MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

II. THE ACTION OF THE CHLORIDES OF LEAD, MERCURY, COPPER, IRON, AND ALUMINUM ON THE PROTOPLASM OF AMOEBA PROTEUS.

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(Accepted for publication, June 22, 1926.)

The effects on protoplasm of some of the cations found in physiological systems have been reported in a previous communication (1). This study utilized the micrurgical technique and the advantages of such a method over that of simple immersion were pointed out in detail. Only by combining the results of injecting substances into the living cell with those obtained by the immersion method can one obtain proper conceptions of such important physiological problems as those of permeability, site of toxic action, antagonism of ions, and protoplasmic consistency.

Our knowledge of the action of heavy metals has been limited because of the difficulty in localizing the effect of salts in definite parts of the cell. The rapidity with which a cell or tissue reacts has usually been considered to mean ease of penetration (2, 3). But this is not necessarily the case. Even where some change has been noted inside the cell one cannot be certain that it might not be due to a surface effect which involved a secondary change within the interior or that it was not caused by the abstraction of some substance from the interior of the cell.

In a review of the work dealing with the action of disinfectants, especially HgCl₂, Cohen (4) points out that previous contributions deal mainly with the course of the process and not with the mechanism concerned. The micrurgical method is especially adapted to a study of the mechanism of the reactions between salts and protoplasm because of the ease with which substances can be brought into direct contact with definite parts of the cells. The importance of many
of the metals in certain physiological (5-7) and pathological (8-10) problems suggested their study by means of micrurgy. With the aid of microdissection and injection, therefore, the effects of the chlorides of lead, mercury, copper, iron, and aluminum on *Amoeba proteus* were investigated. It is the purpose of this paper to present the results of immersing and tearing amebæ in solutions of these salts and of injecting such solutions into these cells.

The manipulation of the apparatus, the general experimental technique, and the terms used, have been fully described in our former paper (1) and the reader is referred to that report for the details of the procedure. The only general modification of the method used in the experiments described in this paper was that in most of them fewer amebæ were used. This seemed justifiable because of the relatively constant quantitative results with several thousand amebæ from the same stock in former experiments.

1.

**Immersion Experiments.**

*PbCl₂.*—*PbCl₂* undergoes gradual hydrolysis in solution, with increase in acidity, and not until a dilution of M/5500 is reached can a solution be maintained at pH 5. If amebæ are immersed in decreasing concentrations of such solutions of *PbCl₂*, they undergo a slow change in shape. They gradually retract their pseudopodia and assume the elliptical form of the so called *Limax* type. The surface is stiffened and the ameba becomes very sluggish. Finally the cell becomes rounded and then dies. The curve labelled *PbCl₂* in Fig. 1 illustrates the relatively slow action of *PbCl₂*. It is toxic eventually in even very great dilutions. Amebæ cannot survive longer than 5 days until a dilution of M/22,000,000 is reached.

*HgCl₂.*—Amebæ immersed in *HgCl₂* die very rapidly compared to those in most of the other salts (Fig. 1). In solutions stronger than M/8000 they are converted into small round masses. In solutions ranging from M/8000 to M/250,000 the plasmalemma breaks and the contents begin to scatter but solidify rapidly. Fig. 1 shows that the curve of toxicity for *HgCl₂* is relatively steep. A solution of M/125,000 is toxic in 1 hour, but amebæ survive more than 5 days in M/500,000. That the immediate effect may not be due entirely to
the acidity which develops when HgCl₂ is dissolved in water is shown by the fact that amebae can live normally in water at pH 6 but die rapidly in the presence of an m/64,000 solution of HgCl₂ of the same pH.

CuCl₂.—The reaction of an m/1000 solution of CuCl₂ is pH 5.5 1 day after the salt is dissolved. After 3 days the acidity increases to pH 4.8. Immersion of amebae in these solutions of CuCl₂ causes the amebae to become rounded and the contractile vacuole to increase in size. During the first 3 days of immersion amebae die in a considerable range of dilutions (Fig. 1). In solutions weaker than m/4,100,000 toxicity decreases abruptly.

FeCl₃.—The acidity of solutions of FeCl₃ increases rapidly on standing, the greatest amount of change, however, occurring early. For example, a solution as dilute as m/66,000 changes from pH 6.2 to pH 5 in 4 days. These solutions of FeCl₃ cause immersed amebae to become rounded and the crystalloid granules within the ameba become blackened. FeCl₃ is relatively non-toxic during the first 2 days of immersion after which time the toxicity increases rapidly (Fig. 1). Thus, amebae can live in a more dilute solution than m/500 for 2 days but cannot live for 5 days until an m/128,000 solution is reached.

FeCl₄⁻—Solutions of FeCl₄⁻ gradually increase in acidity (e.g. m/325,000 has a reaction of pH 6.2 when first made and is more acid

\footnote{In preparing solutions of FeCl₄⁻ the molecular weight was taken as 540.44 (Fe₂Cl₆, 12H₂O).}
than pH 5 in 5 days). FeCl₃ is much more toxic but produces the same visible effect as the salt of divalent iron. The amebae become rounded and the crystalloid granules blacken. The maximum toxicity is approached after 1 day of immersion (Fig. 1). Subsequently the curve of toxicity rises more steeply than for any of the other salts used in these experiments.

AlCl₃.—Solutions of AlCl₃ are acid in reaction over a considerable range (an m/64,000 solution has a pH of 5). This salt, in concentrations of m/400 and higher, causes the amebae to become round rapidly. In solutions from m/400 to m/32,000 the amebae continue to move about until their surfaces break. The most striking effect of the salt is the tremendous enlargement of the contractile vacuole (Fig. 2). This occurs in concentrations of m/2,000,000 and stronger. The granules are pushed against the plasmalemma by the distended vacuole. In very dilute solutions, in which the amebae may live beyond 5 days, the large contractile vacuole may persist for 2 or 3 days but then decreases to its normal size and the ameba recovers completely. The curve of toxicity of AlCl₃ is fairly steep (Fig. 1).

II.

Injection Experiments.

PbCl₂.—In solutions stronger than m/1000, PbCl₂ is changed rapidly into the insoluble carbonate by the abstraction of CO₂ from the air. The injection of PbCl₂ in concentrations ranging from m/1000 to m/20,000 (Fig. 3) causes the gradual appearance of an irregular, glassy mass containing very few granules. The streaming movements in the rest of the ameba are very active and the glassy mass is
extruded. A second injection immediately after the first results in no solidification. If a large amount is introduced, the surface of the cell may break. If a second injection is made 20 minutes or more after the first, a second solidification and extrusion of the affected region may occur. Subsequent injections have no effect on the viability of the ameba, whether extrusions do or do not occur.

The effect of PbCl₂ in causing a delayed coagulation after the first injection and no coagulation after an immediate second injection, suggest that in the reaction between PbCl₂ and the protoplasm the lead uses up some cellular constituent which gradually forms anew in the cell. This harmonizes with the suggestion made by Aub and Reznikoff (11) that lead, in the small concentrations used in these experiments, unites with the phosphates or carbonates of the cell. The delay in the formation and the peculiar glassy appearance of the solidified mass in the cytoplasm also point to a different type of reaction between protoplasm and lead than those obtained with the other coagulating ions studied.

HgCl₂.—A solution of 1/5 to 1/100 HgCl₂ causes an immediate solidification of the internal protoplasm (Fig. 3). With solutions of 1/100 to 1/1600 the surface breaks and some of the contents of the ameba flows out. Small amounts of 1/300 HgCl₂ cause a disruption of the surface with subsequent recovery. Injections of small amounts of 1/600 produce no break and the ameba readily recovers from the
breaks caused by larger injections. A moderate quantity of $m/1600$ causes no break but the ameba may pinch off the somewhat solidified injected region. An injection of an $m/2400$ solution even in large quantities causes no disruption of the plasmalemma.

The results indicate that both very high and very low concentrations of $HgCl_2$ act principally on the interior of the cell. Moderate concentrations erode the plasmalemma.

$CuCl_2$.—The injection of solutions of $CuCl_2$ causes a solidification of the affected region and a disintegration of the adjacent surface. With $m/16$ the ameba can pinch off the affected region (Fig. 3). Stronger solutions solidify the entire cell. Not until $m/8000$ is reached does this solidification process cease. Each successive injection of $CuCl_2$ in concentrations ranging from $m/16$ to $m/8000$ causes the pinching off of the solidified area with its disintegrated surface.

After the solidified area is pinched off, the remnant is apparently normal except for a temporary moderate enlargement of the contractile vacuole. Some enlargement of the contractile vacuole also occurs when very dilute solutions are injected with no resulting local solidification.

$FeCl_2$.—Solutions of $FeCl_2$ stronger than $m/32$ solidify the internal protoplasm with which they come into direct contact (Fig. 3). With $m/32$ the affected portion is usually pinched off. $m/64$ causes a quiescence and partial solidification of the injected region, which is subsequently reincorporated by the active portion of the ameba. Large amounts of $m/512$ may sometimes cause death, but moderate quantities of $m/32$ to $m/64$ as a rule are not fatal. The method by which the ameba constricts off the solidified part when $FeCl_2$ is injected is somewhat different from that observed with most other solidifying agents. Instead of a sharp constriction between the injured and healthy portions (1), the ameba forms a line of demarcation between the healthy and affected area and the living part flows around the solidified region so that the latter lies in a deep depression and is slowly extruded as the depression is everted (Fig. 4). The surface of the extruded region has no definite pellicle in some places. The living portion appears normal except for a slightly enlarged contractile vacuole.
FeCl₃.—FeCl₃ is much more toxic than the divalent chloride of iron to the internal protoplasm (Fig. 3). A small injection of M/160 solidifies almost the entire ameba and the streaming movements of the unaffected region, which are apparently attempts to pinch itself off, soon cease and the whole cell is killed. From M/320 to M/1280 pinching off usually occurs after each injection. Occasionally, after the introduction of M/320 the ameba may incorporate the affected mass. With M/5120 an extrusion of a small mass from the injected region may occasionally occur after repeated injections. FeCl₃ causes the ameba to become sluggish and no typical water effect (1) is obtained until a dilution of M/10,240 is reached. Immediately after injection the vacuole of the ameba enlarges to some extent.

AlCl₃.—The introduction of AlCl₃ has a very striking effect on the ameba. In strengths of M/16 and stronger the injected area is solidified (Fig. 3). M/8 affects the entire ameba but when a solution of M/16 is introduced only the portion injected is solidified and is subsequently pinched off by the unaffected part of the ameba. The injection of M/32 also causes solidification, and pinching off may occur if the injection is made near the edge of the cell. However, in many such cases the ameba may reincorporate the affected mass after it is almost separated from the living portion, as though it were a foreign body. The solidified portion, held by a narrow band is finally en-

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**Fig. 4.** Injection of M/32 FeCl₃ into ameba; a, before injection; b, solidification of the region injected; c, d, e, f, and g, extrusion by sliding around solidified area; h, complete separation of living remnant from dead area.
gulped by the ameba and is soon completely absorbed. The various steps of this process are illustrated in Fig. 5. One of the most marked features of the injection of AlCl₃ in concentrations ranging from 1/32 through 1/250 is the tremendous enlargement of the contractile vacuole (Fig. 2). This is identical with the results obtained in the immersion experiments. The ameba recovers from this condition, which may last from a few hours to several days, depending upon the concentration of AlCl₃ injected. The similarity in appearance of the internal protoplasm in immersion and injection experiments indicates a high degree of permeability which is in accord with the findings of Michaelis (12).

**FIG. 5.** Injection of 1/32 AlCl₃ into ameba; a, before injection; b and c, uninjured portion flowing away from injected area and beginning to pinch it off; d, almost complete pinching off and beginning of engulfment of injured region; e, completion of engulfment; f, incorporation; g, return to normal state.

**III. Tearing Experiments.**

Because of the marked toxicity of even dilute solutions of the heavy metal salts to immersed amebae, it is very difficult to maintain them alive and in good condition long enough to react visibly to a tearing operation. Thus, in HgCl₂ and FeCl₃ the rapidity of action of the salts is so great compared to that in salts like AlCl₃ and PbCl₂ that the amebae are dead before the needles can be brought into use. Therefore the results of these experiments merely show the dilution at which the immersed ameba remain alive long enough to react to a marked trauma of the needle. This, of course, varies with the rate of the action of the salt on the plasmalemma.
With this limitation in view the following table indicates the reparability of the torn surfaces of amebae immersed in these salts.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Greatest concentration of salts in which repair of torn plasmalemma takes place.</th>
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<tbody>
<tr>
<td>AlCl₃</td>
<td>M/320</td>
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<tr>
<td>PbCl₂</td>
<td>M/1,000</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>M/2,000</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>M/3,200</td>
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<tr>
<td>FeCl₄</td>
<td>M/10,200</td>
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<tr>
<td>HgCl₂</td>
<td>M/24,000</td>
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DISCUSSION.

These experiments present some evidence as to the actual mechanism involved in the effect on protoplasm of the salts tested. An attempt to group the entire action of all the heavy metals into a common process cannot be justified. There is, however, one common feature which merits consideration. All salts used in this work, PbCl₂, HgCl₂, CuCl₂, FeCl₃, FeCl₄, and AlCl₃, hydrolyze to form strong acids. Moreover, this process of hydrolysis takes place over an extended period of time. This has been studied for lead salts by von Ende (13). Coincident with the increase in acidity the amebae gradually die and it is probable that this liberation of acid is at least one important factor in their death.

The marked toxicity of these salts when amebae are immersed in them as compared with the results obtained when the salts are injected into the amebae indicates that the lethal action of these substances is on the surface of the cell. In no case was the solution sufficiently acid to produce an effect on the internal protoplasm by its acidity alone. This has been shown by experiments previously reported (1), in which repeated injections of a solution of HCl at a reaction of pH 3 had no effect on the internal protoplasm but immersion in HCl at a reaction of pH 5 was lethal in a short time. This lends further support to the view that the action of the salt when injected is due to the cation alone, and that when the ameba is immersed in the solution the effect on the surface is due to the presence of the acid, which is being constantly produced, as
well as to the metal cation. It might, however, be suggested that the buffers in a cell can effectively neutralize any acid formed by hydrolysis. But the possibility must be considered that local effects may occur, for example on the surface, and cause irreversible changes before buffering of the acid takes place. This has been suggested by Aub and Reznikoff (11) as a possible mechanism in the action of lead on cells.

That the relative non-toxicity of the salts on the internal protoplasm may be due to their outward diffusion is not probable. No direct evidence for such outward diffusion was found in the case of NaCl (1), and the salts used in these experiments form much more stable compounds with protoplasm than does NaCl.

Some of these salts seem to have an effect on a specific part of the cell, depending upon their concentration. This is particularly true for HgCl₂ as has been found also by Bechhold (14) with red blood cells and by MacInnes (15) with *Aspergillus niger*. There is an indication, therefore, that various chemical combinations may be formed between the toxic substance and the different constituents of the cell depending upon the relative concentration of the toxic agent used. Krahé (16) suggests that the action of HgCl₂ is due not only to its ionization but, in certain concentrations, to its lipid solubility.

The gradual increase in toxicity of FeCl₂ in the immersion experiments may be associated with its gradual oxidation to the trivalent iron salt which is rapidly toxic. In this connection it is interesting to note that Buschke, Jacobsohn, and Klopstock (17) believe that the "oligodynamic" action of metals depends to a great extent on the ionization of their salts and on an oxidation process.

PbCl₂, in the concentrations used in these experiments, probably acts by uniting with the phosphates or carbonates of the cell and thus liberating free acid. Such a secondary reaction is indicated by the slow rate of toxicity.

A striking feature brought out by these experiments is the greater variation in the viability of different amebae in these solutions when compared to that which occurs in salts such as NaCl, KCl, CaCl₂, and MgCl₂ (1).

In attempting to determine the mode of action of a toxic substance on a cell it is necessary to consider all the possible mechanisms in-
involved. A toxic agent may (a) affect the plasma membrane only, (b) affect both the plasma membrane and the internal protoplasm, (c) leave the plasma membrane unharmed and injure the internal protoplasm, or (d) may not enter the cell but affect it by abstracting a necessary constituent. In considering the third possibility, (c), there is no evidence available, so far, to support the belief that a substance may pass through the plasmalemma in a non-toxic form and by some chemical alteration may change into a toxic form inside the cell or may be harmful to the internal protoplasm only. A consideration of the other possibilities, (a), (b), (d), suggests that a substance either abstracts a necessary constituent from the cell or primarily affects its surface. All visible evidence obtained so far points to the fact that toxic agents affect the surface of the immersed cell. So consistent is this result that the suggestion may be made that the maintenance of the surface membrane in a normal state is necessary for the life of the cell.

CONCLUSIONS.

I. Plasmalemma.

1. The order of toxicity of the salts used in these experiments on the surface membrane of a cell, taking as a criterion viability of amebæ immersed in solutions for 1 day, is HgCl₂, FeCl₃ > AlCl₃ > CuCl₂ > PbCl₂ > FeCl₂.

Using viability for 5 days as a criterion, the order of toxicity is PbCl₂ > CuCl₂ > HgCl₂ > AlCl₃ > FeCl₃ > FeCl₂.

2. The rate of toxicity is in the order FeCl₃ > HgCl₂ > AlCl₃ > FeCl₂ > CuCl₂ > PbCl₂.

3. The ability of amebæ to recover from a marked tear of the plasmalemma in the solutions of the salts occurred in the following order: AlCl₃ > PbCl₂ > FeCl₃ > CuCl₂ > FeCl₂ > HgCl₂.

II. Internal Protoplasm.

4. The relative toxicity of the salts on the internal protoplasm, judged by the recovery of the amebæ from large injections and the range over which these salts can cause coagulation of the internal protoplasm, is in the following order: PbCl₂ > CuCl₂ > FeCl₃ > HgCl₂ > FeCl₂ > AlCl₃.
5. AlCl₃ in concentrations between m/32 and m/250 causes a marked temporary enlargement of the contractile vacuole. FeCl₂, FeCl₃, and CuCl₂ produce a slight enlargement of the vacuole.

6. PbCl₂, in concentrations used in these experiments, appears to form a different type of combination with the internal protoplasm than do the other salts.

III. Permeability.

7. Using the similarity in appearance of the internal protoplasm after injection and after immersion to indicate that the surface is permeable to a substance in which the ameba is immersed, it is concluded that AlCl₃ can easily penetrate the intact plasmalemma. CuCl₂ also seems to have some penetrating power. None of the other salts studied give visible internal evidence of penetrability into the ameba.

IV. Toxicity.

8. The toxic action of the chlorides of the heavy metals used in these experiments, and of aluminum, is exerted principally upon the surface of the cell and is due not only to the action of the metal cation but also to acid which is produced by hydrolysis.

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