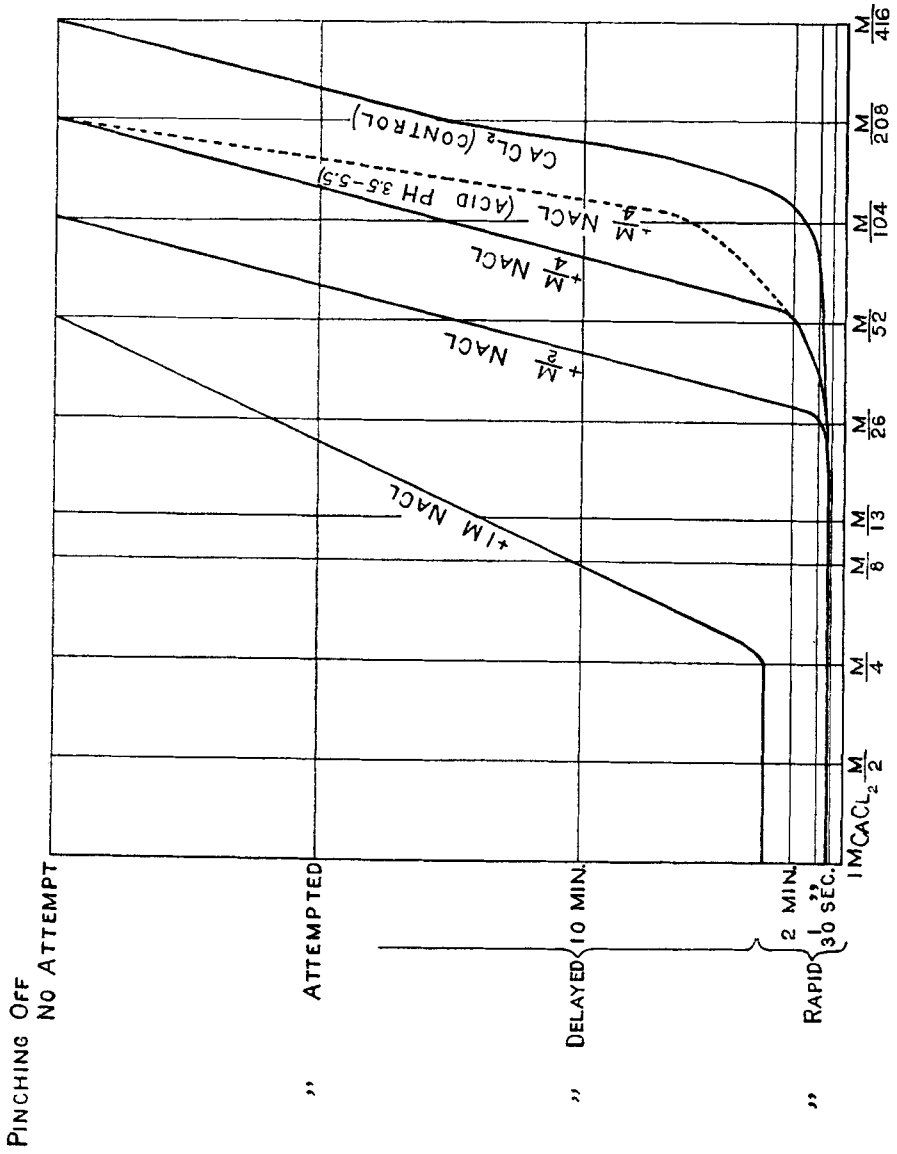

FIG. 12. Antagonism of 1 M, M/2, and M/4 NaCl to decreasing concentrations of $CaCl_2$ judged by modification of pinching-off effect when mixtures of these salts are injected into the ameba. The $CaCl_2$ curve of Fig. 11 is repeated here as a control. The broken line curve represents the effect of acidifying the mixture of $CaCl_2$ + M/4 NaCl (pH 3.5–5.5).

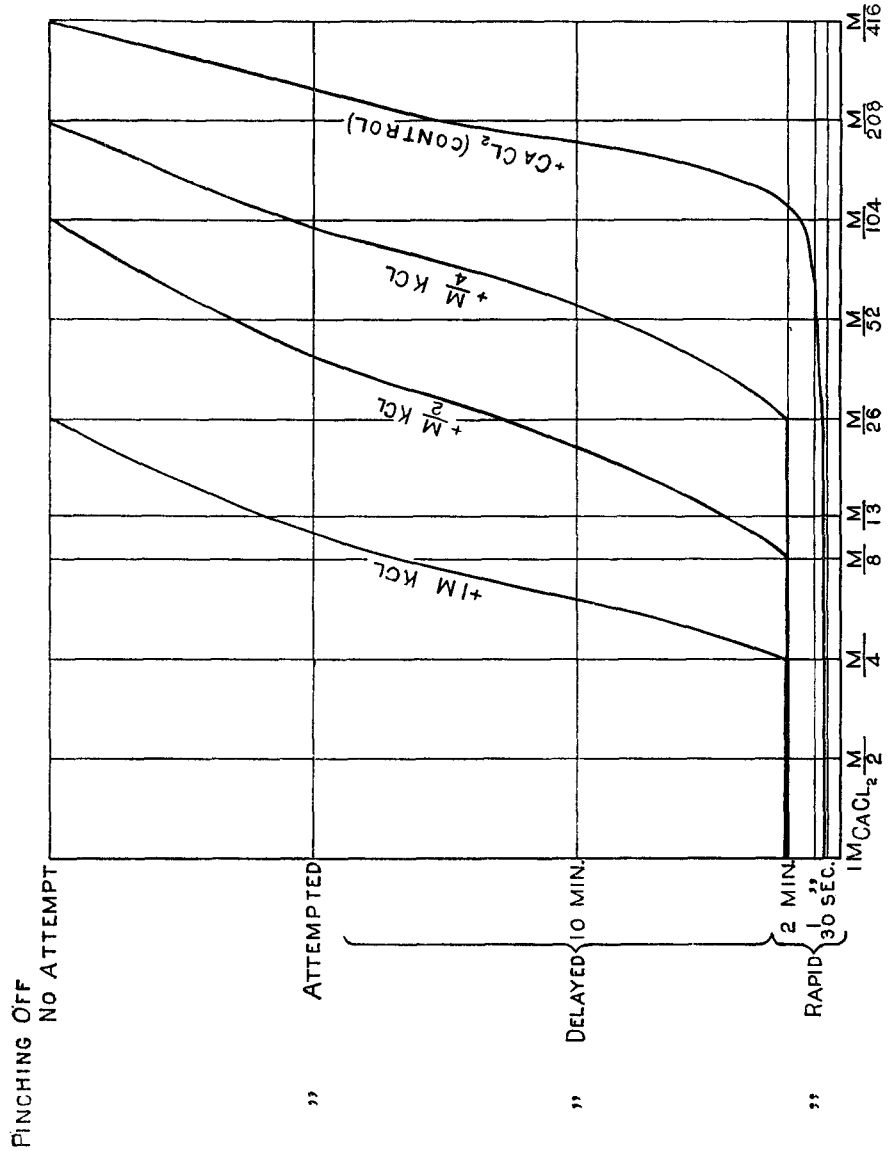

FIG. 13. Antagonism of 1 M, M/2, and M/4 KCl to different concentrations of $CaCl_2$ judged by modification of pinching-off effect when mixtures of these salts are injected. The $CaCl_2$ curve of Fig. 11 is repeated here as a control.

necessary to antagonize completely the effect of $CaCl_2$ on the interior of the ameba is fifty-two times that of the $CaCl_2$. As might be inferred from the chart, M/8 NaCl was found to be too dilute to modify the $CaCl_2$ effect.

Fig. 13 shows the delay in the pinching-off process when KCl is combined with $CaCl_2$. As is the case for NaCl, here also no antagonism occurs with M/8 KCl. However, 1 M KCl appears to be more effective than 1 M NaCl in antagonizing $CaCl_2$. It has already been noted (page 372) that KCl makes the plasmalemma sticky, in marked contrast to NaCl. When the region injected with $CaCl_2$ plus KCl is being pinched off, its surface tenaciously adheres to the plasmalemma of the living portion and, on being pushed away by the microneedle, strands of viscid material are seen extending between the two surfaces. This adhesiveness may explain the apparent delay in the pinching-off process with the concentration of 1 M KCl.

These injection experiments show that a liquefying dose of NaCl or of KCl and a concentration of $CaCl_2$ which, by itself, causes localized solidification can be combined so that the specific effect of each salt is completely neutralized.

h. Variations in the Hydrogen Ion Concentration of Solutions Injected.—The injection of water plus NaOH of pH values from 7 to 9.8 has no effect other than that of distilled water. An immediate solidification of the injected region occurs with M/12 HCl (pH 1.8), and the ameba reacts by pinching off the affected region. One injection of M/100 HCl (pH 2.2) has no appreciable effect but a second injection closely following the first causes a solidification and a pinching off of the affected region. The ineffectiveness of only one injection of M/100 HCl is possibly due to the neutralization of the acid by buffers in the cell, and the solidification caused by subsequent injections would, therefore, be due to the absence of buffers to neutralize the acid.

The solidifying action of HCl antagonizes the liquefying action of NaCl. Thus, when M/4 NaCl at pH 1.8 is injected into an ameba solidification and pinching off do not occur, neither is there any sinking of the cytoplasmic granules typical of the NaCl effect. By acidifying the medium we can also favor the action of $CaCl_2$ in its antagonism to NaCl. This is shown in the broken curve, Fig. 12. With a mixture containing M/4 NaCl and M/104 $CaCl_2$ at pH 7 the pinching-off

process was merely begun but never completed. However, at pH 3.5 to 5.5 the pinching-off reaction occurred within approximately 3 minutes. Therefore, this shift to the $CaCl_2$ effect is apparently due to the antagonism between acid and NaCl.

III.

Recovery from Surface Tears of Amebæ Immersed in Salt Solutions.

Amebæ from a stock culture were placed in a large amount of the salt solution to be tested and, within a few seconds, were transferred in a drop of the solution to a cover-slip. The cover-slip was then inverted over the moist chamber and the amebæ were operated upon within 1 to 2 minutes after their immersion in the solution.

a. Recovery from Tears in Distilled Water.—In order to control the specific action of the salt solutions the extent of recovery from tears in distilled water was first determined. The region around the tear tends to close over the gap and, within a few seconds, the repair is complete (Fig. 14, *a* to *c*). If the tear is extensive there is usually an outflow of some of the granular contents which is discarded during the repair. The marked degree of the ability of the plasmalemma to recover from surface tears is shown when the ameba is brought to the air-water surface of its medium. The plasmalemma bursts, entirely disappears, and the contents of the ameba begin to scatter. If the scattering does not occur too rapidly, a new plasmalemma may form suddenly and will persist if the ameba is pushed back quickly into the drop. From this it may be assumed that plasmalemma formation is a reaction between the internal protoplasm and the surrounding medium.

b. Recovery from Tears in NaCl Solutions.—In concentrations of NaCl of M/13 and stronger the amebæ have already begun to retract their pseudopodia and to cease movement within the minute that elapses before operation. In these concentrations no repair occurs even from the slightest surface tears. The contents begin to pour out, the plasmalemma rapidly disintegrates, and the granular cytoplasm scatters (Fig. 15, *a* to *c; cf.* Fig. 4, Plate 3). The destructive action of the NaCl on the membranes is to be seen not only in the disintegration of the plasmalemma of the torn ameba but also in the dissipation

of the membranes surrounding the various exposed vacuoles. In solutions weaker than M/13 repair takes place with increasing ease as the concentration is diminished. In an M/104 solution the ameba recovers from an extensive tear as easily as it does in water.

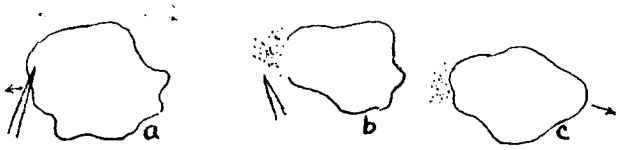

FIG. 14. Effect of tearing ameba in water; *a*, needle inserted into ameba preparatory to tearing in direction of arrow; *b*, plasmalemma torn with slight outflow of granules; *c*, rapid repair.

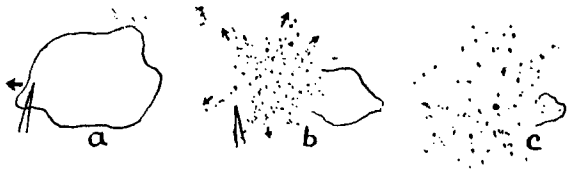

FIG. 15. Effect of tearing ameba in M/13 NaCl. Rapid dissipation of surface with scattering of granules.

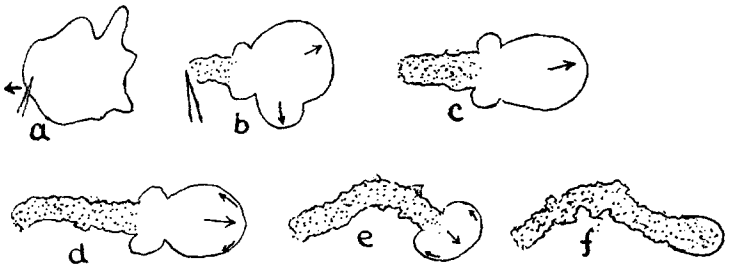

FIG. 16. Effect of tearing ameba in M/13 $CaCl_2$; *a*, before tear; *b*, *c*, *d*, and *e*, ameba flowing from region the plasmalemma of which has been torn. The inward diffusion of the $CaCl_2$ and the retreat of the ameba is converting the protoplasm into an ever lengthening column of coagulated material until, *f*, the entire ameba is set and killed.

c. Recovery from Tears in KCl Solutions.—Amebæ torn in KCl react like those torn in NaCl except that the plasmalemma becomes very sticky and does not disintegrate so easily. However, KCl appears

to be more toxic than NaCl in solutions more dilute than M/52 and has to be brought to a dilution of M/312 before repair occurs readily from extensive tears.

d. Recovery from Tears in $CaCl_2$ Solutions.—In concentrations of M/6.5 $CaCl_2$ and stronger, death results from the slightest tear. This occurs in a characteristic manner (Fig. 16, *cf.* Fig. 5, Plate 4). The cytoplasm in the region where the plasmalemma is torn immediately solidifies. The rest of the ameba begins to flow away and attempts to pinch off the involved region while the solidifying process rapidly spreads inward. This reaction results in a steadily lengthening column of solidified material with the living part at the end farthest from the tear actively flowing and attempting to pinch itself off. Finally, this part also succumbs and the ameba is converted into a solid column of dead material. The plasmalemma over this column is changed into a brittle pellicle. The several constrictions of the column indicate where the ameba had attempted to pinch off the region involved. This striking phenomenon is best demonstrated in concentrations of $CaCl_2$ from M/5 to M/10. With more extensive tears or in stronger solutions the inward extension of the solidifying process is usually so rapid that the ameba has little time to flow away before it is completely solidified. With each further dilution, or with a diminution of the traumatic injury in stronger solutions, the attempts to pinch off become increasingly successful (Fig. 6, Plate 4), until with M/104 the involved region is rapidly discarded even when extensive tears are made. The inward diffusion of $CaCl_2$ is thus stopped by the creation of a barrier in the form of an impermeable membrane.

In brief, the specific reaction to a tear of the ameba in $CaCl_2$ is: (*a*) an immediate solidification of the torn region, (*b*) a vigorous and active movement away from the region involved, and (*c*) a pronounced attempt of the plasmalemma to produce a constriction between the healthy and the affected portions.

e. Recovery from Tears in $MgCl_2$ Solutions.—Amebæ are less able to recover from tears in $MgCl_2$ than in solutions of any of the other salts. In solutions of M/52 or stronger no repair is even attempted, and there is no flow of the ameba away from the injured region. The cytoplasm where the tear is made jellies, and the streaming movements within the rest of the ameba become irregular and indefinite, then slow down

and stop as the solidifying process spreads from the region of the tear. Repair may occur from small tears in solutions more dilute than M/52, but recovery from extensive tears does not take place until a dilution of M/832 is reached. The greater toxicity of $MgCl_2$ as compared with $CaCl_2$ is probably due to the fact that no pinching-off reaction opposes the coincident solidifying effect of the salt so that the entire ameba readily becomes involved. Another difference between $MgCl_2$ and $CaCl_2$ is in their effect on the plasmalemma. In $CaCl_2$ the plasmalemma is very active and able to repair itself, whereas in $MgCl_2$ no repair and no movement of the plasmalemma occur.

f. Comparison of the Effects of the Four Salts in the Tearing Experiments.—In NaCl solutions the plasmalemma of the ameba becomes non-adhesive and delicate. It is readily torn and disintegration spreads rapidly over the surface from the spot of injury. The interior of the ameba then disperses. In KCl solutions, the plasmalemma becomes viscid and when torn disintegrates much less readily than in NaCl. But the interior, as it pours through the tear, disperses as in NaCl.

In both $CaCl_2$ and $MgCl_2$ there is no spread of disintegration over the surface from the spot of injury. In $CaCl_2$ the plasmalemma tends to be readily repaired when torn. In $MgCl_2$ it does not. It is noteworthy that these salts have no solidifying effect on the protoplasm so long as an intact plasmalemma intervenes. On the other hand, the internal protoplasm, whether solidified or not, is freely permeable to them.

A comparison of the recovery from surface tears of amebæ immersed in different concentrations of each of the four salts is shown in Fig. 17. The order of toxicity of the salts in their action on the recovery of amebæ from surface tears is $Mg > K > Na > Ca(?)$. Ca stands in a category by itself owing to the fact that the plasmalemma adjacent to the tear appears to assist actively in the repair by the peculiar pinching-off reaction. This makes it impossible to determine the rôle played by the denuded protoplasm in the process of surface reformation when the plasmalemma is torn in $CaCl_2$.

g. Recovery from Surface Tears in Binary Combinations of the Salts.—The prevention of repair of the torn surface in toxic concentrations of NaCl and of KCl can be antagonized by $CaCl_2$. Fig. 18 illustrates

the results on tearing amebæ in solutions containing different concentrations of $CaCl_2$ combined with M/13 NaCl. In M/13 NaCl alone amebæ cannot recover from even the slightest tear. However, in a combination of this concentration of NaCl with M/52 to M/208 $CaCl_2$ the amebæ recover from extensive tears. Fig. 19 illustrates similar

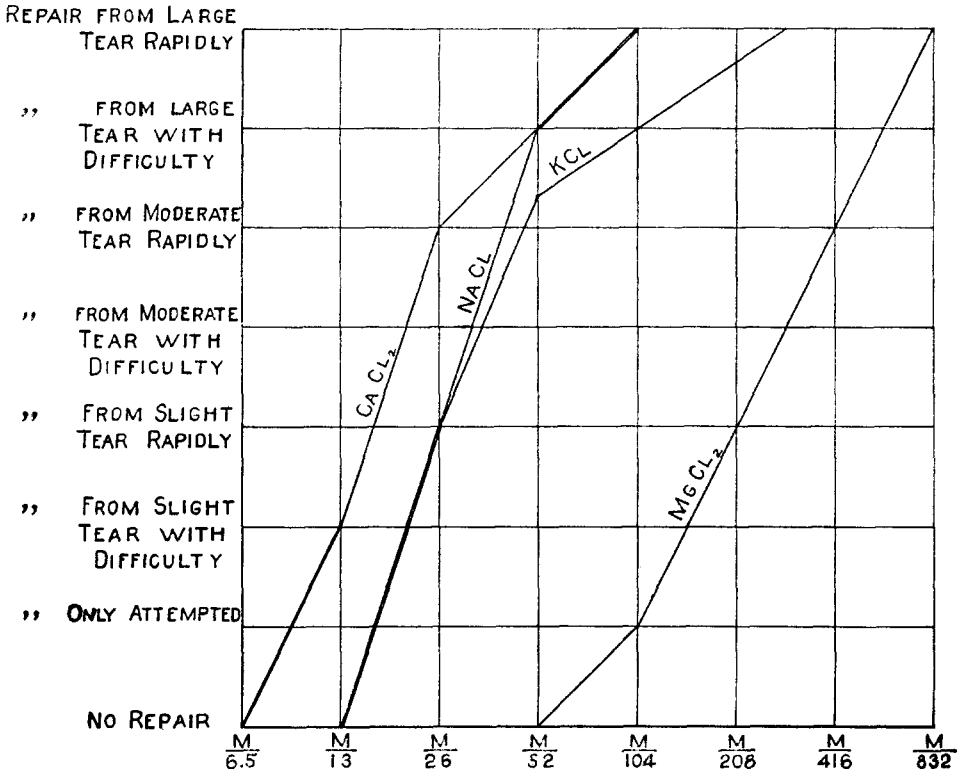

FIG. 17. Recovery from surface tears of ameba immersed in decreasing concentrations of $CaCl_2$, NaCl, KCl, and $MgCl_2$ solutions.

results obtained with combinations of M/13 KCl and different concentrations of $CaCl_2$. It will be seen that a toxic concentration of M/13 KCl is completely antagonized only by M/208 $CaCl_2$.

It is noteworthy that the range of maximum antagonism between NaCl and $CaCl_2$ in regard to repair of the surface is greater than that between KCl and $CaCl_2$. This is also true for the extreme ranges

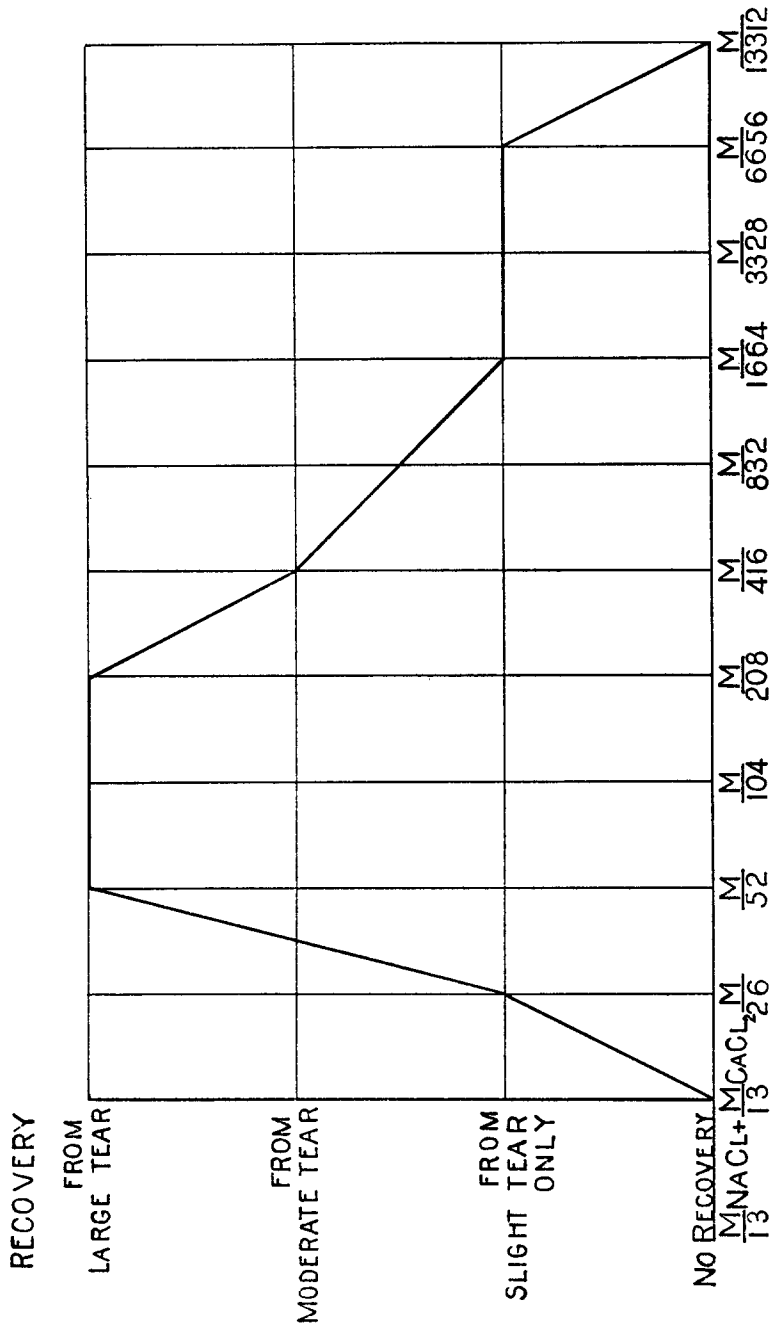

FIG. 18. Antagonism of decreasing concentrations of $CaCl_2$ to M/13 NaCl judged by the recovery of surface tears of amebæ immersed in mixtures of these solutions.

of antagonism which, in the case of M/13 NaCl, extends to M/13,312 $CaCl_2$ and, in the case or M/13 KCl, only to M/1,664 $CaCl_2$. In the case of M/13 NaCl appreciable antagonism fails when the $CaCl_2$ is present in concentrations greater than M/52, while with KCl the antagonism ceases at concentrations greater than M/208 (*cf.* Figs.

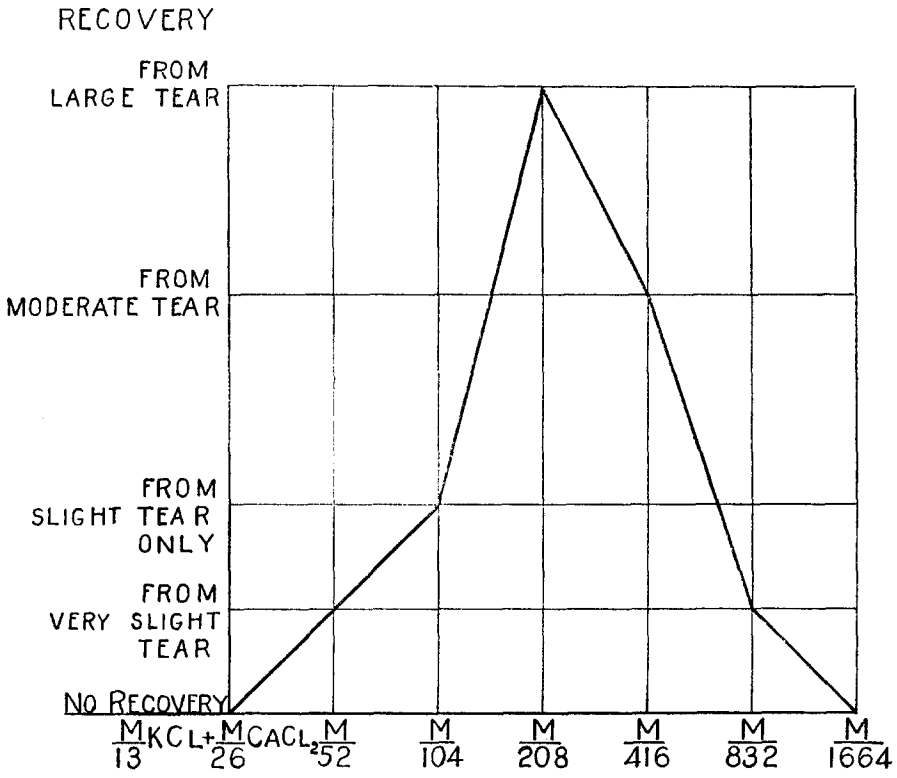

FIG. 19. Antagonism of decreasing concentrations of $CaCl_2$ to M/13 KCl judged by the recovery of surface tears of amebæ immersed in mixtures of these solutions.

18 and 19). This lack of antagonism of the higher concentrations of $CaCl_2$ against the toxic effect of NaCl and of KCl does not appear to be merely a function of the excess $CaCl_2$ present. This can be seen on comparing the toxicity of $CaCl_2$ alone (Fig. 17) with that of combinations of $CaCl_2$ and NaCl or KCl (Figs. 18, 19).

DISCUSSION.

The liquefying action of NaCl and of KCl and the solidifying action of $MgCl_2$ and of $CaCl_2$ on the internal protoplasm of the ameba closely simulate the behavior of these salts with proteins and soaps (6, 7). Also Mangin (8), Hansteen-Cranner (9), and True (10), have shown that changes in the consistency of the cellulose walls of plant cells can be produced by variations in the amounts of K, Na, and Ca pectin compounds. Analogous results have been obtained by Lillie (11) with the jelly which surrounds starfish eggs. He found that the jelly swells and dissolves in NaCl but is unaffected by $CaCl_2$.

The microdissection method also throws light on the action of the various electrolytes upon the plasmalemma of the ameba. The disintegration of the plasmalemma of amebæ immersed in NaCl is similar to the observations of Lillie (12), who noted the breakdown of cilia and of the plasma-membrane of marine cells in NaCl. In contrast to the monovalent salts, $CaCl_2$ and $MgCl_2$ exert no appreciable effect on the intact plasmalemma when they come into contact with it from the exterior.

The order of the toxicity of these salts for the repair of surface tears is the same as that for the internal protoplasm and not that for the plasmalemma in the immersion experiments. This supports the suggestion that the reformation of the plasmalemma is a function of the interior protoplasm, and not solely of the preexisting plasmalemma as recently stated by Edwards (13). The fact that no membrane is formed when water or aqueous solutions are injected into the interior of an ameba may be due to an insufficient concentration within the interior of the ameba of membrane-forming material, which presumably is present in high concentration at the periphery.

Salt antagonism is too well known to require detailed discussion. Loeb (14) and many others (8–10, 15) have found that the monovalent and polyvalent cations antagonize each other's action on marine eggs and organisms, plant tissues, etc. The experiments recorded in this paper afford evidence for a similar antagonistic action between salts on a variety of protoplasmic properties in the ameba. Presumably the delicate balance in the physical state of the protoplasm of *Amœba* is due to the presence of definitely proportioned amounts of different electrolytes.

The antagonistic action of the salts is also to be seen in their action on the plasmalemma. One important fact must be considered in connection with the effects of the salts on the membrane and its permeability. When an ameba is immersed in a mixture of $CaCl_2$ and NaCl in antagonistic proportions, the environment approaches more nearly a normal one than it does in a pure NaCl solution. The action of the non-antagonized NaCl is injurious to the plasmalemma and the consequent increase in permeability is due to an abnormal state of the membrane.

Loeb, in one of his later papers (16), states that NaCl, which usually increases permeability, will in moderate concentrations decrease permeability or inhibit increase of permeability exactly like $CaCl_2$. He bases this statement on his findings that NaCl prevents the entrance of HCl into the *Fundulus* egg. Our experiments show the existence of a definite antagonism between NaCl and HCl in their effects on the physical state of the internal protoplasm. Loeb's results may have been due not so much to an action of NaCl in decreasing the permeability of the membrane to HCl but rather to the antagonism of the salt to the toxic action of the acid in the interior of the cell.

To explain the toxic effect of the monovalent ions in the immersion experiments two assumptions may be made. Either the plasmalemma is the part most susceptible to injury or the injury is due to the accumulation of the salt within the ameba by continued penetration. If the latter assumption be true it would mean that in the injection experiments the ameba recovers because the salt diffuses out into the surrounding medium. The improbability of such an explanation for recovery is shown by the experiments in which amebæ were injected with NaCl while they were immersed in a concentration of the salt equal to that introduced into the interior. The fact that they recover warrants the conclusion that the salt did not diffuse out and that the presence of the salt within the ameba is not necessarily toxic. Since it is necessary to have a balanced salt solution (NaCl plus $CaCl_2$) as the surrounding medium to maintain viable conditions, there are two alternatives to explain recovery from injection. The recovery may be due either to some change in the chemical combination of the Na in the cell or to the antagonism of the Na effect by the ultimate

penetration into the ameba of $CaCl_2$ from the external medium of the mixed salts.

In the immersion experiments death from NaCl always occurs with a disruption of the pellicle. Further evidence that the primary site of the lethal action of Na is on the surface is seen when very concentrated solutions are injected into the cell. In such cases, if the ameba dies, the plasmalemma becomes extremely delicate and breaks spontaneously. All the experimental evidence points to the probability that the lethal action of NaCl and the antagonizing effect of $CaCl_2$ to NaCl occur at the surface of the cell. This is in accordance with Clowes' work on water and oil emulsions (7) in which he points out the probability that salts of Ca promote the formation of a protoplasmic membrane and salts of Na exert the reverse effect, while balanced solutions are those in which antagonistic electrolytes are present in such proportions as to give to the protoplasmic membrane that measure of permeability most favorable for cell life.

In a recent article Michaelis (17) discusses the permeability of membranes to electrolytes. He used purely physical membranes and warns against the use of physiological membranes because of the complications involved. However, the results obtained in the permeability experiments recorded in this paper agree with those found by Michaelis. This promises that the material and the method used in these experiments may be serviceable for an extended study of physicochemical problems with a physiological membrane. Finally, the ability to discriminate experimentally between different regions of the protoplasm of a cell is a distinct advance beyond past work on protoplasm in general.

CONCLUSIONS.

By means of micro-dissection and injection *Amœba proteus* was treated with the chlorides of Na, K, Ca, and Mg alone, in combination, and with variations of pH.

I. *The Plasmalemma.*

1. NaCl weakens and disrupts the surface membrane of the ameba. Tearing the membrane accelerates the disruption which spreads rapidly from the site of the tear. KCl has no disruptive effect on the membrane but renders it adhesive.

2. $MgCl_2$ and $CaCl_2$ have no appreciable effect on the integrity of the surface membrane of the ameba when applied on the outside. No spread of disruption occurs when the membrane is torn in these salts. When these salts are introduced into the ameba they render the pellicle of the involved region rigid.

II. *The Internal Protoplasm.*

3. Injected water either diffuses through the protoplasm or becomes localized in a hyaline blister. Large amounts when rapidly injected produce a "rushing effect".

4. HCl at pH 1.8 solidifies the internal protoplasm and at pH 2.2 causes solidification only after several successive injections. The effect of the subsequent injections may be due to the neutralization of the cell-buffers by the first injection.

5. NaCl and KCl increase the fluidity of the internal protoplasm and induce quiescence.

6. $CaCl_2$ and $MgCl_2$ to a lesser extent solidify the internal protoplasm. With $CaCl_2$ the solidification tends to be localized. With $MgCl_2$ it tends to spread. The injection of $CaCl_2$ accelerates movement in the regions not solidified whereas the injection of $MgCl_2$ induces quiescence.

III. *Pinching-Off Reaction.*

7. A hyaline blister produced by the injection of water may be pinched off. The pinched-off blister is a liquid sphere surrounded by a pellicle.

8. Pinching off always takes place with injections of HCl when the injected region is solidified.

9. The injection of $CaCl_2$ usually results in the pinching off of the portion solidified. The rate of pinching off varies with the concentration of the salt. The injection of $MgCl_2$ does not cause pinching off.

IV. *Reparability of Torn Surfaces.*

10. The repair of a torn surface takes place readily in distilled water. In the different salt solutions, reparability varies specifically with each salt, with the concentration of the salt, and with the extent of the tear. In NaCl and in KCl repair occurs less readily than in water. In $MgCl_2$ repair takes place with great difficulty. In $CaCl_2$ a proper estimate of the process of repair is complicated by the pinching-off phenomenon. However, $CaCl_2$ is the only salt found to

increase the mobility of the plasmalemma, and this presumably enhances its reparability.

11. The repair of the surface is probably a function of the internal protoplasm and depends upon an interaction of the protoplasm with the surrounding medium.

V. *Permeability.*

12. NaCl and KCl readily penetrate the ameba from the exterior. $CaCl_2$ and $MgCl_2$ do not.

13. All four salts when injected into an ameba readily diffuse through the internal protoplasm. In the case of $CaCl_2$ the diffusion may be arrested by the pinching-off process.

VI. *Toxicity.*

14. NaCl and KCl are more toxic to the exterior of the cell than to the interior, and the reverse is true for $CaCl_2$ and $MgCl_2$.

15. The relative non-toxicity of injected NaCl to the interior of the ameba is not necessarily due to its diffusion outward from the cell.

16. HCl is much more toxic to the exterior of a cell than to the interior; at pH 5.5 it is toxic to the surface whereas at pH 2.5 it is not toxic to the interior. NaOH to pH 9.8 is not toxic either to the surface or to the interior.

VII. *Antagonism.*

17. The toxic effects of NaCl and of KCl on the exterior of the cell can be antagonized by $CaCl_2$ and this antagonism occurs at the surface. Although the lethal effect of NaCl is thus antagonized, NaCl still penetrates but at a slower rate than if the ameba were immersed in a solution of this salt alone.

18. NaCl and HCl are mutually antagonistic in the interior of the ameba. No antagonism between the salts and HCl was found on the exterior of the ameba. No antagonism between the salts and NaOH was found on the interior or exterior of the ameba.

19. The pinching-off phenomenon can be antagonized by NaCl or by KCl, and the rate of the retardation of the pinching-off process varies with the concentration of the antagonizing salt.

20. The prevention of repair of a torn membrane by toxic solutions of NaCl or KCl can be antagonized by $CaCl_2$.

These experiments show directly the marked difference between the interior and the exterior of the cell in their behavior toward the chlorides of Na, K, Ca, and Mg.

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EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Ameba injected with sublethal dose of 1 M NaCl and photographed just after rounded, quiescent period; pseudopodia beginning to reform. The specimen was rolled over on its side and photographed while in this position. Note effect of sedimentation with formation of an upper, narrow, hyaline; a middle, extensive, finely granular; and a lower, condensed, coarsely granular zone.

FIG. 2. Ameba injected with lethal dose of 1 M NaCl and photographed after complete sedimentation of granules. Viewed from its side just before disruption of extremely delicate pellicle.

FIG. 3. Injection of M/104 $CaCl_2$ with pinching off: *a*, before injection; *b*, localized blister forming at injected region; *c* and *d*, blister at rear of advancing ameba being pinched off; *e*, pinching-off process being completed. Note in *b*, *c*, *d*, and *e* that granules in living part of ameba are blurred because of the flowing movement; in the dead, coagulated blister, they are stationary and distinct.

FIG. 4. Tearing of ameba in 1 M NaCl: *a*, ameba with needle in place before operation; *b*, beginning of radial scattering of region torn and remainder of ameba contracting in attempt to recover; *c*, complete dissipation of plasmalemma and scattering of granular contents; *d*, scatttered granules.

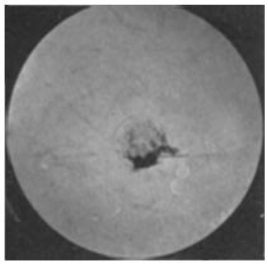
PLATE 4.

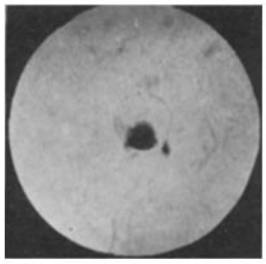
FIG. 5. Tearing of ameba in M/13 $CaCl_2$: *a*, 10 seconds after tear with column of solidified material and rapidly moving, living portion of ameba attempting to pinch off affected region; *b*, *c*, *d*, and *e*, ever lengthening column of solidified material and diminishing living portion; *f*, entire ameba dead.

FIG. 6. Effect of slight tear in M/13 $CaCl_2$ resulting in pinching off. *a*, before tear, needle in place; *b*, *c*, *d*, and *e*, successive steps in flow of ameba away from ever lengthening affected region which it is attempting to pinch off; *f*, final completion of pinching-off process and recovery of living portion of ameba.

Injection of 1 M NaCl.

Subleth-
al dose.

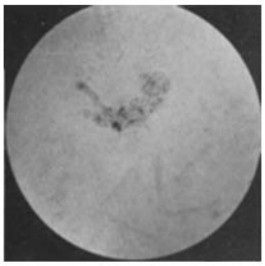




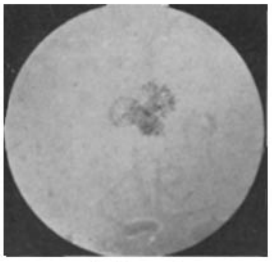
Lethal
dose.

FIG. 1. FIG. 2.

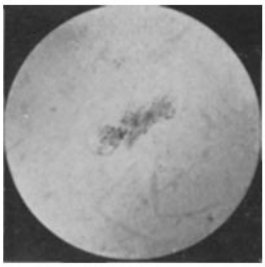
Injection of M/104 $CaCl_2$ with pinching off.



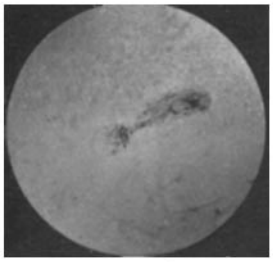
a (Before.)



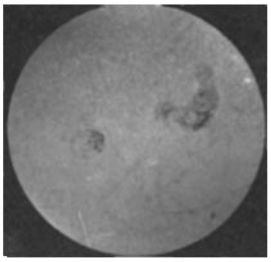
b (5 sec.)



c (25 sec.)



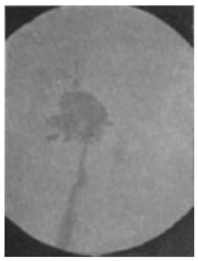
d (45 sec.)



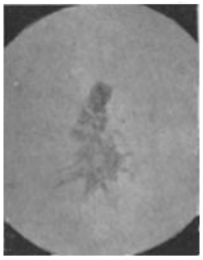
e (1 min.)

FIG. 3.

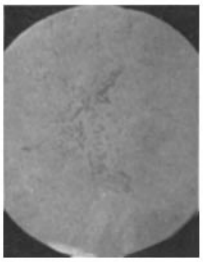
Torn in 1 M NaCl.



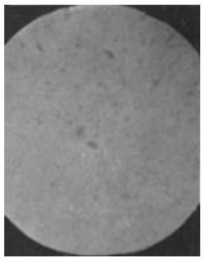
a (Before.)



b (5 sec.)



c (20 sec.)

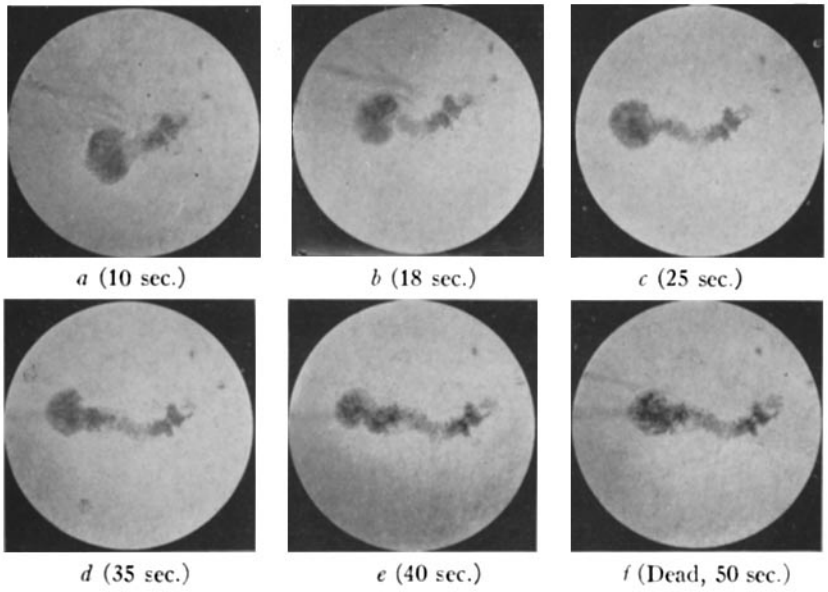


d (40 sec.)

FIG. 4.

(Chambers and Reznikoff: Cell physiology. I.)

Large tear in M/13 $CaCl_2$.

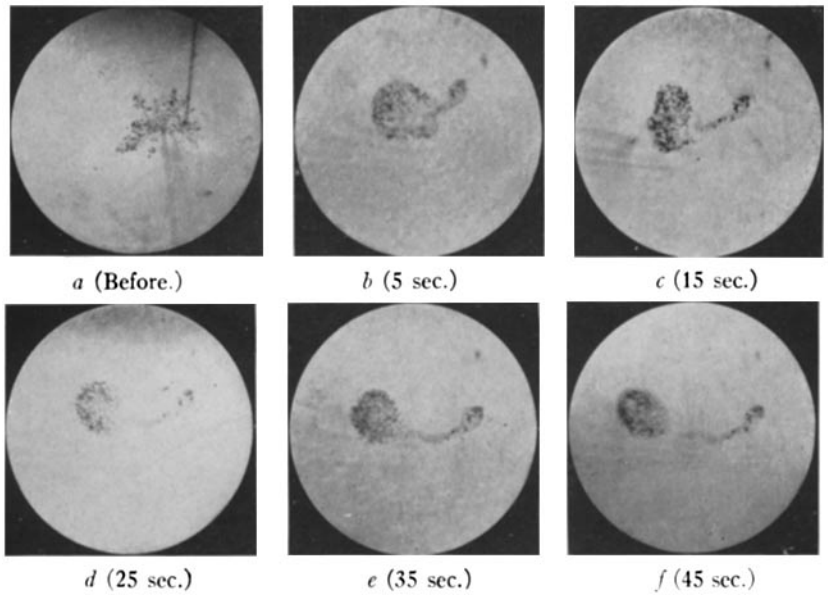


a (10 sec.) *b* (18 sec.) *c* (25 sec.)

d (35 sec.) *e* (40 sec.) *f* (Dead, 50 sec.)

FIG. 5.

Slight tear in M/13 $CaCl_2$ with pinching off.



a (Before.) *b* (5 sec.) *c* (15 sec.)

d (25 sec.) *e* (35 sec.) *f* (45 sec.)

FIG. 6.

(Chambers and Reznikoff: Cell physiology. I.)