THE MECHANICAL PROPERTIES OF THE CELL SURFACE

IV. THE EFFECT OF CHEMICAL AGENTS AND OF CHANGES IN pH ON THE UNFERTILIZED SEA-URCHIN EGG

By J. M. MITCHISON

Department of Zoology, University of Edinburgh, and Stazione Zoologica, Naples

(*Received* 7 *March* 1956)

INTRODUCTION

The earlier papers of this series (Mitchison & Swann, 1954*a*, *b*, 1955) described measurements of the rigidity of the cell surface of sea-urchin eggs which were carried out with an instrument called a 'cell elastimeter'. The present paper describes experiments using the same technique on unfertilized sea-urchin eggs which had been subjected to the action of various chemical agents and to changes in pH. The aim of this work was to discover something of the structural components of the cell membrane or cortex by testing the action of chemicals which might be expected to affect these components. If, for example, there were sulphydryl groups and disulphide links in the structural protein of the membrane, a reducing agent might be expected to decrease the rigidity and an oxidizing agent to increase it. There is also the possibility that the maintenance of the membrane structure might require energy from respiration, and therefore that respiratory inhibitors might alter the rigidity. Considerations such as these largely dictated the choice of the chemicals that were used, though a few others were tested for different reasons.

It should be said at the outset that the results of this work are difficult to interpret in view of our present lack of knowledge about the components of the cell surface. Many of the chemicals did not affect the membrane rigidity, and of those that did so only some changed it in a way that would be expected from simple theory.

MEASUREMENTS ON THE EFFECT OF CHEMICAL AGENTS

The materials used for these experiments were the unfertilized eggs of two species of Mediterranean sea-urchin, *Sphaerechinus granularis* and *Paracentrotus lividus*. No difference was found between the eggs of these two species in their behaviour towards any of the chemicals tested. The jelly was removed by pre-treatment with acidified sea water.

In the initial tests one sample of eggs was placed in the solution of the chemical agent and another sample from the same female was left in sea water as a control. After 3 hr. the rigidity of five eggs from each sample was measured with the cell elastimeter, using the technique described by Mitchison & Swann (1954*a*). If the difference between the average rigidities of the two samples was less than 20% it

Table 1. *Effect of chemical agents on the rigidity of the cortex of unfertilized eggs*

M.E. = Elevation of fertilization membrane.
Cyt. = Cytolysis.

Agent	Effect on rigidity	Max. conc. tested (molarity, unless otherwise stated)	Comments
1. Oxidizing agents:			
Hydrogen peroxide	No	10^{-2}	M.E. with 10^{-1}M
Sodium metaperiodate	No	10^{-4}	M.E. with 10^{-3}M Ca- and Mg-free sea water
Sodium iodosobenzoate	No	Sat. soln.	
Oxidized glutathione	No	10^{-2}	
Cystine	No	Sat. soln.	
Iodine in potassium iodide	Yes	10^{-4}	
2. Reducing agents:			
Sodium sulphide	Yes	10^{-2}	
Dithiopropanol (B.A.L.)	Yes	10^{-1}	
Sodium thioglycollate	Yes	10^{-2}	
Reduced glutathione	No	10^{-2}	
Cysteine (+sodium cyanide)	No	10^{-2}	Cyt. with 10^{-1}M
3. Mercaptide-forming agents:			
Phenyl mercuric acetate	No	Sat. soln.	
p-Chloromercuric benzoate	No	Sat. soln.	
Sodium arsenite	No	10^{-1}	
Sodium cacodylate	No	10^{-1}	
4. Alkylating agents:			
Chloracetophenone	Yes	10^{-2}	
n-Ethyl maleimide	No	10^{-4}	M.E. with 10^{-2}M
5. Respiratory inhibitors:			
Sodium cyanide	No	10^{-1}	
Sodium azide	No	10^{-1}	
Sodium fluoride	No	10^{-2}	Cyt. with 10^{-1}M Ca- and Mg-free sea water
Sodium malonate	No	10^{-1}	
Sodium iodoacetate	No	10^{-2}	Cyt. with 10^{-1}M
Iodacetamide	No	10^{-2}	
2, 4-Dinitrophenol	No	5×10^{-2}M	
6. Detergents:			
Cetyltrimethyl ammonium bromide	Yes	0·001 %	Cyt. with 0·01 %
Sodium dodecyl sulphate	Yes	0·001 %	Cyt. with 0·1 % M.E. with 0·01 %
$H(CH_2)_{10}(CH_2.CH_2.O)_8H$	Yes	0·01 %	Cyt. with 0·1 %
7. Salts:			
Sodium chloride	No	0·55	
Potassium chloride	No	0·55	
Magnesium chloride	No	0·37	
Calcium chloride	No	0·37	
Zinc chloride	Yes	—	
Cupric chloride	No	—	
Lead chloride	No	—	
Ferric chloride	No	—	
8. Miscellaneous:			
Formalin	Yes	10^{-1}	
Trypsin	Yes	0·1 %	
Ether	No	10 % sat. soln.	Cyt. with sat. soln.
Chloroform	No	10 % sat. soln.	Cyt. with sat. soln.
Glycine	No	10^{-1}	
Versene	No	10^{-2}	
Heparin	No	0·1 %	
Hyaluronidase	No	10 units/ml.	

was assumed that the test was negative and that the chemical had no effect on the membrane rigidity.

Table 1 gives a list of the chemical agents used and the maximum concentrations at which they were tested, and it also shows whether or not they affected the rigidity. The chemicals are divided into eight groups of which the first four are those that affect sulphydryl groups or disulphide bonds. The separation into the groups is somewhat arbitrary, since some of the respiratory inhibitors affect sulphydryl groups (e.g. iodoacetate) and vice versa. All the chemicals were made up in sea water except for sodium fluoride and metaperiodate. These precipitated in ordinary sea water and had to be made up in an artificial sea water without calcium and magnesium and containing only sodium and potassium chloride. The controls for these chemicals were also placed in this artificial sea water. The pH of all the solutions was measured with a glass electrode and, when necessary, adjusted to pH 7·5–8·0 with NaOH or HCl. The maximum concentration which was tested is given in most cases as molarity, but one-tenth saturated solutions were used with ether and chloroform, and fully saturated solutions with chemicals of low solubility (iodoso-benzoate, cystine, phenyl mercuric acetate and chloromercuric benzoate). In the case of zinc, copper, lead and iron, the chloride was made up at 10^{-2}M, but most of the metal precipitated as hydroxide when the pH was raised to 8·0 and the final concentration, after filtering off the precipitate, was probably very low. The chlorides of sodium, potassium, magnesium and calcium were made up in solutions which were approximately isosmotic with sea water. Table 1 also shows that some of the chemicals produced cytolysis or caused the elevation of fertilization membranes.

Further measurements were made with the eleven chemicals that produced definite changes in rigidity. An experiment was carried out with each of these chemicals to find the change in rigidity with time at different concentrations. Eggs were placed in a series of solutions of ten-fold dilution (e.g. 10^{-2}, 10^{-3}, 10^{-4}M) and in sea water as a control, and then measured (average of five eggs) every 30–60 min. for a period of 3–4 hr. The results for sodium thioglycollate are shown as an example in Fig. 1. There is no significant effect at a concentration of 10^{-3}M (or at lower concentrations which are not shown), but at 10^{-2}M there is a fairly steady rise in the rigidity. As in the earlier papers of this series, the rigidity is given as 'corrected stiffness' (dynes/cm.^{2}/μ deformation for the standard condition of 100μ diam. egg and 50μ diam. pipette). Some of the eggs were removed from the 10^{-2}M-thioglycollate after 130 min. and washed three times with sea water. They were then left in sea water and measured later. The rigidity dropped to a value nearly as low as the controls, thus showing that most of the effect of thioglycollate could be reversed by washing.

Of the remaining ten chemicals, seven caused an increase in rigidity and three caused a decrease. Excluding for the moment the case of trypsin, the general shape of the curves was similar to those with thioglycollate. They have not therefore been reproduced in detail, but the most important information from them is given in the first two columns of Table 2. The 'minimum concentration' is the most dilute of the ten-fold dilutions which showed an effect on the rigidity. This is not strictly

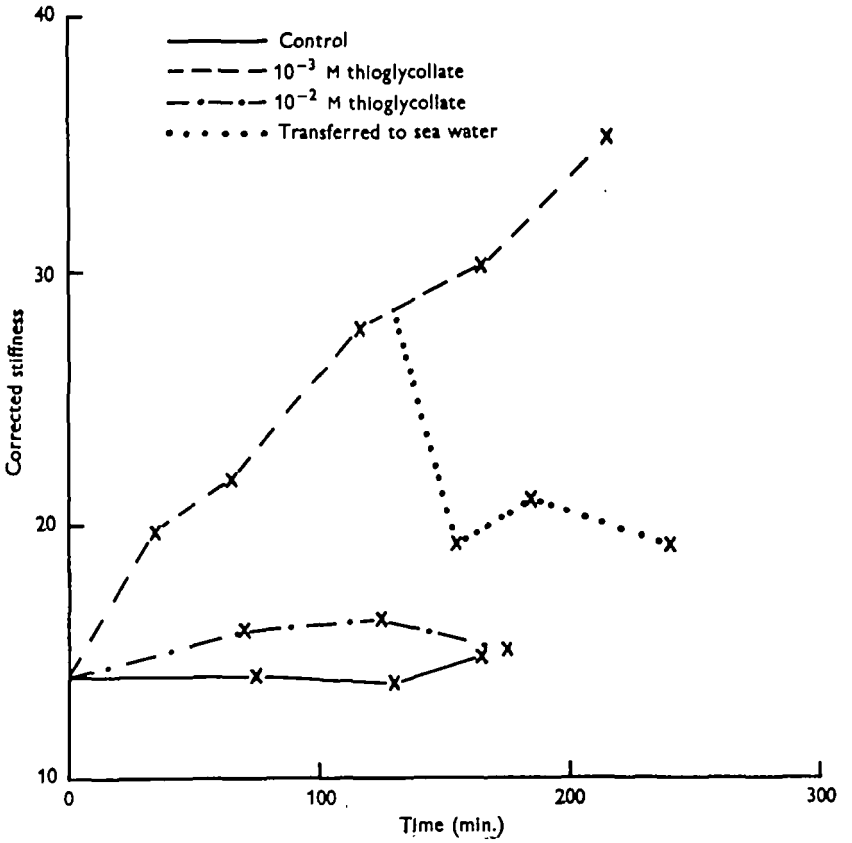

Fig. 1. Changes of membrane rigidity with sodium thioglycollate. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for 100 μ diam. egg and 50 μ diam. pipette.

Table 2. *Chemical agents with positive effect on cortical rigidity*

Agent	Min. conc. for effect	Change in rigidity after 3 hr. in min. conc.	Reversibility	Increased cytoplasmic rigidity
Oxidizing agent:				
Iodine in potassium iodide	10^{-4}M	×5·3	No	Yes
Reducing agent:				
Sodium sulphide	10^{-2}M	×2·2	No	Yes
Dithiopropanol (B.A.L.)	10^{-2}M	×6·7	Yes	No
Sodium thioglycollate	10^{-2}M	×2·1	Yes	No
Alkylating agent:				
Chloracetophenone	10^{-2}M	×3·8	No	No
Detergents:				
Cetyltrimethyl ammonium bromide	0·0001 %	×0·59	Yes	No
Sodium dodecyl sulphate	0·001 %	×0·44	Yes	No
$H(CH_2)_{10}(CH_2.CH_2.O)_6H$	0·01 %	×1·6	Yes	No
Salt:				
Zinc chloride	—	×1·6	Yes	No
Miscellaneous:				
Formalin	10^{-2}M	×8·3	No	Yes
Trypsin	0·001 %	×0·23	No	No

speaking the true minimum concentration, since there is a factor of ten between this concentration and the next more dilute concentration which showed no effect. At this minimum concentration the rigidity increased (or decreased) fairly steadily, though there was usually a more rapid increase in the first hour than there was subsequently. The second column of Table 2 shows the change in corrected stiffness after 3 hr. at this concentration as compared with the controls. With concentrations lower than the minimum, and with the controls, the rigidity remained nearly constant throughout the experiment. With concentrations higher than the minimum either the eggs showed larger changes in rigidity or measurements were impossible because of cytolysis or membrane formation.

The third column of Table 2 shows whether or not the rigidity change could be reversed by washing. A sample of eggs was removed from the minimum concentration of the chemical after a measurement at $2\frac{1}{2}$ hr., washed three times, left in sea water for a further hour and then measured again. If the rigidity had decreased (or increased) to the value for the controls the effect was regarded as reversible, while if it remained at its original value the effect was regarded as irreversible.

If the measured rigidity decreases this is almost certainly due to a decrease in the elastic modulus of the surface, since the interior cytoplasm of an unfertilized egg is so fluid that a decrease in its 'elasticity' would be most unlikely to affect the elastimeter readings. If, however, the measured rigidity increases, it might be caused by an increase in the elastic modulus either of the membrane or of the cytoplasm. It was therefore necessary to test whether those chemicals which affected the measured rigidity also caused an increase in cytoplasmic rigidity. The eggs were left in the minimum concentrations of the chemicals for 2 hr. and then centrifuged in a sugar gradient (1·1M-sucrose) at 7000 g. for 10 min. They were examined under a microscope and the degree of stratification of the granules was compared with that in control untreated eggs. With iodine, sodium sulphide and formalin, there was no stratification, thus showing that the cytoplasmic rigidity had increased. With the other chemicals there was no difference between the treated eggs and the controls. These results are presented in the last column of Table 2.

The effect of trypsin is shown in Fig. 2. These results differed in two respects from those with any of the other chemicals. First, an increase in the concentration made only a small difference to the decreased rigidity which was produced. Secondly, there was little change in the rigidity with time after the initial decrease. These effects will be discussed below.

MEASUREMENTS ON THE EFFECT OF CHANGE IN pH

The results of three experiments on effect of changes in the pH are shown in Fig. 3. Measurements of rigidity were made (average of five eggs) on samples of eggs from one female which had been left for 100 min. in sea water whose pH had been altered with NaOH or HCl and measured with a glass electrode. The first two experiments were made without buffers and there were slight rises in the pH (maximum of 0·2) during the course of the experiment. The pH given in Fig. 3 is the final pH of the solutions. The untreated sea water had a pH 8·0–8·1. The third

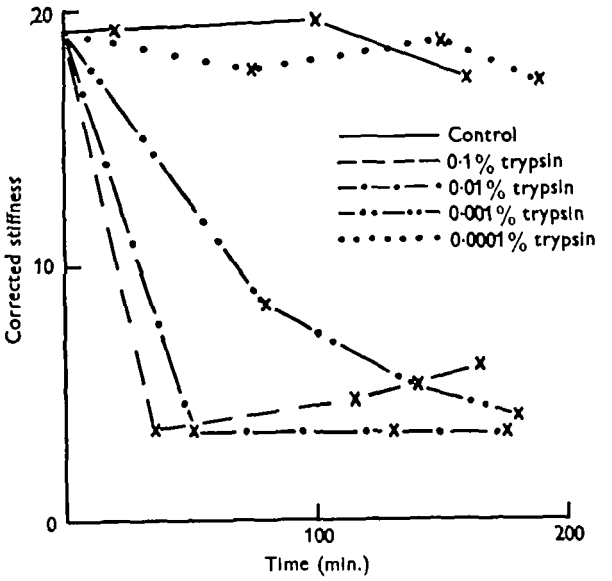

Fig. 2. Changes of membrane rigidity with trypsin. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for 100 μ diam. egg and 50 μ diam. pipette.

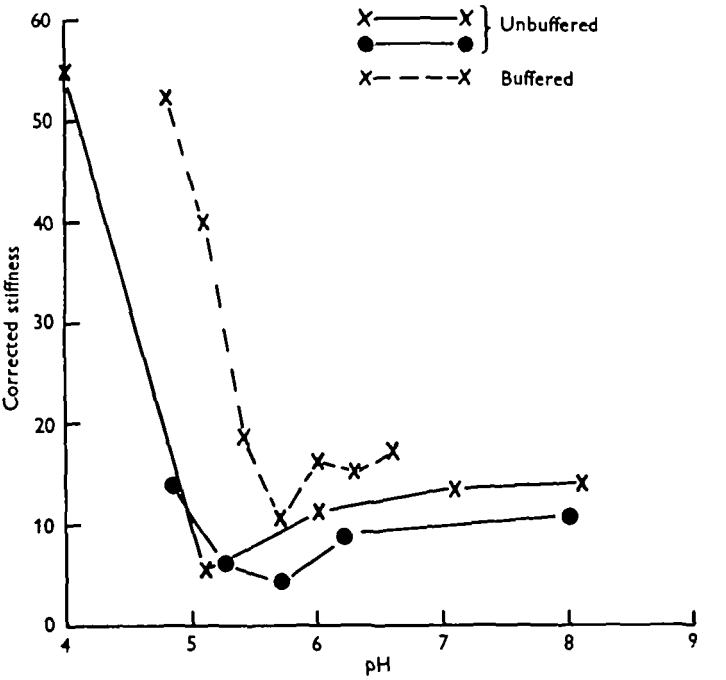

Fig. 3. Changes of membrane rigidity with pH. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for 100 μ diam. egg and 50 μ diam. pipette.

experiment was carried out with an artificial Ca- and Mg-free sea water and citrate-phosphate buffers. These solutions had constant pH values but were not very satisfactory media for the eggs, since there was an appreciable amount of cytolysis.

These results show a minimum rigidity about pH 5·7, and a sharp rise in rigidity in acid solutions with pH values of about 5·0 or less. The changes with time, which are not shown in Fig. 3, resembled those with trypsin in having a large initial change and thereafter little alteration. The high rigidity in acid solutions could not be reversed by washing.

DISCUSSION

Of the chemicals which affect the rigidity, formalin, iodine and sulphide cause an irreversible increase in the general rigidity of the cytoplasm and probably also in that of the cell membrane. This action is not surprising in the case of formalin, since it is well known to increase the rigidity of protein gels. It is worth pointing out that this method of determining rigidity might prove useful in a general study of fixation, since it provides quantitative measurements on a cellular level of one of the most important actions of a fixative.

The other chemicals which affect the rigidity appear to act mainly if not exclusively on the cell membrane or cortex. Apart from zinc chloride, they can be divided into three groups; SH reagents, detergents and trypsin. They will be discussed in this order.

Of the SH reagents that were tested, two of the reducing agents (dithiopropanol and thioglycollate) and one of the alkylating agents (chloracetophenone) affect the membrane rigidity. Dithiopropanol and thioglycollate are stronger reducing agents than cysteine or reduced glutathione and might be expected to have a more marked effect. It is, however, surprising that they increase the membrane rigidity. The simplest expectation would be that they would decrease the rigidity by breaking disulphide cross-links. The absence of any effect with the oxidizing agents suggests that there are few if any SH groups which can be converted into disulphide links, but if this is so it is difficult to explain the action of chloracetophenone which should react with free SH groups. It seems better not to draw any conclusions from these results in view of the fact that only a few of the SH reagents affect the rigidity and those that do affect it behave in an unexpected way.

Three detergents were tested; a cationic one (cetyltrimethyl ammonium bromide), an anionic one (sodium dodecyl sulphate) and a nonionic polyethanoxide ($H(CH_2)_{10}(CH_2.CH_2.O)_8H$). They all affect the membrane rigidity reversibly, though the cationic detergent is more effective than the anionic, and the anionic is more effective than the nonionic. This is the same order as that shown in their bactericidal properties (Putnam, 1948). The polyethanoxide increases the rigidity whereas the other two detergents decrease it. This may perhaps be connected with the fact that both cationic and anionic detergents combine with globular protein and cause a partial unfolding of the molecule (Few, Ottewill & Parreira, 1955). It is worth pointing out that all the detergents affect the membrane at concentrations between 1/10 and 1/100 of those which cause cytolysis.

The effect of trypsin differs from that of all the other reagents in that it appears to cause an 'all or none' reaction. As long as the initial concentration of trypsin is above about 0·0001%, the membrane rigidity drops irreversibly to a low value which is more or less independent both of time and of concentration of trypsin. This result would agree with the suggestion put forward by Runnström, Monné & Broman (1943) that trypsin digests away a vitelline membrane round the surface of unfertilized eggs. On the other hand, there is as yet no sign of a vitelline membrane under the light microscope or the electron microscope (Mitchison, 1956), so it may be that trypsin acts by disorganizing an outer layer of the cortex rather than by digesting away a separate membrane.

The experiments with varying pH show that there is a minimum rigidity at a pH of about 5·7. This may indicate that the isoelectric point of the membrane protein is at this value but, as with SH reagents, this is not the result that would be expected. At the isoelectric point there will be a maximum number of charged groups on the proteins, and these should produce an increased rigidity due to electrostatic forces between the molecules. On the other hand, Gerngross (1926) found that the rigidity of a gelatine gel was independent of pH over a range of about three pH units near the isoelectric point. This suggests either that the total number of charged groups is not greatly altered over this range, or that electrostatic forces are not of primary importance in determining the rigidity of a gel. In any case, however, there is always the difficulty with living cells that changes in the external pH may not produce equivalent changes in the pH of the membrane protein within the cell's permeability barrier.

Most of the reagents which were tested had no effect on the rigidity, but it is worth emphasizing two points about these negative results. First, the fact that none of the respiratory inhibitors affect the membrane rigidity indicates that the maintenance of the membrane structure does not depend on a supply of energy. Secondly, the fact that the rigidity remains unchanged both in solutions containing large amounts of calcium (e.g. 0·37M-$CaCl_2$) and in solutions free of calcium (e.g. 0·55M-NaCl) indicates that the main structural protein of the membrane is unaffected by changes in the external concentration of calcium ions. There is, however, a warning which should be given about all the negative results. The absence of an effect may simply be due to the fact that the reagent does not penetrate the egg. It is likely that the respiratory inhibitors penetrate since they stop division in a fertilized egg, but even this argument is open to the objection that the permeability of an unfertilized egg is much less than that of a fertilized one.

Kriszat (1953, 1954) has also investigated the effect of a number of reagents on the rigidity of the cell membrane of sea-urchin eggs. He used the degree of elongation and of stratification of eggs in a centrifuge microscope as measures of the cortical and cytoplasmic rigidity. His results differ from those presented in this paper since he found that glutathione, thioglycollate and periodate decrease the rigidity of the membrane.

SUMMARY

Measurements were made with the cell elastimeter on the effect of a number of reagents and of changes in pH on the rigidity of the cell membrane of unfertilized sea-urchin eggs. The membrane rigidity was increased by dithiopropanol, thioglycollate, chloracetophenone, a polyethanoxide and zinc ions. It was lowered by trypsin, cetyltrimethyl ammonium bromide and sodium dodecyl sulphate. Most of the other reagents, including respiratory inhibitors and calcium ions, had no effect. The membrane has a minimum rigidity at a pH of about 5·7.

I should like to express my thanks to Dr J. H. Schulman, of the Department of Colloid Science, Cambridge, for his kindness in supplying the detergents. I should also like to thank the Director and Staff of the Stazione Zoologica, Naples, for their assistance in this work.

REFERENCES

FEW, A. V., OTTEWILL, R. H. & PARREIRA, H. C. (1955). The interaction between bovine plasma albumin and dodecyltrimethylammonium bromide. *Biochim. biophys. Acta*, **18**, 136–7.

GERNGROSS, O. (1926). Säurewirkung und H'-Konzentration bei Leim und Gelatine. *Kolloidzschr.* **40**, 279–86.

KRISZAT, G. (1953). Die Wirkung von Perjodat auf den Zustand der Plasmamembran des Seeigeleis. *Exp. Cell. Res.* **5**, 420–6.

KRISZAT, G. (1954). Zentrifugierversuche über den Einfluss befruchtungsfördernder und hemmender Substanzen auf den Zustand der Oberfläche des Seeigeleis. *Exp. Cell. Res.* **7**, 103–10.

MITCHISON, J. M. (1956). The thickness of the cortex of the sea-urchin egg and the problem of the vitelline membrane. *Quart. J. Micr. Sci.* **97**, 109–21.

MITCHISON, J. M. & SWANN, M. M. (1954*a*). The mechanical properties of the cell surface. I. The cell elastimeter. *J. Exp. Biol.* **31**, 443–60.

MITCHISON, J. M. & SWANN, M. M. (1954*b*). The mechanical properties of the cell surface. II. The unfertilized sea-urchin egg. *J. Exp. Biol.* **31**, 461–72.

MITCHISON, J. M. & SWANN, M. M. (1955). The mechanical properties of the cell surface. III. The sea-urchin egg from fertilization to cleavage. *J. Exp. Biol.* **32**, 734–50.

PUTNAM, F. W. (1948). The interaction of protein and synthetic detergents. *Advan. Protein Chem.* **4**, 80–122.

RUNNSTRÖM, J., MONNÉ, L. & BROMAN, L. (1943). On some properties of the surface layers in the sea-urchin egg, and their changes upon activation. *Ark. Zool.* **35**A, no. 3.