MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

VI. Calcium Ions in Living Protoplasm.

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In studying the chemistry of protoplasm it is essential to permit as little variation from the normal state of the cell as possible. The mass of data accumulated on the analysis of dead tissues reveals little information concerning its actual state during life. A striking example is afforded in studying protein precipitation in living cells and in extracts of dead cells. An alcoholic solution of picric acid, for example will precipitate protein from its aqueous extracts of dead cells within wide ranges of pH, whereas one may inject such a picric acid solution, in comparatively large quantities, into the living ameba with practically no toxic effect (1). Another example of the difference in the chemistry of living and dead or injured cells is seen in the study of the hydrogen ion concentration (2, 3), in which it has been shown that the pH of the normal cytoplasm of echinoderm ova is about 6.8, while the injured cytoplasm has a pH of about 5.4, and the dead mass assumes the pH of its medium.

The application of chemical tests to the living cell is subject to many limitations. First, the reagent must react at the pH of the cytoplasm. Secondly, color tests are to be preferred because of the difficulty of watching precipitations in the cell with ordinary illumination. Third, the reagent should be relatively non-toxic. Fourth, the reagent must be very sensitive.

The recently reported values for the pH of the ameba lie between 6.9 (4) and 7.6 (5, 6). This restricts the reagents that can be used in the ameba to those that are most efficient in a medium close to neutrality.

It was found that alizarin sulfonate will precipitate calcium ions

quantitatively while conforming to the above conditions. The resulting compound, calcium alizarinate, can be seen under the microscope in the form of purplish red crystals. When the pH is close to neutrality and there is an excess of calcium one would expect no appreciable interference from the possible presence of magnesium in the cell. The toxicity of this reagent is discussed in the experimental section below.

Alizarin has been used (7) for the study of bone growth, by feeding it in the form of madder root to experimental animals. The justification for this practice seems to be that alizarin combines with calcium in the bone to form red colored insoluble calcium alizarinate. Alizarin has also been used recently to determined blood calcium (8).

EXPERIMENTAL.

1. Injection of Alizarin.

By means of the micrurgical technique (9) varying quantities of a saturated aqueous solution of sodium alizarin sulfonate were injected into *Amœba dubia* and *Amœba proteus*.

The injection of a moderate quantity (1/4 the volume of the ameba) of a saturated aqueous solution of this reagent (reddish brown in color) causes a temporary cessation of movement. The ameba rounds up and the larger crystals and granules may settle to the bottom. A close examination of the cytoplasm shows fine purplish red granules scattered throughout the cell, and the hyaline cytoplasm itself is diffusely colored pale red.

If the ameba attempts to put forth a pseudopod as evidenced by a slight lifting of the membrane a shower of these purplish red granules are seen to appear in this area and the pseudopod formation is immediately stopped. During recovery the diffuse red color gradually disappears but there is an appreciable increase in the number of the purplish red granules.

The return to normal activity is gradual, generally lasting over a period of 2 to 3 hours. A peculiar phase in the process of recovery is the elevation of the plasmalemma and the appearance of a prominent hyaline zone between it and the condensing granuloplasm. The surface boundary of the granuloplasm soon breaks down and a portion

of the granuloplasm starts to flow into the hyaline zone, slowly at first, then with increasing rapidity. The movement then stops, the granuloplasm again separates itself from the hyaline zone, and the process is repeated. Reznikoff and Chambers (10) have called this phenomenon a pseudomembrane formation.

If an ameba is killed during the injections or is torn by the micro needles in a medium containing alizarin, the large crystals normally present in the ameba and some of the coagulum which is produced upon death will also take on the purplish red color characteristic of calcium alizarinate.

The nucleus may also be affected by the injection. When it is affected it loses its normal granular appearance and appears as a hyaline body with a pinkish brown stain. The ameba may extrude such a nucleus and recover to the extent of actively moving for some time.

2. Injection of Calcium Following the Injection of Alizarin.

The quiescence which is induced after an injection of alizarin may be due to a removal of calcium of the protoplasm from the sphere of action. The idea suggested itself that a subsequent injection of a solution of calcium chloride should aid in the recovery of the ameba.

It has been shown (11) that the injection of calcium chloride solutions in concentrations stronger than M/208 causes an injury in the form of a local coagulation (11). Therefore the concentrations used were limited to M/208 and less.

When an ameba which has previously been injected with alizarin is injected with an M/208 calcium chloride solution, active flowing movements appear almost immediately which subside in a very short time. Another injection of the calcium solution into the same ameba has the same effect. Because of the resulting trauma no more than two successive injections were tried on any one ameba. The time usually required for complete recovery after an alizarin injection is shortened from about 2 to 3 hours to $\frac{1}{2}$ to 1 hour.

3. Injection of Other Calcium-Precipitating Anions.

The similarity of the effects of the injection of the phosphates, carbonates, and sulfates as observed by Reznikoff and Chambers (10)

to those observed on the injection of alizarin is very close. This is of interest, since these three anions also form insoluble salts with calcium. To further investigate this uniformity of action of the calcium precipitants, injections were made of the salts of two other organic anions whose calcium salts have relatively low solubility products, *viz.*, tartrate and oxalate.[1]

It was found that the effect of these injections was practically the same as that of alizarin. The ameba could recover from a moderate injection of M/8 solution of sodium potassium tartrate or of M/18 solution of sodium oxalate after going through the stages of quiescence, rounding, and pseudomembrane formation. Concentrations as low as M/128 of sodium potassium tartrate and M/620 of sodium oxalate still called forth a pseudomembrane reaction. If the nucleus became hyaline, the ameba would not recover unless it extruded the hyalinized nucleus.

DISCUSSION AND SUMMARY.

The quiescence, rounding, sinking of the granules, and paling of the nucleus are similar to the effects seen after the injection of potassium and sodium chloride (11). Since the sodium salts of the anions were used, it might be inferred that the sodium is the active agent in the injected solutions. This is not entirely the case, however, for the effective concentrations of NaCl required are many times greater than those required in the case of the sodium salts of the calcium-precipitating anions. The fact that practically the same effects can be obtained in both cases leads one to suspect that there is a relation between the results of an increase in sodium ions and a decrease in calcium ions. It has been shown that a M/416 $CaCl_2$ solution will antagonize a M/1 NaCl solution and even a more concentrated solution of KCl inside the ameba (12). Therefore the reduction in amount of calcium may leave a comparatively high concentration of unantagonized sodium and potassium.

The fine, purplish red granules resulting from the injection of the alizarin are, no doubt, the insoluble calcium alizarinate. Recovery of an ameba from such an injection may be explained by the postulate

[1] I take this opportunity to thank Dr. Ruth B. Howland for her kind help with these injections.

that the free calcium ions in the living ameba are in equilibrium with a reserve supply of unionized calcium. The equilibrium is upset when the free calcium is removed by precipitation or by other means, and the system may possibly react in such a way as to counteract the effect of the change imposed. By mobilization of the calcium from a reserve supply the ameba can therefore gradually resume its normal activity. The time required for the recovery depends on the amount of alizarin injected. The diffuse red color which is seen immediately following the injection of alizarin probably represents that extra amount of dye which was not used in precipitating the immediately available calcium. Then, as the calcium is being liberated from the reserve, it is taken up by this surplus alizarin, resulting in a gradual loss of the diffuse coloration and an increase in the number of purplish

TABLE I.

Salt injected	Toxic concentration of injection of ¼ volume of ameba	Water effect	Solubility product of corresponding calcium salt
Na_2SO_4	M/2	M/64	6×10^{-5}
NaK Tartrate	M/8	M/250	7.7×10^{-7}
Na_2HPO_4	M/16	M/1280	5.4×10^{-7} $Ca_3(PO_4)_2$
Na_2Oxalate	M/16 M/18	M/1860	1.7×10^{-9}
$NaHCO_3$	M/32	M/256	1×10^{-8}

red calcium alizarinate granules. Only when all of the injected dye has been precipitated can the mobilized calcium be used to carry on the normal physiological processes of the organism.

The need of calcium to effect ameboid movement has been shown by Pantin (13) in a series of immersion experiments. This fact is quite suggestive, because the first effect of the injection of any of the calcium precipitants is absolute quiescence. Furthermore, there is no return to normal movement until the calcium apparently becomes available to the protoplasm.

In support of the conception of a reserve supply of calcium is the presence of the large crystals which give a positive reaction with alizarin for calcium on the death of the ameba. Schewiakoff (14), from crystallographic studies, claims that they are calcium phosphate.

The effect of the injection of the calcium-precipitating anions on the calcium of the protoplasm may be shown in another way. In determining the relative toxicity of these salts an arbitrarily standardized injection, about one-fourth of the volume of an ameba, was used. This was introduced because of the necessity to avoid effects due to variable amounts of the solvent, *viz.*, water. Thus the water effect was kept constant, and the variations in actual amount of salt injected were obtained by using a graded series of concentrations.

Arranging the sodium salts of these anions in order of increasing toxicity in one column, and the *in vitro* solubility products of the corresponding calcium salts in another column, it is seen that as the toxicity increases, the solubility product decreases (Table I). This fact strongly suggests that the toxicity depends on the ability of the salt to remove calcium ions from the protoplasm. The apparent deviation of the carbonate from the rule can be explained by the specific effect of CO_2 (10) which is always present from the hydrolysis of the carbonate.

CONCLUSIONS.

1. The injection of alizarin sulfonate gives a color test which demonstrates an appreciable amount of free calcium ions in the living ameba.
2. The toxicity of the anions: phosphate, sulfate, tartrate, and oxalate is related in some measure to the solubility product of the corresponding calcium salt. The carbonate does not fall in line.
3. The ameba has a calcium reserve which serves as a mechanism of recovering from the effects of sublethal doses of these anions and of the alizarin sulfonate.
4. The post mortem color reaction with alizarin sulfonate gives evidence of the presence of calcium in the large crystals in the ameba.
5. The behavior of the ameba is practically the same when the intracellular calcium ion concentration is diminished or when the sodium or potassium ion concentration is increased.

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