

The Acclimatization of Chaos Diffluens
to Sodium Chloride Solutions

by

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Introduction

Just what do we mean when we say that an organism has become acclimated to a change in its environment? If we subject amebas to new environments how shall we measure their acclimitization to those new environments? If an organism dies---but again, what is death? Just when can we say an organism ceases to live? In the case of amebas, is it when the spherical form is assumed and the granules have gone to the center of the sphere, leaving a clear layer on the outside of the sphere, or has death come when the last small pseudopod has been drawn in, and the granules are uniformly scattered throughout the spherical ameba? Does lack of movement signify that the ameba is dead? Perhaps the ameba is dead only when it has entirely disintegrated, whether it has first formed a perfectly spherical form or not. How shall we measure the limits of acclimitization? Even though we do not yet know just when an organism is dead, at least, we do have some positive measurements as to whether it is living and adjusting itself to environmental changes; for, if there is growth followed by reproduction, the ameba is living and thriving, in some manner, under the new conditions to which it has been subjected.

Thus, this experiment has been conducted from the positive rather than from the negative point of view.

For years, in such books as Davenport's Experimental Morphology (p. 86), T. J. Parker's Elementary Biology (p.22), and Calkins' The Protozoa (p. 297) the general reader has been led to accept V. Czerny's ('69) version of the acclimatization of "Ameba princeps" ("A. diffluens?") Chaos diffluens Mueller (see Schaeffer '26, p. 42) to sodium chloride reported by him in the Arch. f. mik. Anat. V, 158-163, under the title of: "Einige Beobachtungen uber Amöben". Other writers also have accepted his conclusions without question that fresh water amebas can be acclimated within a comparatively short time to a 4% sodium chloride solution.

A careful reading of this report will however, soon disclose the crude and unscientific way in which this experiment was carried out, and, since the results of this paper have been accepted so widely and for so long a time, without apparent question, it is necessary to quote fully the extent and the results of his experiment, at this time.

Czerny found in his preliminary experiments:

- (1) that resistance to NaCl among the amebas varied, for in 7/4% NaCl many of the amebas died, while others could stand the concentration up to 2% NaCl;
- (2) that the slower moving animals had a greater resistance to NaCl solutions than the faster moving

ones; (3) the ball-like form either appeared at once, or after the animals had put out wart like growths and then burst, leaving nothing but an outer covering like a thin sac; (4) the ameba sometimes swelled up before bursting; (5) very fine growths were sent out when the NaCl became effective. These processes arose close together, grew longer quickly and became knotty, often bending and quivering; (6) occasional-ly the amebas put forth long strong pseudopodia which drew together like a rosary and then the end dropped off entirely; (7) if the concentration of NaCl was small (concentration not stated) the amebas began their usual motion when put back into fresh water again. One of these divided, ^{he says,} "before my eyes".

He really began his experiment on March 13, 1869 when he put some Chaos (Amoeba) diffluens into 1/3% NaCl solution. He found that the movement of the granules grew less and less and finally ceased altogether, but pseudopods were spread out and these assumed the spiral form. Some of the amebas became round. After some time he added fresh water again and the two amebas he was experimenting with drew in their pseudopods and took on the round form, soon, however, they put out pseudopods and the movement of the granules became normal. He saw one of these two divide, and altho he could not see the nuclei he saw that the contractile vacuoles were still present.

At this time, he also saw what he erroneously thought might be a conjugation of amoebas. He goes on to say that he kept amoebas in a watchglass in which he first had $1/4\%$ (or .25 of 1%) NaCl. After 24^{to}-48 hours he changed this solution increasing the concentration $1/6\%$, so that 9 days later he had increased it to $7/6\%$, and the amoebas then living showed almost no granular movement and were slower than the amoebas which existed originally in the fluid. These amoebas put out knobby pseudopods; they did not stick to the bottom of the watchglass, but could be swept away by every jar of the dish. On account of the presence of these amoebas he renewed the water for several days (every day?) with $4/3\%$ and then some more amoebas developed which he calls radiosa (!) This form, he found died in distilled water as well as in a 2% NaCl solution. In both cases the spherical form was assumed and they burst ten to twenty minutes later. They also often swelled increasing from 11^{to}16 units of measurement. Several stretched out and then burst. He suggested that these reactions were due to individual differences. By the 20th day he had reached a concentration of $1\ 2/3\%$. Altho he did not change this concentration for several days, the number of amoebas daily decreased, and on the 24th day none was to be seen. Since he had observed their disappearance

Pantin, C. F. A. - - - - - - - -1923

Edwards, J. G. - - - - - - - -1924-'25

Reznikoff P. & Chambers, R - - -1924-'25
2 papers

Schaeffer, A. A. - - - - - - - -1924

Pantin, C. F. A. - - - - - - - -1924

Adolph, E. F. - - - - - - - -1926

Botsford, E. F. - - - - - - - -1926

Schaeffer, A. A. - - - - - - - -1926

Pantin, C. F. A. - - - - - - - -1926
(In manuscript) 2 papers

Criticism of Czerny's Work

Czerny correctly judged that amebas could not be acclimated by putting them into solutions so concentrated that it is likely to kill them at once. If he experimented with Chaos diffluens (see Schaeffer '26 p. 42), I doubt that he could increase the concentration of NaCl to such great degrees and as fast as he did and still obtain growth and reproduction of the amebas. I do not think that in successfully acclimating Chaos diffluens he could begin at .25%. According to my experience, that is too high a concentration of NaCl to begin with. Increase of 1/6% far exceeded the increases I found I could use. He says nothing about using a pedigreed line so that he could have as nearly as possible uniform material to begin with.

He gives no record of just how he measured the salt he added, nor just how he calculated the concentration of the solutions the amebas were in. We are not given a clear understanding as to just what technique he used. We are left to conjecture as to just how and when he changed the concentrations and also to guess as to whether he removed all the amebas to a new clean culture dish, with the newly prepared NaCl solution in it when he says he "changed the concentration", or whether he merely took off some of the fluid and added more fluid. If he did change the amebas to a new dish each time he changed the NaCl concentration, how could he have obtained a new species, radiosa when he originally started out with Chaos diffluens? After he discovered the species radiosa however, he goes on with the experiment and we find that before he is thru he has mentioned at least 4 species of amebas tho' he started out with Chaos diffluens! This leaves us to wonder just what he means by his report. Does he conclude that our fresh water species Chaos diffluens has become acclimated to 4% NaCl solution, or, does he mean that any of the four species mentioned can be acclimated to 4% NaCl? As far as experimenting with Chaos diffluens is concerned, I think his experiment is vitiated as soon as, or before, he discovers radiosa amebas present.

In the latter part of his experiment where he

changed the Lemna and Sphagnum because he thought the dying of the plants had something to do with the disappearance of the amebas, he perhaps forgot that the Lemna and Sphagnum might act as buffers to the NaCl solution and thus change the concentration. He records that not before the 6th day could he find any amebas in this last prepared culture. Were the amebas he found on the 6th day first present as cysts? We know that there could be no spontaneous generation. He does not say how many amebas he found on the 6th day nor does he say how many and in what condition those were which he found in the culture at 4% NaCl. He does not state in this latter case by what steps he increased the concentrations of the solutions and when, altho' he has gone from 5/3% to 4% NaCl. We do not know what species he found living at 4% NaCl concentration.

Some of his results however, correspond with those of others who have exposed amebas to NaCl solutions.

G. F. A. Pantin has probably done the best work in experimenting with amebas in various salt solutions. He used the Marine limax types of amebas of which he gives the descriptions (Pantin '23). His drawings of the amebas, (Pantin '26) which were made to show the effect

of the change of the concentration of sea water, show decided changes in the shapes of the amebas and in the position of the granules. The amebas swelled when he put them into diluted sea water, and sometimes the pseudopods had irregular projections on them. Czerny ('69) observed the same results with some fresh water amebas. Pantin also found that amebas could still recover if returned to "outside" sea water from 0.2 strength sea water. If however, the .2 strength sea water was replaced by distilled water, they were incapable of recovery; they might not undergo immediate cytolysis but they swelled until almost spherical. When he introduced "type A", into hypertonic solutions of sea water (1.7 to 1.8 strength) he found that the pseudopods might even extend to several times the body length. The ameba appeared, took on the shape resembling a radiosa. Increases above 2 times sea water strength caused great shrinkage. Cytolysis did not take place for some hours even in 3 times sea water strength and recovery could take place if the ameba were brought slowly back to normal sea water. Great swelling, usually followed by cytolysis, occurred if amebas were suddenly transferred from 3 times sea water strength to .1 strength sea water. Pantin ('24) thinks that besides the solutions in which the

amebas were and the p \bar{H} of the solution, high temperature destroys something that is necessary for ameboid movement. In his as yet unpublished experiments (Pantin '26) he found that pure isotonic solutions of NaCl (also K \bar{Cl} MgCl or CaCl) will not support ameboid movement. In NaCl or K \bar{Cl} the amebas swelled after a few minutes, the protoplasm tended to become more fluid and cytolysis occurred most rapidly in K \bar{Cl} . He discovered also, that within certain limits the ameba was viable in isotonic mixtures of any two of the salts NaCl, K \bar{Cl} , Mg Cl and Ca Cl (p H 7.-7.2) except in those of NaCl and K \bar{Cl} . He believes that Ca stabilizes the cell membrane by preventing the great permeability found when in NaCl alone (Pantin '26). When he put the amebas into solutions which approached the composition of sea water where all the four salts NaCl, CaCl, Mg and K were present in the sea water proportions, they remain normal. Sr. can replace Ca for movement, but movement is only, maintained for long if the cell membrane be stabilized and stabilization is probably connected with reduction of permeability which prevents both penetration of the medium into the cell and loss from the cell of certain necessary substances, particularly Ca. The cell surface, he believes, is evidently an important part of the mechanism of movement.

Mg and Ba are well able to prevent the increased permeability and cytolysis seen in pure NaCl solutions.

In 1916 Schaeffer (Notes on specific and other characters of A. proteus Pallas Leidy, A. dubia spec. nov and A. discoids spec. nov.) correctly described and pedigreed the ameba that is used in this experiment. He managed to clear up the true nomenclatorial facts concerning this particular species, which, up to that time, had been confused with various other species, since no one had taken time ~~ex-the't~~ to discover its true characters by the pedigree method. The main features of this ameba are longitudinal ridges and a discoidal nucleus. Schaeffer agrees with Pantin that there is a relationship between the action of salts and the changes in ameboid movement (Schaeffer '26) because he found in experimenting with amebas in dilute sea water that the speed of movement is often increased for some of them, even tho sea water is their normal habitat and they cannot live in fresh water even for a few minutes (Schaeffer '24). All chemicals, thus far, which have been used in treating the common large fresh water amebas, have had the effect of slowing down movement where there was any effect at all. (Schaeffer '25) In work with amebas at Tortugas (1919) Schaeffer found that different species of amebas are variously sensitive to dilutions of sea water.

TABLE I

Effect of aduted seawater on marine amebas — Schaeffer '26
(N = returns to normal)

Ameba	100%	75%	50%	25%	10%	5%	1%	Fresh water
<i>T. sphaerum</i>			dies and breaks					
<i>T. gumia</i>					nearly sphere		sphere	
<i>T. pallida</i>				becomes more fluid				
<i>M. fulvum</i>					sphere begins		sphere	N
<i>F. crassa</i>					sphere begins		sphere	N
<i>F. citata</i>						not much change		N
<i>F. pellucida</i>				vacuoles		more vacuoles		N
<i>M. conips</i>					★ sphere begins	sphere		N
<i>N. auræ</i>					almost sphere	wrinkles and dies		no recovery
<i>S. tardus</i>								burst
<i>T. orbis</i>					★ slight movement	sphere opaque		no recovery
<i>T. hilla</i>					sphere in 10 min.			no recovery
<i>T. rugosa</i>				ovoid		spherical transparent		no recovery
<i>H. unda maris</i>					motion slight		sphere	N
<i>H. elegans</i>						motionless	nearly sphere	N
<i>H. caeruleus</i>						motionless		N
<i>Flamella magnifica</i>					several large vacuoles	motionless		N
<i>C. gulosum</i>				sphere begins	c. vacuoles slight increase			sphere N
<i>C. clarum</i>					not much change	change		N

★ In certain cases the nucleus was brot out very clearly during this process

He also found individual differences (Schaeffer '26). His experiments with one species of ameba Striolatus tardus, shows this. This ameba had been partially acclimated since the sea water had evaporated until it was 130% sea water. When one of these amebas was bro't into fresh water it burst at once, the rest remained for 2 minutes in fresh water before bursting, and one individual did not burst until 25% sea water was added, after it had been in fresh water first. He records the effects of changes in concentration of sea water on several marine amebas. See Table I.

Zuelzer ('10) who wished to find out whether and how far rhythm of the contractile vacuole is dependent upon sea water, chose The Ameba (Amoeba) Verucosa for her experiments, using for food the alga Gleocapsa, which lives very well in salt water. She isolated a number of the amebas and during four experiments tried them out in various concentrations of sea water. In order to get a higher concentration of the liquid she allowed the water to evaporate slowly by keeping the sand in the moist chamber a little dryer. She made up the deficit caused by evaporation by using diluted sea water. In the end, the experimental animals were put into water of this final concentration. The temperature was 17 C. She started them in .3 of 1% salt except in experiment No. 4 where she used 3/5 of 1%. She found that the animals lived well in this concentration of sea salts.

but that as soon as the sea water culture became more concentrated by evaporations, the animals began to show the appearance of shrinking. After the first 8 or 10 days, the amebas began to shrink in .3 to .5 sea water concentration. This process kept on, and the animals began to form knobs on their surfaces. The ability to stick to the substratum and the differentiation of ectoplasm and endoplasm was lost. The forward movement was slowed down, and the animals accepted less and less food. The diameter of the contractile vacuole decreased and pulsations became slower and slower, and at the time between systole and growth often the contractile vacuole seemed absent entirely. The amebas rolled about when shaken and streaming was hardly visible. They contained digested and undigested algae, so they had not entirely lost the ability to accept food. Part of the animals died during the beginning of the experiment when in a knob^y shrunken condition. They became spherical, swelled, and burst leaving a sac (also Czerny('69) found sac ^{shape} condition) behind. The final disappearance of the pulsating vacuole was first observed after 20 days when they were in 1 1/2% NaCl solution. The amebas varied among themselves in their reactions to the solutions, depending upon the length of time and the concentration. After 34 days (Exp. 4) where she used pure sea water) no contractile vacuoles were left in any of the shrunken amebas.

She reports that the amebas stood $3/4\%$ seawater well, tho' she never could see divisions in the salt water solutions, while the control amebas divided very often. Amebas that had been in sea water 3^{to}8 weeks were put into fresh water by gradually diluting the sea water drop by drop. They swelled up, the contractile vacuole reappeared considerably faster than it had disappeared and it increased in size until it became normal like the rest of the ameba, so that by the 6th day the ameba resembled the normal even in movement, and also in staining. She agrees with Czerny (869) that individual amebas which have moved more slowly seemed to adapt themselves more quickly to salt water changes than the faster moving ones. Zuelzer found that on staining the amebas which had been subjected to sea water solutions that the chromatin filled the nucleus and could not be stained as easily as the nucleus of controls. The cytoplasm of those in the salt solutions stained more heavily and the nucleus stained lighter than that of controls. She says that .3-.3/5% of sea water is probably isotonic with the body of The-amoeba verrucosa since higher concentrations cause decrease of fluid from body. She considers that the amount of water in the ameba has diminished since the contractile vacuole pulsates more slowly as the concentration of sea water is increased and finally disappears and does not reappear in $1\ 1/2\%$ ~~2~~ $1/2\%$ salt unless the individual is gradually returned to a fresh water medium.

Hogue ('23) in experimenting with Flabellula (Vahlkamfia) calkinsi (parasitic in the digestive tract of the oyster where the salt content is probably equivalent to seawater) finds that they gain, within a few days, from 1²⁴ contractile vacuoles, and that their rate of pulsation depended upon the amount of agar in the medium. The amebas did not reproduce in the change of environment. There was variability among the amebas, for while one encysted on the 9th day, another, under the same conditions, did not encyst until the 14th day. Similiar to Zuelzer's ('10) experiment, Hogue found that F. calkinsi seemed to feed, for it had food vacuoles full of bacteria. It formed pseudopodia and moved out over new medium. This is quite contrary to the results of Czerny and Zuelzer with the fresh water amebas where the power of locomotion was lost in too high salt concentration solutions. Hogue carried out the experiment several times keeping the amebas as long as 5 or 6 days on an agar medium made up with tap water and distilled water. She concluded, among other things, 1. that the older the culture of the amebas the slower was the rate of contraction of the vacuoles, 2. that the more active the ameba is, the more rapid is the pulsation of its vacuole.

In determining the effect of alkali chlorides on the rate of locomotion of amebas, J. G. Edwards and

H. S. Forgrave Jr. found that, 1. the rate of locomotion in N/100 (.058%) was greater for NaCl thruout the whole experiment than for any one of the other three salts used, 2. the decrease in rate of locomotion is a function of the length of time during which the amebas are in the solution, and is not conditioned by some factor such as absence of food, 3. the rate of locomotion increases with the decrease in the concentration of the salt. The optimum for NaCl was found to be N/900 or .0064%, 4. the rate of locomotion differs with ^{different} salts and this is due to the cation. In NaCl N/100 (.058%) the locomotion was 0.1476 mm per minute the first hour, while on the 4th day it was only 0.08 mm. per minute.

Reznikoff and Chambers (1924 & '25) found that "Ameba" lives for less than 1 hour in M/6.5 NaCl. (.89%)^x Amebas which died in the NaCl showed sinking and clumping of the granules and resembled amebas into which the salts had been introduced by injection. They concluded, 1. that amebas dead in NaCl apparently had a loss in the "integrity of the pellicle" (This did not occur with the other salts); 2. NaCl and KCl are much more toxic to amebas when in contact with their external surface than when injected and furthermore, there is evidence that NaCl and K can penetrate fairly easily while Ca and Mg cannot. Ca Cl in a non-lethal dose antagonized the toxic effects of NaCl.

Amebas torn even slightly in M/13 (.447%) NaCl did not recover, while with NaCl weaker than M/13 repair took place with increasing ease. The amebas could recover from extensive tears only in M/312 (.0178%) NaCl. Of the 4 salts studied only NaCl had a disintegrating action upon the surface of the amebas.

Botsford ('26) found that the mechanism of systole of the contractile vacuole of Chaos diffluans is related to surface tension. The ameba can regenerate a new vacuole if the old one is cut off.

Adolph ('26) discovered that when the influence of salt solutions M/20 NaCl, KCl, CaCl and urea was studied by measuring the vacuoles of single individuals, both in culture fluid and the M/20 salt solutions, in all cases the duration of the vacuoles increased markedly, but the rates of volume increase were not effected to any extent. He also found that in estimates of the total water metabolism 4¹/₃₀ hours are required for the elimination of a volume of water equivalent to the body volume.

Edwards ('24) reports that the ectoplasmic surface of amebas ruptures readily in strong solutions of bases and salts, the time required varying with the concentration. The time of inactivation or death in N/300 NaCl = 266 hours; in N/100 NaCl = 124 hours. He found that the cations have the most marked effect where the salt is ionic. NaCl solutions induce the formation of food cups (Edwards '25) in such suitable concentrations

as N/300 or N/500. No appreciable difference exists in either concentration as to the size or the number of food cups formed. These feeding reactions also occur if NaCl is added to a dilute acid or base.

Degen ('05) believes that the contractile vacuole is simply an osmotic system which works against an over inhibition of water, but can still support respiration, excretion and perhaps circulation. Environmental changes, he believes, cause disturbances in the frequency of pulsations and the condition of permeability. Pulsations of the contractile vacuole are retarded by such things as temperature, passing away from 34°C and also by neutral substances such as cane sugar, NaCl etc. He found in experimenting with infusorians that NaCl becomes least effective between .75% and .02%. The greatest distortions are caused by Na salts on the one hand and Cl salts on the other hand. The reagents used affected the protoplasm and the cell membrane in the same way, but to different degrees. If the affecting agency was washed out in time the dilated vacuole could be brought back to normal. In ".1 mole" (M/10) Degen reported that the infusorians look entirely distorted. The concentration of the salt affects the motility of the animals greatly. NaCl works most violently even down to .05 mole. Only from .M/.025 on down is mobility to be seen. Of

K_2CO_3 , only Cl makes the animals loose the power of movement about to $M/.75$. Na_2SO_4 and $NaNO_3$ the infusorians were found able to move more or less at $M/.10$.

Material and Methods

One line of amebas was used throught this experiment. This line was an isolation pedigree developed from a mass pedigree culture of the amebas now known as Chaos diffluens Mueller (Schaeffer '16). I began this experiment by isolating several individuals of Chaos diffluens from a culture which had been kept in the laboratory continually, by careful culture, for at least ten years. I used Chilomonas paramecium (Ehrenberg) grown in dilute hay infusion, as food for the amebas. Ocassionally some hypotrichs and Paramecium caudatum were in the food culture. The food culture fluid was strained thru a piece of duck cloth into a beaker and later portioned to the various watch glass cultures. The amount of food present, even in the sodium chloride solutions, always far exceeded the needs of the ameba placed in the culture. The watch glasses used were of the ordinary Syracuse type.

A simple method was used in numbering the amebas in the pedigreed line. This method consists in numbering the original ameba 1 and the daughters resulting from it, 1_1 1_2 and the daughters of 1_1 as 1_{11} and 1_{12} and

so on until the row of numbers becomes too cumbersome to handle, at which time the numbers can be simplified to 1, 2, 3, 4, etc. and continued as at first. These cultures, stacked up one upon another, were kept at room temperature in diffused day light. The temperature ranged from 19° to 25° C, averaging about 21° or 22° C. From these isolated individuals the ameba showing the most rapid rate of division was finally selected as the pedigreed line for the experiment.

The stock solution of NaCl was prepared by carefully weighing out 10 gms. of Squibb's c. p. NaCl, and then dissolving this in 90 c. c. of distilled water. This was kept in a sterile bottle and labeled "10% NaCl solution". A second solution (1% solution) was made from the first solution by measuring out 10 c. c. of the stock NaCl solution, and adding to it 90 c. c. distilled water. This 1% solution was in daily use. Both solutions were always kept tightly corked and out of direct sunlight.*

Foot Note *

Schlesinger, p. 362, says "since the Na and the Cl. are unquestionably charged, ionization, in a certain sense of the word, has occurred before the NaCl is dissolved. The fact that solid NaCl is a non-conductor of electricity is due in part to the fact that the crystals are so rigid that the ions cannot flow into the current," and p. 199, "NaCl is a strong

electrolyte, which, when in aqueous solution, may be looked upon as 100% ionized. In fact, it is considered to be ionized in the solid state. The strong electrolytes and their ions do not appear to obey the law of chemical equilibrium. In crystals of salts, like NaCl, the material points, which constitute the corner of chemical structure, are not molecules of NaCl as one would expect if NaCl were ionized in the solid state, but seem to be the Na and Cl ions. Finally, in dilute aqueous solutions of strong electrolytes, there are no known properties, which can be ascribed to the ionized molecules" (199).

A glass pipette rack was made by cementing strips of glass across two longer strips in such a manner that several spaces large enough to hold the pipettes were left between the shorter strips. This was then placed at an angle in a large glass dish, at a sufficient height to keep the pipettes from touching the floor of the dish, so that they could be rinsed and then placed in the rack to drain and dry for use the next day. There were two pipettes for NaCl solutions one drawn to a very fine point and used for the transference of an ameba in NaCl solution from one watch glass to another, and the other plain pipette was dipped into the 1% NaCl solutions. There were other pipettes used for the controls and for the Chilomonas food culture fluid. The pipettes were all of the same size, in order that all the concentrations should be uniform since the measurements were all made by holding the pipette vertically and counting the drops.

TABLE II

Showing the gradual increases of NaCl solutions
from .1 of 1% up to .2 of 1%

Amount of 1% NaCl solution in drops	Amount of culture in drops	Total amount of solution in drops	Calculated percentage by weight
1	19	20	.05 of 1%
2	18	20	.10 of 1%
2	16	18	.111%
2	14	16	.125%
3	18	21	.143%
3	17	20	.150%
4	22	26	.154%
3	15	18	.1665%
4	18	22	.1815%
4	16	20	.20%

NaCl solution made up as follows: 10 grams of NaCl by weight to every 90 cc. distilled water = 10% sol. 10cc of above solution to 90cc. distilled water = 1% sol. used in above table.

It was necessary to keep a supply of dry watch glasses on hand, since drops of water adhering to recently washed watch glasses would change the NaCl concentrations. This solution, measured in drops, generally averaged about twenty drops (See Tab. II.) so every additional drop made a considerable difference.

The contents of the watch glasses were daily examined with a binocular microscope, and records of the amebas kept in the note book. The number of the ameba (in lead pencil) and the amount of NaCl concentration (in red glass pencil) were written on the watch glasses and changed as a division occurred. For example: No. 1 in red meant .1 of 1% NaCl; .05 meant five hundredths of 1% NaCl; 15 meant .15 of 1% and so on, the numbers indicating a certain number of drops of NaCl to a certain number of drops of culture fluid, as seen in the chart of calculations (Table II). The watch glasses in which the regular amount of NaCl and culture fluid had been put, were mechanically agitated to hasten diffusion, tho' this was probably unnecessary owing to the dilute concentration of NaCl used in the experiment. Often the cultures were prepared only a minute or two before, I changed the ameba into the new solution. After having removed the ameba to the newly prepared culture, I always looked to be sure that the ameba had not been lost in transit.

All solutions, controls as well as NaCl solutions, were changed daily to insure uniformity among all the

amebas throughout the experiment.

As the^a preliminary to the experiment, Chilomonas were tested out in varying NaCl solutions (4%, 3%, 2%, and .1 of 1% up to 1%) to see whether they could be used as food for the amebas. The amebas were also tried out in NaCl concentrations from .1 of 1% to 1%.

Many amebas were used in these experiments for they soon died in the concentrations above .15% so some had to be tried in still weaker concentrations of NaCl.

Experimental Results

According to Czerny, one could begin at 1/4% (.25%) NaCl and increase the concentration of the solution every 24^{to}48 hours at the rate of 1/6% (.16%), so that by the ninth day one should have reached 7/6% or 1.16%. This is taking for granted, of course, that his measurements for NaCl were correct. He says that after he had reached 1.16% there were many amebas present intimating that there had been divisions during the time he was raising the concentration of the NaCl solution in which the amebas were living. I found, however, by trial and error method, that .25% is too high a concentration in which to successfully acclimate Chaos diffluens as will be shown later.

Chilomonas tried out in 1%, 2%, 3%, and 4% NaCl solutions all slowed down in movement at once; in 30 minutes those in 1% were still slower, those in 2% very slow and mostly dead or dying, while in the 3% and 4% solutions all the Chilomonas were dead. By the next day there were a few living, moving Chilomonas only in the 1% solution. Chilomonas were next tried out in tenths of a 1% solution--.1 of 1%, 2 of 1% etc. on up to .9 of 1% NaCl. I found that some of the Chilomonas lived in all of the solutions, but as the concentrations increased, the Chilomonas tended to form larger and larger clumps, somewhat similar in

appearance to agglutinating bacteria. This clumping began in .2 of 1% where movement was noticeably slowed down and the Chilomonas slightly clumped tho most of them still swim actively thru the culture. In .1 of 1% the Chilomonas appeared to move much as they did in control cultures.

The p H. of the food used was measured on March 30th and found to be 7.2 by the test with Bromthymol blue as an indicator. In April it ranged from about 6.8 to 7.0.

Preliminary testing of Chaos diffluens in 1% NaCl solution convinced me, after watching the effect it had on the ameba for just two hours, at the end of which time it had become more or less spherical, that I surely could never expect it to acclimate itself to this solution to the extent that an initial division could be made in this high a concentration. Czerny ('69) and Zuelzer ('10) had similiar experiences when placing amebas in solutions much too highly concentrated for them. I decided to follow a plan whereby the increases would be made by tenths of one percent. Before this, however, I tried the amebas in .1 of 1%, .2 of 1% etc., up to .9 of 1% NaCl solutions with Chilomonas, and I found that above .2 of 1% the general appearance of the ameba after 3-4 to 24 hours in this solution was far from normal. Even in .1 of 1% and .2 of 1% the amebas did not fasten themselves to the substratum as did the Con-

controls. Czerny and Zuelzer also found in their experiments with amebas that the power of sticking to the substratum is lost as the NaCl concentration rises. This power of sticking to the substratum is also lost when the ameba is enucleated, Calkins ('10) Phelps ('26). Mast thinks that the power to adhere to the substratum is due to the excretion ~~to adhere to the substance or to the excretion~~ of an adhesive substance or to a state of the "plasma lemma" (=-plasma membrane). Only rarely have I found amebas which have divided in .1 of 1% and are living it, fastened to the substratum, and on these occasions it was mostly when there had apparently just been a division, and I found them greatly branched out with perhaps two or three long, slender pseudopods fastened at or near their tips to the substratum. On one occasion, I saw an ameba divide in .1 of 1% NaCl. It had been in a round form, normally indicative of preparation for division for 2 days. Division took place normally (Phelps '26). The daughters stuck fairly well to the substratum for a time, at least. The daughters flowed apart then backed together and after that, flowed apart again, one individual especially, moving with its body highly bent above the substratum while only the tips of a few pseudopods stuck to the bottom of the dish. The next day neither stuck to the bottom of the dish.

In .05 of 1% the amebas were closely adherent to the substratum similiar to the controls. Perhaps this

concentration of NaCl may be considered isotonic for Chaos diffluens.

I found that, as the charts and graphs which follow will show, C. diffluens grows normally in .05% NaCl with the same reactions and rate of division as do the controls. In .1 of 1% acclimatization becomes increasingly difficult and division delayed, with very few amoebas sticking even slightly to the substratum. From this concentration it becomes necessary to increase the NaCl concentration by smaller degrees, so I increased the solutions as the chart for solutions shows, by hundredths of a percent to see if I could gradually acclimate the amoebas at least to .2 of 1%. The step from .1 to .12% is possible without putting the amoeba into .11% first as I found out by experiment. Only very "hardy" amoebas can withstand being put into .2 or even .15% suddenly. In only three cases, did they divide in .2 of 1% (see Tab. III. and Figs. 1 and 2) and after they had done this they were not able to divide in .2 of 1% again, but took on shrunken forms and did not recover. The amoebas in .1 of 1% in practically all cases, divide sooner or later even if, as in one case, at least, an amoeba remained in .1 of 1% 12 days before it divided. This amoeba was very large for some days before it divided. The few that were put into .3% from a division in .2% existed on that concentration for from 3 to 6 days.

Table III. gives a rough percentage estimate of the first divisions among the amoebas in the various concen-

TABLE III

31

Showing the divisions occurring once in the various concentrations of NaCl.

Percentage of NaCl solution	No. of individuals tried	No. of divisions once	% of divisions
.05	6	6	100.
.10	31	30	96.8
.125	15	9	60.
.15	18	4	22.2
.20	31	3	9.68

TABLE IV

Showing the number of divisions among those put directly into the solution without gradual acclimitization

Percentage of solution	No. of individuals put in directly	No. of divisions once
.05	6	6
.10	29	27
.125	0	0
.15	1	1
.20	6	2

Fig. I

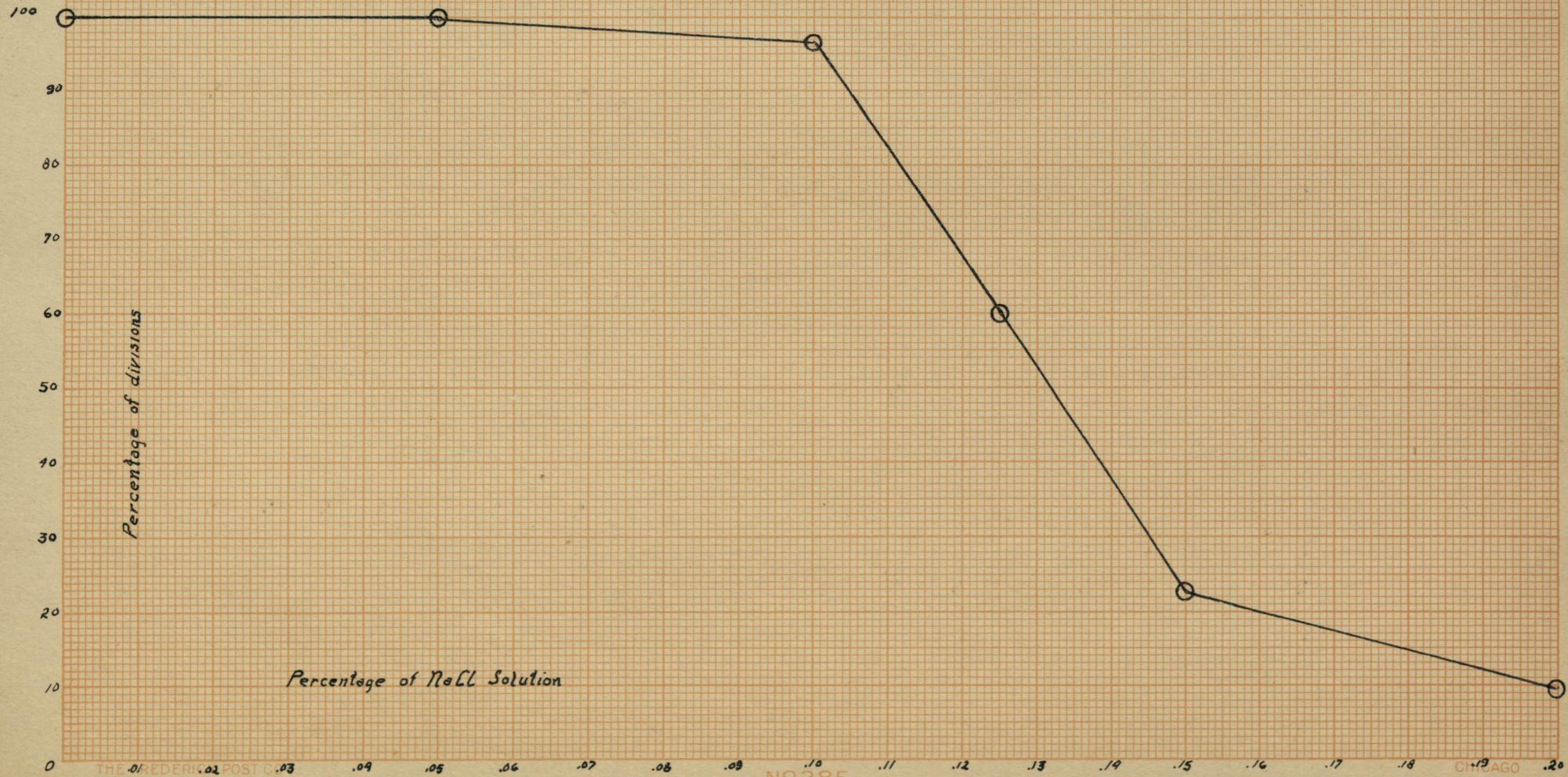


Fig. 2

Showing the time when most divisions occurred
in different concentrations of NaCl.

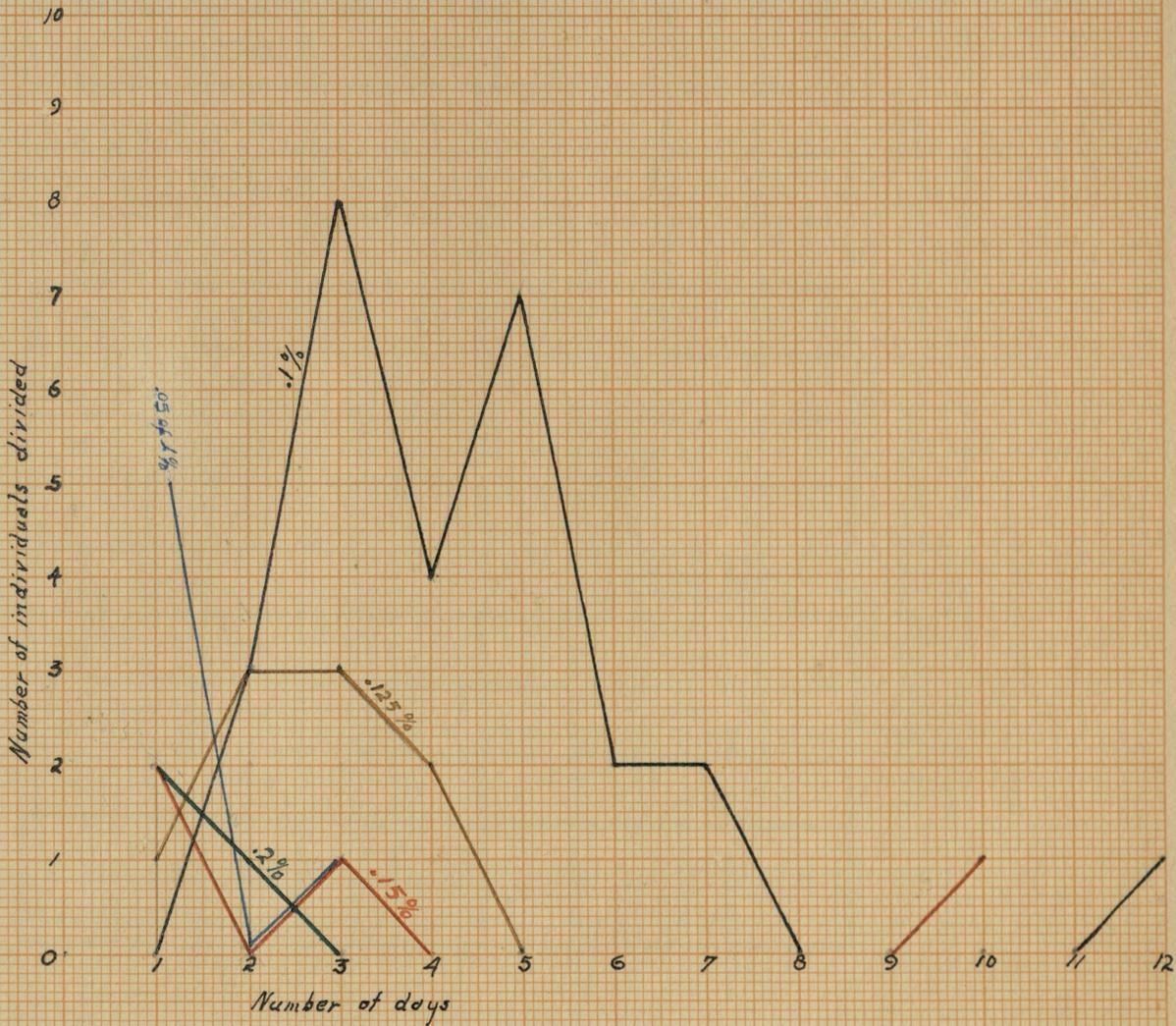


TABLE V

Typical table of results obtained in passing from a low to a higher concentration of NaCl

AMOEBA	.05%	.10%	.111%	.125%	.143%	.15%	.2%	.30%
1	2-d	9-d		2-d-8				
2	1-d	3-d		2-d-9+				
3	1-d-2-d-1-d	8-d						
4	1-d	3-d		2-d	8+			
5		3-d	2-d-1-d	4?				
6		3-d		5-d	4+			
7		2-d				3-d-6+		
8		1-d				1-d		
9						returned to normal 12 fluid, but did not recover.		
10		3-d					5+	
11		4-d					10+	
12		2-d					7+	
13							1-d	5+
14							8+	

Signs: d = first division ; d' = second division ; d'' = third division.

The number before the "d" indicates the number of days before division.

"+" = shrunken condition from which amoeba did not recover.

trations. Fig. I. is based upon the results in Table III. showing the decrease in divisions as the concentration is increased. Table IV shows the divisions in the solutions when the amebas were put into the solutions directly without first being put into lower concentrations. In this chart the ameba that was placed directly into .15% was an extraordinarily "hardy" ameba, it appears, for it not only divided once but twice in .1%. I could not, however, get it to divide the third time. It became smaller and shrunken beyond recovery. The other individuals concerned in this division were put into .2% and .1665% NaCl and they also became very small and shrunken within a few days. The divisions in .2% appear to be "hang over" divisions for they never divided in .2% again. [¶] Figure 2. shows the difference in the rate of division of those amebas in the various concentrations of NaCl. We see here that amebas in .05% divided very quickly and uniformly, in fact their rate of division is comparable to the rate of division among the controls. By the third day all had divided. In all other ways, such as sticking to the substratum and movement these amebas were normal. The wide range in rate of first divisions of amebas in .1% is very apparent, the most of them had divided for the first time by the end of the fifth day. The short range in the case of those in .12 may be accounted for by the fact that all of them had divided at least once in

.1%, tho the rate of division in .125% was not more rapid when the individual had divided twice instead of only once in .1%. For instance one individual which had divided twice in .1% divided in .125% on the 3rd day it was in that solution while another individual which had divided only once in .1% divided on the first day it was placed into .125%. In both cases the individuals were put into .125% on the day that they had divided in .1%.

The short range, in Figure 2I, covered by the amebas dividing for the first time in .15% and also those in .2% can possibly be explained by the fact that they were probably cases of hang over divisions, tho' they might have been exceptionally hardy individual amebas also.

Table V. was made to show a typical picture of results obtained by treating the amebas with NaCl solutions. These actual results were chosen as typical examples of the results found when the individual amebas were passing from a low to a higher concentration of NaCl.

The difference between the shapes of the amebas living in the different NaCl solutions and the controls, was decidedly noticeable. (See Fig. 3). Czerny ('69) Zuelzer ('10) Schaeffer ('26) Pantin ('26) and others tell of various changes in shape when amebas are immersed in salt solutions. Of course, amebas in .05 or 1% showed no difference in shape or ^{degree of} adhesion

to the substratum as compared with the controls; those living in .1 of 1% however, were almost always loose and branched, sometimes with pseudopods extending outward from the body of the ameba like so many fine rays. L. Loeb ('21) believes that this type of pseudopod is formed because of liquefaction at the advancing tip of the pseudopodium, followed by gelation at the sides, since in these solutions the ameba is more gelled because the loss of water has caused an increase in consistency. The ray like pseudopods were sometimes extremely thin and almost hyaline. After some days in NaCl solution, when the ameba was not faring well, the pseudopods seemed to be withdrawn more and more, extending shorter and shorter distances from the body and often being branched. The pseudopods became gradually less extended until the ameba assumed a knobby appearance and disintegrated or took on spherical form. When the ameba assumed a knobby appearance I found that it never seemed to move any distance from the center of the dish where it had been placed, on changing, the day before. It changed shape slightly and almost imperceptibly. (See Fig. 3) Calkins ('10) says that in all cases of harmful action upon the Rhizopoda, the reaction is expressed by the withdrawal of the pseudopodia, rounding out of the body and final disintegration. These, in brief, were the results I obtained by subjecting Chaos diffluens to increasing NaCl concentrations. Zuelzer ('10) also, found that

T. verrucosa shrank and took on abnormal shape in dilute sea water. Sometimes I have seen, in amebas the NaCl solution, a pseudopod which is clear for several minutes and then suddenly granular fluid is shot out into it.

The amebas feed in .2% of 1%, for on April 15th, I plainly saw a Chilomonas paramecium struggling in the food cup of an individual in .2% of 1% NaCl solution. This was an ameba that had been put directly into .2% and had been in it for 36 hours. It became spherical four days later.

There was ^{some} variability among the daughter amebas, two of the same division with the same treatment showing different susceptibility to the same NaCl solution.

According to Schwitalla ('26) the optimum temperature for the locomotion of the amebas he worked with is 23.5°C. Altho the temperature averaged very near this during this experiment I found that with increasing NaCl solutions, movement was slowed down, so that in concentrations of even .2% the movement slowed down more and more, as the time spent in that concentration passed, until movement ceased entirely and the ameba disintegrated or became spherical. Edwards and Forgrave ('23) obtained similar results and that the optimum movement for Chaos diffluens was between M/100 (.058%) and M/900 (.0064%) while I found that the amebas divided normally and stuck to the substratum at least up to .05%. Hogue ('23) obtained movement in

experimenting with F. Calkinsi, but could get no reproduction.

On examination of the amebas under high power lens, it was found that in case of those amebas in NaCl solutions up to .15% of 1% there was a contractile vacuole present. The rate of pulsation was not obtained however, because in those cases which were investigated the granules in the ameba were so numerous that the contractile vacuole was frequently lost sight of. It was seen sometimes at the end of a pseudopod as well as in the body of the ameba. In those amebas which were extremely granular and taking a more or less spherical form, I did not see the vacuole. zuelzer ('10) found in the case of T. verrucosa that the contractile vacuole disappeared after 20 days when the amebas were in 1 1/2% NaCl solution. It seems that a vacuole is necessary for the existence of Chaos diffluens for it soon forms one if it loses the one it has Metcalf ('10) (Budington) and Botsford ('26).

The amebas recover from NaCl solutions if they have not become too granular and shrunken before they are put into a normal fluid. Two individuals which were put directly into normal solutions just after their first division in .1% recovered and were perfectly normal appearing amebas adhering closely to the substratum in as short a time as a day. One ameba which had been in .1% divided the first day it was put into normal

culture fluid, another which had divided in .1% ten days after being placed in it, divided in normal fluid 3 days after it had been put in. An ameba which had been put directly into .15% and never divided in it even tho it had been in .15% 8 days, recovered and divided when returned to normal fluid. Another which had divided once in .05%, twice in .10% and once in .125% and been in .15% for 5 days without division, recovered and divided after 2 days in normal solution. One animal which had been in .15% for 12 days and was quite shrunken did not recover in normal fluid. There were a number of other amebas that reacted similiar to these. Amebas will recover from salt water changes, Schaeffer ('26) and Zuelzer ('10), but the change generally has to be gradual.

The Controls of which I always carried at least 3 and at times 6 or 8, were found almost always stuck tightly to the substratum. They divided, on the average, every other day. Among these, as among those living in NaCl solutions, there was, of course, variation. One Control No. 2 divided 21 times in a months, while others averaged 15^{to}17 divisions for a months.

Concerning division among the amebas, which did not occur for Hogue ('23) or Zuelzer ('10) in experiments with their particular amebas, it is interesting to note that while Reznikoff and Chambers ('24) found that amebas could recover from extensive tears only in M/312 (.0178%), I found that they grow and reproduce

in a comparatively higher concentration of NaCl i. e. .05% at least.

The change in shape and the shrinkage of the amebas in this experiment is probably due to the permeability of the cell membrane. NaCl is a strong electrolyte (Schlesinger, '25) so we could expect well defined effects upon an organism not used to a high concentration or even a low concentration of it. Bayle'sss ('24) believes that Na salts are a case where we can expect some chemical action to come into play. In these salts, he says, we know that it is the ions and not the molecules which are concerned because we get actions by solutions so dilute that the undissociated molecules are nearly absent. Pantin ('26) thinks that pure NaCl solutions seem to increase the permeability of the cell membrane so that imbibition results either from penetration of the medium into the cell or from a loss of substance including Ca from the cell. Reznikoff and Chambers (1924-25) also believe that NaCl has a disintegrating effect upon the surface of amebas. The most marked effects upon protoplasm and the cell membrane were found by Degen ('05) to be caused by Na and Cl salts. It is the anion of NaCl which penetrates membranes. Clowes ('16) and Lillie ('09) say that death may be the result of this increase in permeability since an organism cannot live with more than a temporary loss of permeability. According to his view it seems that in this experiment Chaos diffluens

was kept constantly in a death environment, as long as it was in solutions above which it could not react normally.

Conclusions

1. V. Czerny ('69) did not do a scientific piece of work in attempting to acclimate Chaos diffluens to 4% NaCl solution:--

A. He did not begin with a pedigreed line of amebas. He starts the experiment with apparently Chaos diffluens and later mentions at least three other species. How could these other species be obtained from a pure culture of Chaos diffluens? Surely, he did not think they changed from one species into another!

B. He evidently did not give his culture uniform treatment for he makes indefinite statements as to how frequently he increased concentrations of the solutions-- "after 24 or 48 hours", "^{On the} 20th day I had attained a percentage of 1/2-3/4, altho I did not increase the concentration for several days". "I gradually increased the concentration and found a few amebas even at 4%."

C. He does not tell how he measured the salt concentrations. Did he prepare new solutions each day, or did he draw off the liquid from the watch glass and add more? In either case, how did he measure the concentration?

D. He does not give a definite number of the amebas he found, even once. He says merely "many" or "few".

E. He did not explain the possibilities of Lemna and Sphagnum acting as buffers to the NaCl concentration.

F. He does not account for the sudden appearance of the amebas "not before the sixth day". Were they present as cysts up to that time?

G. I do not think he could increase the concentration as fast as he evidently did, and still obtain growth and reproduction of Chaos diffluens, nor do I think that he could successfully acclimate them beginning at .25% NaCl and increase by 1/6%.

1. The results of my experiment show:--

A. It is probable that Chaos diffluens could become acclimated to .125% NaCl solutions to a certain extent, if we can say that the lack of the power to stick to the substratum has nothing to do with the acclimitization of this ameba.

B. Chaos diffluens can not be acclimated to NaCl solutions by starting directly with .25 of 1%.

C. NaCl concentrations cannot be increased by such large amounts as .16%.

D. A successful starting point seems to be at .1 of 1% with increases no greater than .01 or .02%.

E. Lack of food was not a factor in preventing growth and division of the amebas in the NaCl solutions for Chilomonas existed in excess in concentrations at at least as high as .3%.

F. Chaos diffluens will exist (have some apparent protoplasmic streaming) for at least three days in .2% NaCl solution in which it does not divide.

G. Chaos diffluens retains its vacuole in NaCl concentrations up to .15% and the vacuoles do not increase in number.

H. The amebas recover when placed in normal culture fluid on the day of their divisions in .1% NaCl solution. It will also recover from higher concentrations if it has not shrunk too much.

I. Different individuals of the same pedigreed line vary somewhat in their susceptibility to NaCl concentrations.

J. The amebas do not adhere to the substratum in concentrations greater than .1% NaCl.

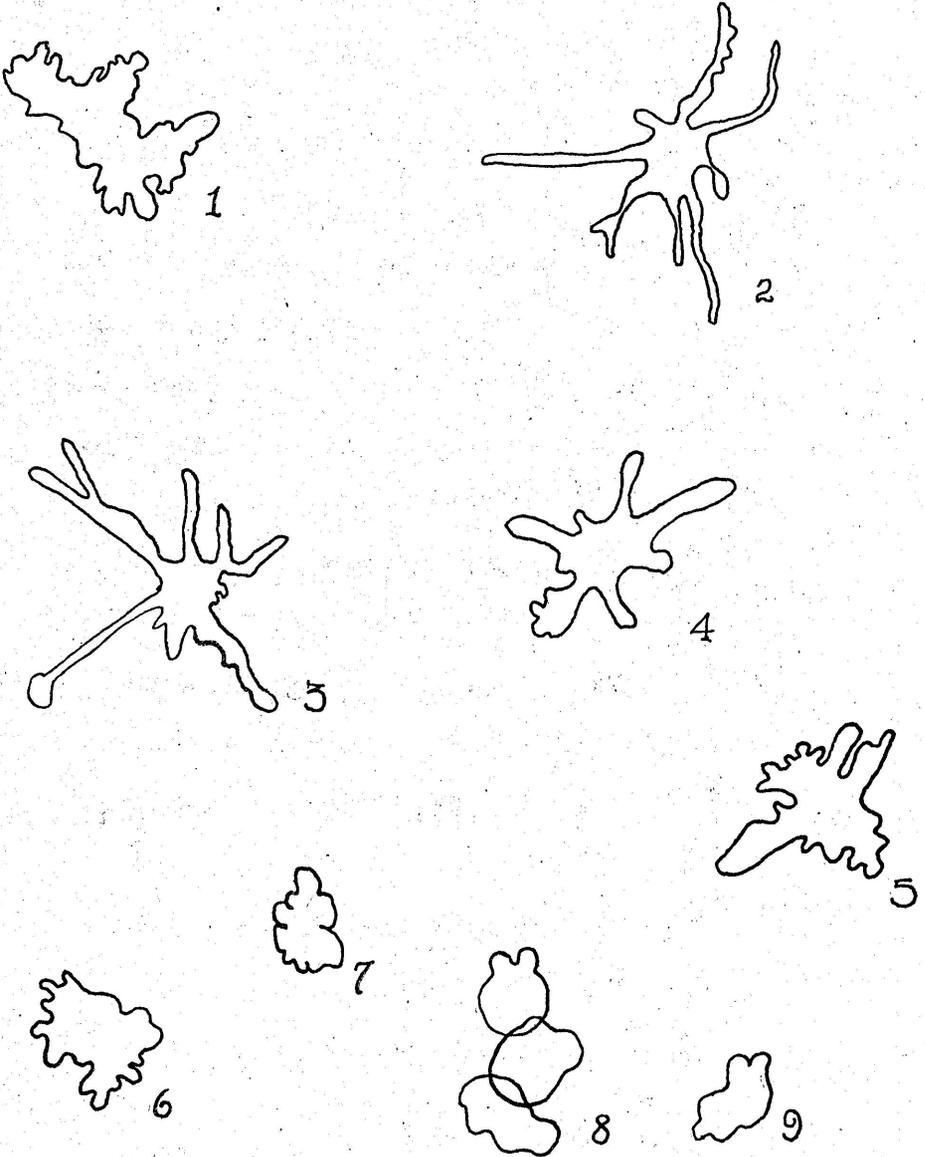
K. Sodium chloride probably penetrates the cell membrane of Chaos diffluens. The shrunken condition is markedly noticeable as the amebas continue to live in concentrations greater than .125% NaCl.

Figure 31.

Camera lucida: all drawn to the same scale
and at the same time.

- No. 1. A Control ameba .
- No. 2. "Radiosa type", has been in .1% for 4 days. Four days later it divided.
- No. 3. "Radiosa type", divided in .1% after 2 days. It was kept in .1% for 5 days, looked unhealthy so was put back into Normal in which it divided after 1 day. It was then put into .15%. The drawing was made at this time it was in 15%. It divided in this .15% solution 4 days later.
- No. 4., divided in .1% twice and was been in .1% for 5 days since without again dividing.
- No. 5. Knobby or "warty" type, divided in .1% twice and was been in .15% for 7 days.
- No. 6., also knobby, divided in .1% after 12 days, and was been in .1% 6 days.
- No. 7., divided once in .1% and was been in .2% for four days.
- No. 8., in three positions--it divided in .1% twice and was been in .2% for 7 days.
- No. 9., divided in .1% and was been in .2 for 6 days.

Figure 3



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