A SIMPLE CASE OF SALT ANTAGONISM IN STARFISH EGGS.

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The freshly laid egg of the common starfish, *Asterias forbesi*, is surrounded by a thin layer of water-swollen or gelatinous material, of about 15 to 20 microns in diameter (one-eighth to one-twelfth the egg diameter of about 160 microns), invisible to ordinary observation, but readily demonstrated by mounting the eggs in a somewhat thick suspension of India ink in sea water and examining on a slide under a moderate magnification. Each egg then appears surrounded by a clear halo outlining the space between the egg surface and the outer limit of the jelly which is impermeable to the ink particles. It is present in all full sized eggs, both mature and immature, but is absent in the smaller ovarian eggs of two-thirds or less the full diameter; evidently the water-swollen material of which it consists is separated from the egg during the later period of ovarian growth. In the starfish egg the jelly is remarkably resistant to solution in ordinary sea water and is not removed by repeated washing in this medium, even with the aid of centrifuging; in this respect the *Asterias* egg differs from the *Arbacia* egg which is surrounded by a jelly otherwise similar but readily removed by washing in sea water. In unfertilized starfish eggs the jelly shows no change after 24 hours in sea water; but it gradually disappears in fertilized cleaving eggs, possibly in consequence of increased separation of CO_2 from the eggs, since, as Garrey[1] has shown, it combines with acids and in so doing acquires increased swelling properties.

[1] Garrey, W. E., *Biol. Bull.*, 1919, xxxvii, 287.

The jelly is readily and rapidly removed from the eggs by washing in pure isotonic NaCl solution (0.54 M). This is most conveniently demonstrated as follows. Sea water containing a suspension of the eggs is gently centrifuged with a hand centrifuge, with ten or twelve slow turns, so as to collect the eggs in the narrow end of the tube; the sea water is poured off and replaced by pure 0.54 M NaCl; the tube is then inverted to suspend the eggs and again slowly centrifuged as before; the solution is poured off and replaced with fresh, and this process is repeated. The eggs are thus exposed to the pure NaCl solution free from all but traces of sea water. If they are then returned to sea water and examined in India ink, the jelly is found to have completely disappeared. There is no evidence of further secretion of jelly by eggs which have thus been freed from it and returned to sea water.

Removal of the jelly leaves the power of fertilization and development unimpaired. Thus, in a typical experiment unfertilized eggs, removed from the animal 45 minutes previously, were exposed for 6 minutes to 0.54 M NaCl with two changes of the solution and centrifuging as above. Examination in India ink showed complete removal of the jelly from all eggs. Sperm was added to part of the eggs 18 minutes after the return to sea water. A majority next day had formed blastulæ; abnormalities were, however, more frequent than in the untreated control eggs and about 20 per cent had died without development; this injurious effect is to be referred to the action of the pure unbalanced solution upon the eggs. Similarly, eggs freed from jelly subsequently to fertilization continued development and formed blastulæ, although showing also a larger proportion of abnormalities than the control.

Arbacia eggs treated as above with 0.54 M NaCl show a similar disappearance of the jelly, but the same effect is seen in sea water and in solutions of NaCl containing $CaCl_2$. In this species the jelly is more soluble than in *Asterias* and the difference between pure and calcium-containing NaCl solutions is not shown by the above method. This difference in the properties of the jelly layer in the two species indicates a specific difference of chemical composition, but so far I have made no attempt to investigate this difference in detail.

In both *Asterias* and *Arbacia* exposure of the eggs to the pure NaCl solution for a few minutes alters the consistency of the cell surface, and apparently also the permeability of the plasma membrane, in a highly characteristic manner. The eggs cohere in small clumps, or agglutinate; this effect is well marked even in eggs exposed to the pure NaCl solution in a finger-bowl without any centrifuging, but the latter process promotes the formation of larger and firmer aggregates. This agglutination, although accompanying the removal of the jelly, is an entirely independent process, and like the removal of the jelly is prevented by the presence of calcium in the solution. A further characteristic effect produced by the pure NaCl solution in *Asterias* eggs (but not in *Arbacia*) is the formation of apparently normal fertilization membranes in a certain proportion of eggs; and in some cases cleavage and development to a blastula stage result.[2] This membrane-forming and activating effect is also antagonized by calcium.[3]

The solution of the jelly in pure NaCl solution is a somewhat gradual process; the jelly gradually incorporates water, and swells, eventually losing coherence and passing into solution. The process of swelling may be arrested at any stage by returning to sea water; but there is no evidence of reversal in the sense of a return of the jelly to its original water content. Eggs were placed in a finger-bowl, the sea water removed as far as possible, and to the remaining mass of eggs (about 1 cc.) 100 cc. of 0.54 NaCl were added. From the NaCl solution eggs were returned to finger-bowls containing sea

[2] Lillie, R. S., *Am. J. Physiol.*, 1910, xxvi, 106; cf. 119.

[3] Lillie, R. S., *Am. J. Physiol.*, 1910–11, xxvii, 289. A further interesting effect of the pure isotonic NaCl solution, also antagonized by $CaCl_2$, is that it prevents the dissolution of the germinal vesicle, and hence the maturation process, in starfish eggs placed in the solution immediately after removal from the animal. If such eggs are left in the pure NaCl solution for from 5 to 10 minutes, and are then returned to sea water, they remain permanently immature. Similar treatment with a calcium-containing NaCl solution (*e.g.*, 95 volumes of M/2 NaCl plus 5 volumes of M/2 $CaCl_2$) leaves them apparently unaffected. The pure NaCl solution thus produces the same effect as weak fatty acid solution or high temperature, either of which, if applied at this time, also prevents maturation (cf. Lillie, R. S., *Biol. Bull.*, 1917, xxxii, 135). Mineral acids appear to have a similar effect (cf. Loeb, J., *Arch. ges. Physiol.*, 1902, xciii, 59).

water at the following intervals: $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, 6, 8, and 12 minutes; after 20 to 30 minutes in sea water, eggs from each bowl of the series were examined on a slide in India ink suspension. It was found that the jelly still remained about eggs that had been exposed to the NaCl solution for so long as 3 or 4 minutes, but that by that time it was swollen to several times its original volume, so that the boundary of the ink suspension was often separated from the egg by a full egg diameter. In eggs exposed to NaCl solution for 6 minutes and examined under the same conditions the jelly had completely disappeared from a large proportion of eggs, though still remaining in a greatly swollen form in most. After 12 minutes exposure the ink was in nearly all cases in contact with the egg surface, indicating complete removal of the jelly.

The removal of the jelly in NaCl solution is completely prevented by the addition of a small proportion of $CaCl_2$. The agglutinating and membrane-forming effects above described are at the same time prevented or greatly decreased. Since these two latter effects are dependent upon alteration of the surface layer of the living protoplasm, this parallelism suggests that the calcium prevents the physiological action of the pure NaCl solution through some characteristic influence on the solubility or hydration of certain compounds which are present in the protoplasmic surface layer and upon which the normal properties of this layer depend.

The following typical experiment (June 18, 1920) will illustrate. Starfish eggs were removed from the animal at 3.06 p.m.; the eggs were normal; all showed the typical jelly envelope in India ink suspension; more than 90 per cent underwent maturation and upon fertilization developed to larval stages. At 3.48 p.m. the two tubes of a hand centrifuge were filled with a suspension of unfertilized eggs in sea water and gently centrifuged until the eggs were settled in a mass about one-third of an inch deep in the narrow part of each tube. The sea water was then poured off and replaced in one tube by pure 0.54 M NaCl (lot A), in the other by a mixture of 250 cc. 0.54 M NaCl plus 15 cc. M/2 $CaCl_2$ (lot B). The solutions were added at 3.49 p.m.; the tubes were then gently centrifuged to settle the eggs and the solution was changed; this procedure was repeated. The eggs were then returned to sea water. The total time

of exposure to the solutions was 6 minutes. Examination in India ink suspension about 15 minutes later showed complete removal of the jelly in the eggs of lot A, of which about 25 to 30 per cent had also formed fertilization membranes. In the eggs of lot B the jelly was unaltered and no fertilization membranes were formed. Part of the eggs of both lots were fertilized with sperm at 4.13 p.m.; the next day about 70 per cent of the eggs of lot A had formed blastulæ, largely abnormal, while almost all of lot B had developed normally.

Other experiments of the same kind give similar results. Exposure to pure NaCl solution for some minutes removes the jelly; it also causes agglutination and injures the eggs, as shown by a certain degree of impairment and abnormality in development, and forms fertilization membranes in a considerable proportion of eggs. All of these effects are prevented by the addition of a little $CaCl_2$ to the solution.

The antagonistic action of calcium is shown in much more dilute solution than the above; *e.g.*, in M/400 $CaCl_2$ (199 volumes of 0.54 M NaCl plus 1 volume of M/2 $CaCl_2$), with the same treatment as above, the jelly remained intact. Further experiments to determine the limit of the effective concentrations were not tried. Magnesium chloride has a similar action in preventing the solution of the jelly, but is less effective.

The question of whether calcium can be replaced in this relation by other polyvalent metals has not yet been experimentally tested except in the case of aluminium. Eggs were placed in pure 0.54 M NaCl (lot A), and in 99 volumes of 0.54 M NaCl plus 1 volume of M/2 $AlCl_3$ (lot B); the solutions were changed twice by centrifuging and decanting as before. On return to sea water after 6 minutes in the solutions the eggs of lot A were found free from jelly and largely agglutinated, while those of lot B retained the jelly, although this was somewhat thinner than in the untreated control eggs; there was no agglutination. The probability is that many other polyvalent cations would be found to have this effect, as in other cases of antagonism.

THEORY.

In the present case of salt antagonism the effect, removal of the jelly, is a direct result of the incorporation of water in pure NaCl solution with consequent swelling until coherence is lost; in the same solution containing a little calcium the jelly retains its normal hydration and remains coherent and water-insoluble. The chemical composition of the jelly is unknown, but the presence of a certain proportion of protein is probable; this is suggested by its acid-combining properties,[1] also by its mode of formation as a cellular secretion, its physical consistency and its variation in properties from species to species. At least it seems clear that some substance is present forming compounds (salts) with sodium and calcium, which differ in their solubilities and in their affinities for water.

Apparently, in the presence of an excess of NaCl, a Na salt with a marked affinity for water is formed; hence the swelling and eventual solution. When $CaCl_2$ is present in the solution a water-insoluble Ca salt is formed; in the presence of a certain proportion of this the jelly as a whole remains insoluble. In other words, the normal coherence and water-insolubility of the jelly depend on the presence of a water-insoluble compound which pervades the system and renders it coherent and water-insoluble. Hence the removal of this by replacement of Ca with Na renders the whole structure soluble.

Antagonisms depending on differences in the solubilities of Na salts (or in general of alkali metal) and Ca salts (or alkali earth and heavy metal) are well known in purely physical systems. In many organic acids the Na salts are highly soluble in water, while the Ca salts are insoluble. The case of soaps is probably the most relevant to the conditions in biological systems. The physicochemical antagonisms recently investigated by Clowes,[4] using salt solutions which were allowed to drop from a stalagmometer through oil containing some fatty acid, depend on the differences between the solubilities of Na soaps and Ca soaps in the oil and the water phases respectively. The antagonisms between the influence of Na and Ca salts on the precipitation of lecithin[5] and heat-denatured egg white[6] are prob-

[4] Clowes, G. H. A., *J. Physical Chem.*, 1916, xx, 407.

[5] Koch, W., *Z. physiol. Chem.*, 1909, lxiii, 432.

[6] Mathews, A. P., *Am. J. Physiol.*, 1905, xiv, 203.

ably in part determined by similar conditions, although factors peculiar to suspensoid systems no doubt also enter in these cases. The properties of soaps, which are at once soluble in water and in many organic solvents, and which are consequently highly surface-active at water lipin interfaces, are probably of fundamental importance in protoplasmic activities. It is noteworthy that the water-combining properties of tissues and cells are influenced by salts in a manner consistent with the hypothesis that soaps or compounds with similar solubilities determine the manner in which the bound water (Overton's "Quellungswasser")[7] is held in the protoplasm. Many years ago Loeb[8] called attention to the parallel between the absorption of water by muscle immersed in isotonic solutions of different salts of alkali and alkali earth metals, and by the soaps of the same metals; thus, the muscle absorbed much more water in isotonic KCl solution than in NaCl, while in isotonic $CaCl_2$ it lost water; when the corresponding soaps are immersed in water a similar order of relative absorption is seen, K soaps (soft soaps) swelling more rapidly than Na soaps, while the water-insoluble Ca soaps do not take up water. Swelling and loss of consistency or turgor in plant tissues immersed in pure NaCl solution are well known phenomena, which have recently been investigated in great detail by Hansteen;[9] the disintegration and loosening of the intercellular coherence which he describes as occurring under these conditions are prevented by the presence of small quantities of calcium salts. Hansteen calls attention to the earlier work of Mangin[10] on the rôle of the pectin compounds in the interstitial substance or middle lamella of plant tissues; the cementing properties of this layer are, according to Mangin, dependent on the presence of an insoluble Ca compound which he calls "Ca pectinate." In the presence of NaCl solution free from Ca this layer becomes soluble and absorbs water and the cells fall apart. Herbst's[11] observations on the loss of coherence of of the blastomeres of sea-urchin eggs in Ca-free sea water probably

[7] Overton, E., *Arch. ges. Physiol.*, 1902, xcii, 115.
[8] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303.
[9] Hansteen, B., *Jahrb. wiss. Bot.*, 1910, xlvii, 289; 1913–14, liii, 536.
[10] Mangin, L., *J. Bot.*, 1893 (cited from Hansteen).
[11] Herbst, C., *Arch. Entwcklngsmechn.*, 1900, ix, 424.

have a similar general significance. The coherence of the ciliated cells in the gill epithelium of mollusca (Mitylus) is similarly lost in pure isotonic solutions of many Na salts, in which also the cells absorb large quantities of water;[12] these effects are prevented by the addition of Ca to the solution. The action of pure NaCl solutions in increasing the permeability of plant tissues to ions,[13] and of structures like the membrane of the *Fundulus* egg[14] to salts and water, is closely related to the above; similarly with the breakdown of protoplasmic structures like cilia and plasma membranes in this solution;[15] here the structural continuity is lost and with it the dependent properties of coherence and semipermeability. In all of these cases an essential part of the protective or antitoxic action of the calcium consists in preventing physical disintegrations of this kind; such disintegrations are apparently the direct result of replacing solid water-insoluble material and structure by water-soluble.

The difference between the water-combining powers of Na and Ca proteinates has been pointed out recently by Loeb in a series of papers on the influence of inorganic salts on the physical properties of proteins;[16] here also characteristic salt antagonisms affecting physical properties such as viscosity, swelling, osmotic pressure, precipitability by alcohol, are observed when the Na and Ca salts are present in certain proportions.[17] It seems probable that certain types of biological salt-antagonisms are to be explained by reference to general facts of this kind; the case of the *Fundulus* egg, where the toxic action of the pure NaCl solution is associated with a destruction of the water-proof character of the membrane or chorion enclosing the egg,[14] appears to exemplify this condition. In the case of antagonisms in living protoplasm, however, not only the proteins but other compounds, and especially the lipoids, appear to be essentially concerned. The salt antagonisms studied by Clowes,[4] which

[12] Lillie, R. S., *Am. J. Physiol.*, 1906–07, xvii, 89.

[13] Osterhout, W. J. V., *Science*, 1912, xxxv, 112; xxxvi, 350.

[14] Loeb, J., *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

[15] Lillie, R. S., *Am. J. Physiol.*, 1903–04, x, 419; 1909, xxiv, 23; 1912–13, xxxi, 259.

[16] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; 1918, xxxiv, 77.

[17] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 3; 1918, xxxiv, 395; 489; and xxxv, 497.

exhibit such a remarkable parallelism with physiological antagonisms, are referable to the formation of soaps or soap-like compounds. It seems probable that variations in the partition of such compounds between the aqueous and the non-aqueous or organic solvents of the protoplasm are important factors in the physiological effects produced by varying the proportion of the inorganic salts in the medium. Clowes attributes the variations in the drop numbers of the solutions flowing through the oil from the stalagmometer to variations in the ratio of the oil-soluble to the water-souble soaps. These soaps have opposite influences on the surface tension factor opposing the detachment of the drop. Oil-soluble compounds like higher alcohols influence the drop numbers in the same manner as Ca salts. The recent observations of Heilbrunn[18] on changes in protoplasmic consistency under the influence of lipoid-solvent compounds also suggest that variations in the partition of compounds between the aqueous and the lipoid phases of protoplasm may influence greatly the type of protoplasmic consistency or structure.

In general, it may be inferred from the above facts that the presence of a certain proportion of solid water-insoluble salt-like compounds in the protoplasmic surface film is necessary to the normal semi-permeability and structural permanence of this structure; similar considerations apply to filamentous solid structures like cilia and to the other solid structural elements of the cell. This view explains the destructive action of pure solutions of alkali salts like NaCl, which have the effect of substituting water-soluble Na-compounds for the water-insoluble Ca compounds (*e.g.*, soaps) normally present. Hansteen's[9] results with plant tissues afford strong evidence that water-insoluble Ca compounds are essential to the stability of the normal protoplasmic structures, and also of cell walls and similar structures formed by protoplasmic activity. He attributes great importance to lipoid compounds present in the protoplasmic surface layers and in the cell walls, and especially to the formation of water-insoluble combinations between these materials (together with pectin) and calcium. He regards these calcium compounds as necessary for the normal coherence of cells, as shown by their presence

[18] Heilbrunn, L., *Biol. Bull.*, 1920, xxxix, 307.

in the middle lamella, and also for the normal properties of the surface protoplasm.[19] According to Hansteen, pure alkali salt solutions attack and alter primarily the lipoid constituents of the cell walls,[20] inducing absorption of water and consequent disintegration; and he cites especially the work of Krefting,[20] who obtained from the intercellular substance of brown algæ an acid material ("Tangsäure") which forms water-soluble salts with alkali metals and water-insoluble salts with alkali earths (Ca, Sr, Ba). This compound apparently corresponds with the Ca pectinate to which Mangin attributed the cohesive properties of the material composing the middle lamella.

Hansteen's further observation that the presence of Ca salts in culture solutions is highly favorable to the branching of the roots and the growth of root-hairs in seedlings also indicates that Ca compounds are necessary to the formation of the solid structures essential to normal growth. For example, the development of root-hairs is greatly promoted in media containing several times the normal concentration of Ca salts, a result which has recently been confirmed by Wiechmann[21] in Höber's laboratory. Wiechmann finds also that strontium, barium and certain heavy metals (Mn, Ni, Co) act similarly to calcium, while magnesium is ineffective. Such data, when considered in relation to those cited above, throw an interesting light on the general significance of calcium in the formation of organic structure. Certain properties of the living protoplasmic structures, such as rigidity, water-insolubility and impermeability to water-soluble substances, seem to require the presence of calcium compounds.[22] The rapid alteration of the superficial protoplasmic

[19] Hansteen, B., *Jahrb. wiss. Bot.*, 1910, xlvii, 374.

[20] Hansteen, B., *Jahrb. wiss. Bot.*, 1913–14, liii, 574.

[21] Wiechmann, E., *Arch. ges. Physiol.*, 1920, clxxxii, 99.

[22] The general view that the presence of water-insoluble materials in the plasma membranes (formed from inorganic salts present) is responsible for their peculiar osmotic properties is by no means a new one, having apparently first been suggested by Traube in 1867 on the basis of his work with precipitation membranes. Recently the subject has been reviewed by Meigs (Meigs, E. B., *Am. J. Physiol.*, 1915, xxxviii, 456), who has studied the effects of impregnating colloidal membranes with various inorganic precipitates. He finds that membranes made by precipitating Ca and Mg phosphates in thin sheets of celloidin show

layer or plasma membrane in pure solutions of Na salts is of a kind which is consistent with this interpretation, since the essential feature of the effect produced is an increase of permeability, allowing the ready penetration of water-soluble substances (sugars, neutral salts, etc.) to which previously the membrane formed a complete barrier.[23]

SUMMARY.

The jelly surrounding the eggs of the starfish, *Asterias forbesi*, is insoluble in normal sea water, but rapidly swells and dissolves when the eggs are washed in a pure isotonic solution of NaCl. In the presence of a small proportion of $CaCl_2$ this solvent and disintegrative action of the NaCl solution is entirely prevented, and in the mixed solution the jelly exhibits the same insolubility and other properties as in normal sea water.

2. This action of $CaCl_2$ in preventing the dissolution of the jelly runs parallel with its action in preventing certain definite effects

many resemblances to plasma membranes in their osmotic properties, especially in their slight permeability to soluble inorganic salts and sugar, and suggests that these phosphates may play a part in determining the properties of the membranes of cells. This possibility should not be overlooked, although the above evidence indicates that insoluble salts formed with the colloidal compounds of the cell are of chief importance.

[23] Apparently the biologically essential properties of an organic membrane are referable chiefly to those of the limiting surface layers. Hence the cases of salt antagonisms at metallic and other surfaces have also an intimate bearing on the present problem. Bredig and Weinmayr (*Z. physik. Chem.*, 1903, xlii, 601) cite cases of such influence in the Hg–H_2O_2 catalysis; thus KOH and KCl may act as antagonists; the rhythm is extinguished by adding KCl and is restored by KOH. Related phenomena are seen in the influence of cations of high oxidation potential, like Ag, in preventing the spontaneous activation of passive iron in solutions of $NaNO_3$ and similar salts (Lillie, R. S., *Science*, 1919, 1, 416). Evidently the permeability and electromotor properties of any interfacial film depend on its composition and physical condition, both of which vary with the internal composition of the two adjoining phases. Both physical and chemical factors are concerned in the formation of the plasma membrane; adsorption, by which (presumably) the surface materials are assembled, is usually classed as a physical process; but the special composition of the membrane depends on the specific character of the cell metabolism, and on the nature of the reactions (oxidations, etc.), occurring at the cell surface.

of the pure NaCl solution on the living egg (agglutination, cytolytic action, membrane formation, prevention of maturation).

3. The inference is that the essential factor in these and similar antagonistic and protective actions is the formation of solid water-insoluble colloidal salts (*e.g.*, soaps and proteinates) of calcium (or other metal) with the structural colloids of the protoplasm. Apparently the presence of a certain proportion of such compounds is necessary to the structural stability of the living protoplasm, and especially to the water-insolubility and semipermeability of its external layer or plasma membrane. When the cell is immersed in the pure NaCl solution, water-soluble Na compounds are substituted for the insoluble Ca compounds which normally provide the necessary insolubility and coherence, and disintegration results.