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Immunochemical Analysis of the Surface of the Sea Urchin Egg - an Approach to the Study of Fertilization

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Introduction

The sea urchin egg constitutes a classical material for the study of fertilization. As in all metazoa, fertilization of the sea urchin egg 'is the incitement of an egg to development by a spermatozoon, together with the transmission of male hereditary material to the egg'[1]. In its first steps, this process involves (1) the attachment of the fertilizing spermatozoon to the egg, (2) the activating stimulus produced in the egg, and (3) the penetration of the egg by the spermatozoon.

As indicated by a variety of incompatability reactions, the first steps of fertilization are characterized by a high degree of specificity. Ultimately, this specificity depends on one or several highly specific interactions between macromolecules from the sperm and from the egg. Hence, for an understanding of the mechanism of the process it is necessary to know the molecular topography of the surface layers of the gametes and the fine structures, in precise chemical terms, of the molecules which interact.

In this situation specific antibodies, used in combination with common biochemical and biological techniques, could be expected to offer analytical tools of a high power of resolution. As is well known, injection of foreign proteins or polysaccharides into mammals leads to slight structural modifications of certain of the globulins in the serum of the injected animals. Thereby these globulins, which we call antibodies, gain the capacity to react chemically with molecules (i.e. antigens) of the same sort as those which had been injected. Since the reactions between antigens and antibodies are highly specific, antibodies may enable us to determine, *in vitro*, various chemical fine structures in complicated mixtures which at present cannot be distinguished by other means. In addition, a study, in antibody-treated cells, of the interference of the antibodies with special cellular functions, may be useful for an elucidation of the molecular mechanisms operating in these functions[2-7].

Immunological investigations in the field of fertilization are not very numerous and will not be reviewed here. In the sea urchin, the use of antibodies has been introduced by TYLER, aiming to prove the importance of certain substances (fertilizin and antifertilizin) for the process of fertilization[8-12]. A discussion of these results falls beyond the scope of the present article.

Some years ago, the present author started an immunological investigation of the macromolecules which constitute the surface layers of the sea urchin egg, and of the possible significance of these molecules for the process of sperm attachment and activation of the eggs. The study was performed with the gametes of the four Mediterranean sea urchins *Paracentrotus lividus, Psammechinus microtuberculatus, Arbacia lixula,* and *Sphaerechinus granularis*[13]. The main object of study was *Paracentrotus lividus.* In this article, the course of the analysis will be described and the main results obtained thus far will be discussed.

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[1] LORD ROTHSCHILD, *Fertilization* (Methuen, London 1956).

[2] W. T. J. MORGAN, in A. A. MILES and N. W. PIRIE, *The Nature of the Bacterial Surface* (Blackwell Scientific Publications, Oxford, England 1949), p. 9.

[3] G. W. NACE, Ann. N. Y. Acad. Sci. *60*, 1038 (1955).

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[7] M. HEIDELBERGER, in Symposium No. 1, IVth Intern. Congress Biochem., Vienna 1958 (Pergamon Press, London 1959).

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[9] A. TYLER, Amer. Naturalist *83*, 195 (1949).

[10] A. TYLER, in B. H. WILLIER, *et al., Analysis of Development* (W. B. Saunders Comp., Publ., Philadelphia and London 1955), p. 170.

[11] A. TYLER and J. W. BROOKBANK, Proc. Natl. Acad. Sci. *42*, 304 (1956).

[12] A. TYLER and J. W. BROOKBANK, Proc. Natl. Acad. Sci. *42*, 308 (1956).

[13] The biological experiments were performed at the Stazione Zoologica of Naples, Italy.

The Morphology of Fertilization

As an introduction to this article, a very brief description of the first steps in fertilization will be given [1,10, 14–17].

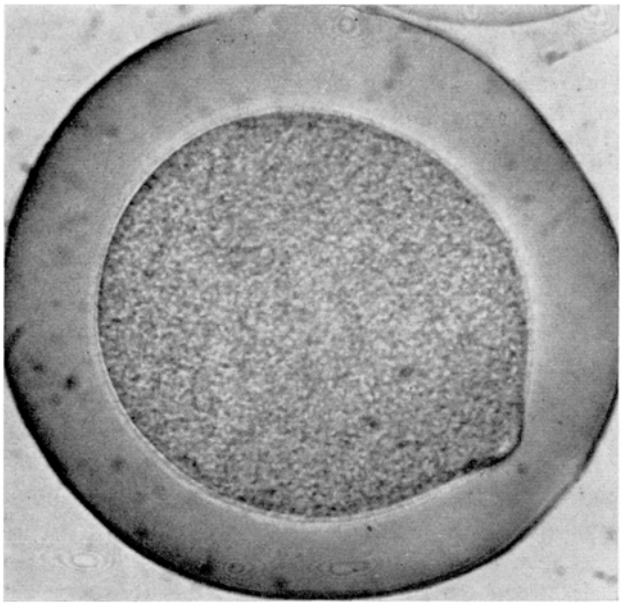


Fig. 1.—Unfertilized sea urchin egg (Echinocardium cordatum). The outer layer is the jelly layer, stained with methylene-blue. The protrusion emerging from the cytoplasm corresponds to the animal pole of the cell. In the eggs of most species, no such protrusions are found. (From IMMERS, unpublished).

Figure 1 shows a mature, unfertilized egg as it occurs free in sea water after shedding. The cell is surrounded by an outer layer, the jelly layer, normally invisible but made visible in the figure by means of staining. The outmost layer of the egg cytoplasm, the cortex, is surrounded by a vitelline membrane and contains the cortical granules; these have been shown to be rich in polysaccharides and possess a species-specific fine structure, apparent in the electron microscope. The layers are shown schematically in Figure 2*a*.

In fertilization, the sperm first passes through the jelly layer. There is evidence that the jelly renders an appreciable proportion of the spermatozoa unfertilizable. Nevertheless, under favorable conditions, some spermatozoa will penetrate the jelly and finally attach to the egg surface. The exact mechanism of this attachment is unknown. However, it seems rather likely that the first contact between sperm and egg is established by means of the tip of the acrosomal filament, a thread-like organella which is expelled from the sperm head at some stage of penetration. Under normal conditions, only one spermatozoon will attach effectively and eventually fertilize the egg. The mechanisms which protect the eggs from polyspermic fertilization are still under discussion.

As soon as the attachment of the sperm is completed a great number of changes are rapidly started in the egg. Thus, from the point of attachment a wave-like change proceeds through the cortex, leading to a continuous expulsion of the cortical granules which are at least partially incorporated in the vitelline membrane. The new membrane, which is lifted up from the egg surface, is called the fertilization membrane. A complete membrane around the egg is normally formed within less than a minute. The space between the membrane and the cortex is called the perivitellin space. Subsequent to the formation of the membrane, a clear, unpigmented layer, the hyaline layer, is formed from the cortex within a few minutes (Figs. 2*b* and *c*; 3).

Simultaneously with these changes of the surface, other marked alterations take place, involving the entire cell. Within 40–50 sec after insemination, the penetration of the fertilizing spermatozoon in the egg can be recorded. At the same time, a total rearrangement

[14] J. RUNNSTRÖM, Adv. Enzymol. *9*, 241 (1949).

[15] Ch. B. METZ, in A. TYLER *et al.*, *The Beginnings of Embryonic Development* (Publ. Amer. Assoc. Advance. Sci., Washington D. C. 1957), p. 23.

[16] A. L. COLWIN and L. HUNTER COLWIN, in A. TYLER *et al.*, *The Beginnings of Embryonic Development* (Publ. Amer. Assoc. Advance. Sci., Washington D. C. 1957), p. 135.

[17] J. RUNNSTRÖM, B. E. HAGSTRÖM, and P. PERLMANN, in J. BRACHET and A. E. MIRSKY, *The Cell*, Vol. I (Academic Press Inc., Publ., New York 1959).

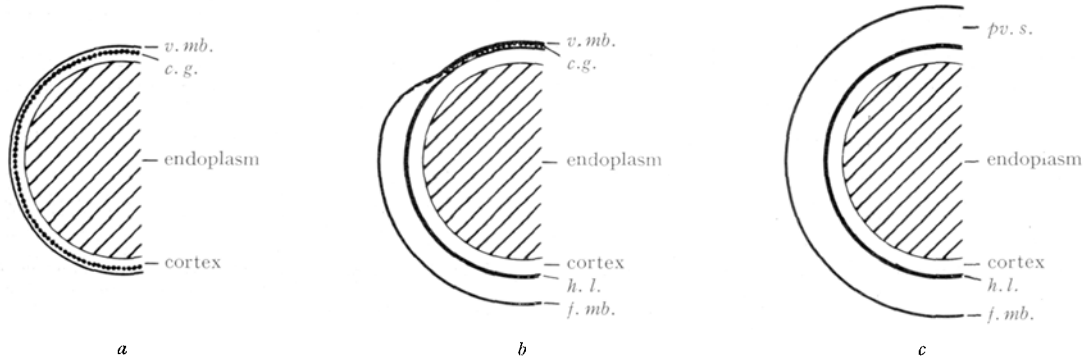


Fig. 2.—Surface layers of the sea urchin egg and their changes upon fertilization. *a*, unfertilized egg; *b*, fertilized egg with partially elevated fertilization membrane; *c*, fertilized egg. – *v. mb.*, vitelline membrane; *c. g.*, cortical granules; *f. mb.*, fertilization membrane; *h. l.*, hyaline layer; *pv. s.*, perivitelline space. – The jelly layer outside the vitelline membrane is not indicated in the Figure. (From RUNNSTRÖM *et al.*[17]).

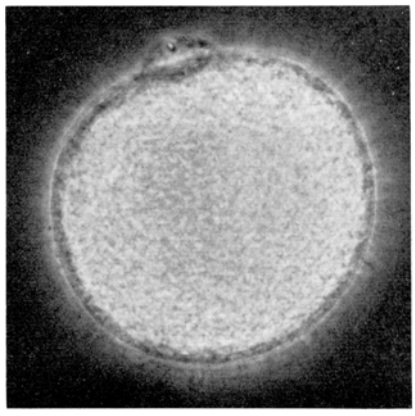


Fig. 3.—Partially fertilized egg of Paracentrotus lividus. Egg and sperm were killed with formalin 10 sec after insemination. By that time only a small membrane blister has been formed. The fertilizing sperm is seen attached to the membrane in that region. (Phase contrast. – From RUNNSTRÖM, unpublished).

of the egg cytoplasm occurs, leading to changes of its optical properties. Subsequent events are aster formation, fusion of male and female pronuclei and, normally after more than one hour, cell division (Figs. 4*a*–*c*).

It must be emphasized that most of the changes described above can also be brought about by various chemical or physical means without the participation of the sperm (*parthenogenetic activation*). Parthenogenesis may lead to membrane and monaster formation and to cell division. However, the ensuing development will frequently be abnormal.

The Immunological Analysis

The Antisera.—The antisera used in the present investigation were obtained from rabbits injected with material from the gametes of various sea urchin species. Antisera, induced by means of injection of *total homogenates from unfertilized eggs*, constituted the most important reagents. Such antisera contain a considerable number of various types of antibodies, directed against the different antigens of the cell. The complexity of the antisera can readily be visualized with the aid of precipitin reactions in agar gel[18,19]. Figure 5*a*, showing the reactions between a saline extract from unfertilized eggs and a number of different anti-egg sera may serve as illustration. The various lines seen in the photograph are each complex and represent a minimum number of specifically different antigen-antibody precipitates[20–23].

The resolving power of the antisera is not restricted to the various macromolecules occurring within the eggs of a particular sea urchin species. Comparison of anti-egg sera obtained with eggs from different species reveal, in addition, a high resolving power of the antibodies with regard to species differences[20,24].

In addition to the anti-egg sera, antisera against *sperm homogenates* or against *isolated egg jellies* were also used. As can be seen from Figure 5*b*, no reactions or at best only very weak reactions took place when extracts from jelly-free eggs were allowed to react with

[18] J. OUDIN, Meth. Med. Res. *5*, 335 (1952).

[19] Ö. OUCHTERLONY, in P. KALLÓS, *Progress in Allergy* (S. Karger, Publ., Basel 1958), p. 1.

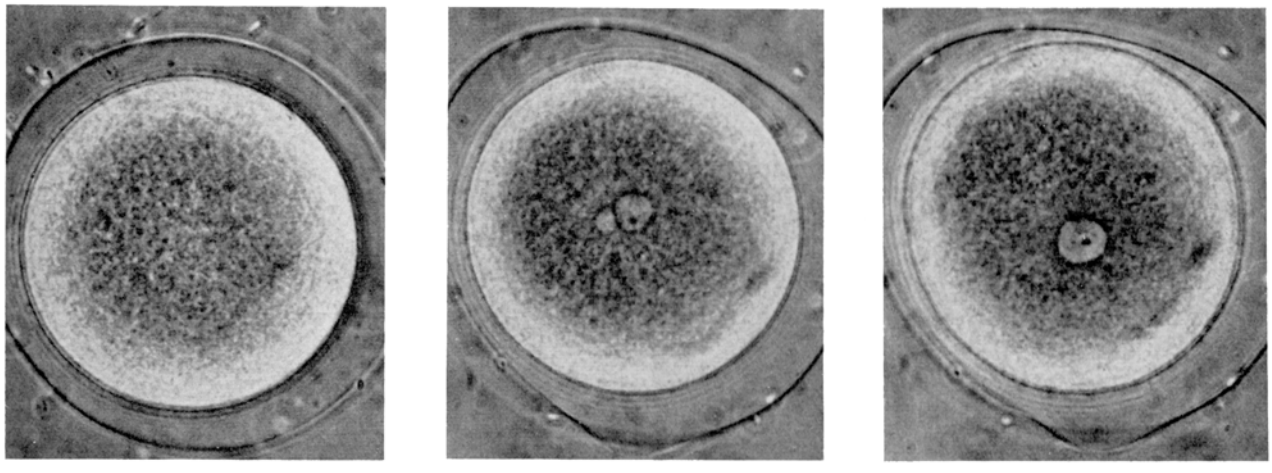
[20] P. PERLMANN, Exp. Cell Res. *5*, 394 (1953).

[21] Among these antigens, 7 soluble proteins could be identified further by a combination of biochemical methods and various agar diffusion techniques. Some of these proteins are localized in the yolk granules of the egg whereas others belong to the non-sedimentable part of the cytoplasm. During embryological development these proteins are consumed and synthesized at individual rates. Preliminary accounts of these results have been given elsewhere[22,23].

[22] P. PERLMANN and J. COUFFER KALTENBACH, Exp. Cell Res. *12*, 185 (1957).

[23] P. PERLMANN and J. COUFFER KALTENBACH, Arkiv Zool. *11*, 121 (1957).

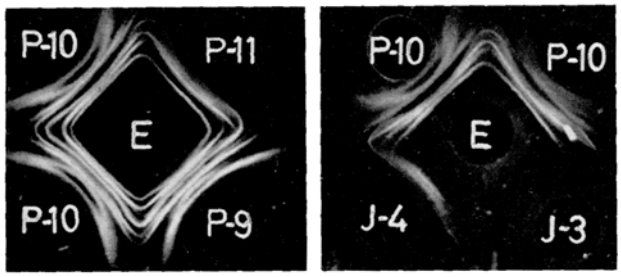
[24] C. V. HARDING, D. HARDING, and P. PERLMANN, Exp. Cell Res. *6*, 202 (1954).



a *b* *c*

Fig. 4.— Fertilization of an egg of Echinocardium cordatum. *a*, Photograph taken 8 min after insemination. Jelly layer is invisible. The protrusion of the fertilization membrane corresponds to the animal pole, seen in Figure 1. Note supernumerary sperm attached to the fertilization membrane; *b*, The same egg, 22 min after insemination. Male and female pronuclei now visible close together near the centre of the cell; *c*, The same egg, 40 min after insemination. The pronuclei have now fused. Approximately 10 min later the zygotic nucleus disapears while dividing. (Phase contrast. – From IMMERS, unpublished).

two such anti-jelly sera. Experiments of this kind demonstrated an appreciable difference in antigenic constitution of egg cytoplasm and jelly layer. Similarly, differences are found when the antigens in extracts from the eggs are compared with those in extracts from the sperm [20].



a *b*

Fig. 5.—Immunological precipitations in agar. Dark field photographs, taken after 2 days incubation at 38° C, followed by 2 weeks in the refrigerator. *E*, soluble part of egg homogenate (*Paracentrotus lividus*), diffusing from reservoirs in the centre; P-9, P-10, P-11, antisera, prepared in different ways, against unfertilized, jelly-free eggs; J-3, anti-jelly serum, obtained with a purified jelly preparation; J-4, anti-jelly serum obtained with a crude preparation, contaminated with egg antigens. Each antiserum is the serum pool from 5 rabbits. The various antisera diffused from the peripheral reservoirs. For further explanations see text. (From PERLMANN [27]).

The Response of the Eggs to Antiserum.—For the study of the antigens concerned with fertilization, living eggs were exposed to antiserum. Antigen-antibody reactions taking place in the eggs then led to morphological or physiological alterations which could be recorded. The procedure is shown schematically in Figure 6.

Exposing a small number of eggs, for example 500, to a standard amount of antiserum, e.g. 0·5 ml, renders them completely unfertilizable; such eggs will not react with the sperm even after removal of the antiserum. Moreover, they will be microscopically altered.

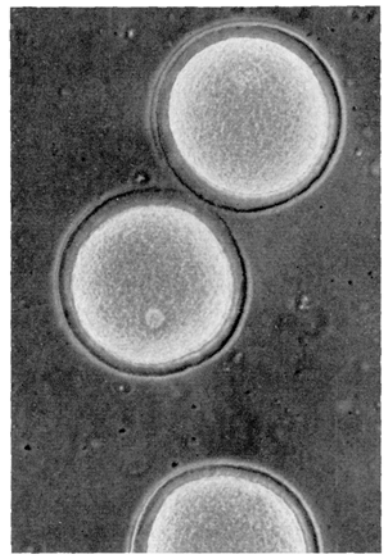


Fig. 7.—Jelly precipitation. Unfertilized eggs of *Paracentrotus lividus*, treated with homologous anti-jelly serum. Jelly layers precipitated and contracted. (Phase contrast).

Thus, frequently a precipitation of the jelly layers is obtained; the affected jellies have changed optical properties and become visible in the microscope. In addition, the precipitated layers very often contract. Jelly precipitated eggs always agglutinate (Figs. 7, 8) [25–27].

[25] P. PERLMANN, Exp. Cell Res. *6*, 485 (1954).
[26] P. PERLMANN, Exp. Cell Res. *10*, 324 (1956);
[27] P. PERLMANN, Exp. Cell Res. *13*, 365 (1957).

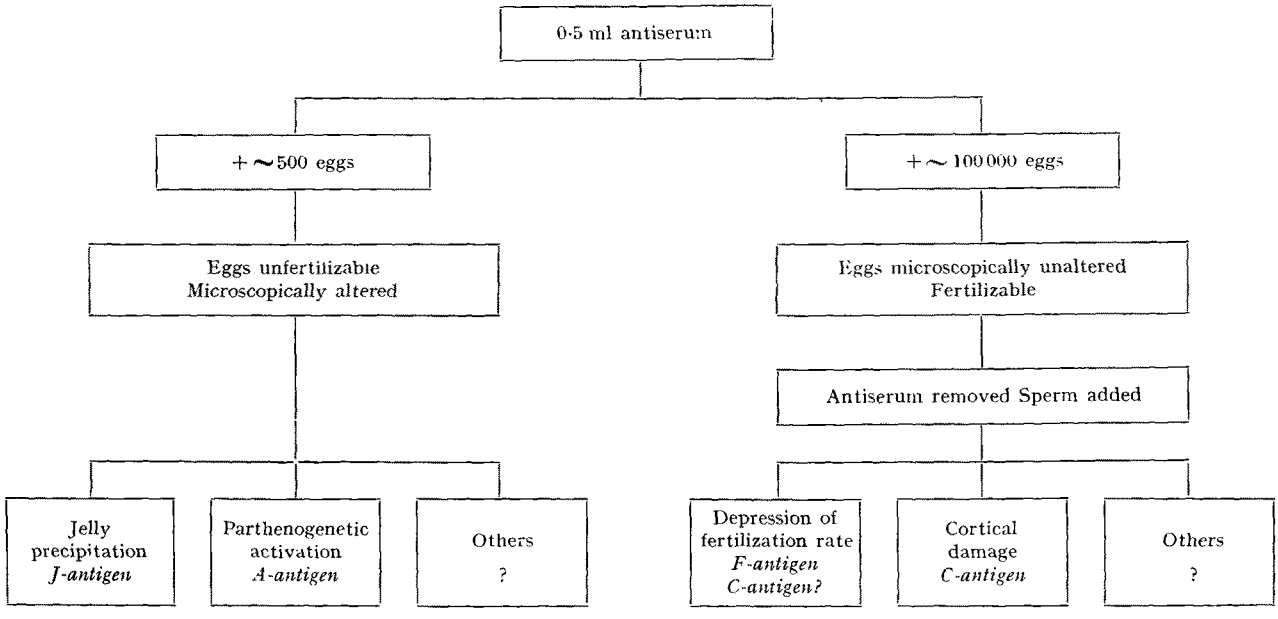


Fig. 6.— Procedure applied for analysis of various surface antigens. Aliquots of, e.g., 0·5 ml antiserum were either mixed with 500 eggs, or with 100,000 eggs. The various response reactions indicated in the Figure served for recording the different activities of the antisera. For further explanations see text.

Exposure of the eggs under the same conditions often leads to another response reaction which may be considered as being the most important in the present connection. In the eggs from a considerable proportion of the females, anti-egg serum induces parthenogenetic activation. Such activated eggs develop cortical reactions, leading to the formation of irregular membranes or membrane blisters, nuclear activation and monaster formation. Frequently, one or more irregular cell divisions take place also (Figs. 9, 10) [25–27].

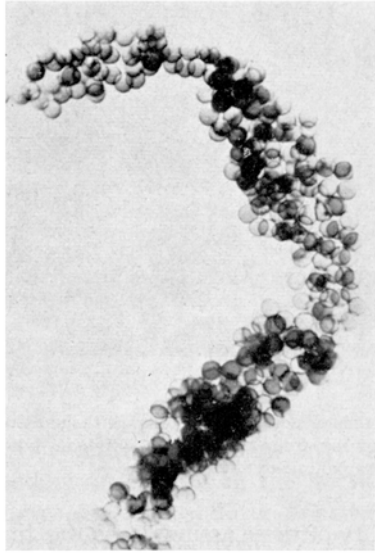


Fig. 8.—Agglutination of jelly precipitated eggs. Eggs from the same experiment as in Figure 7, photograph taken 1 h later. The layers now contracted to very narrow rims, not visible at the magnification used for the photograph. Eggs strongly agglutinated.

The extent of jelly precipitation and activation is strictly dependent on the ratio in which eggs and antiserum are mixed. When these ratios are too high, as shown in the right part of Figure 6, the eggs appear completely normal as judged from microscopic observations. Nevertheless, alterations are found when their behavior in fertilization is studied after removal of the antiserum.

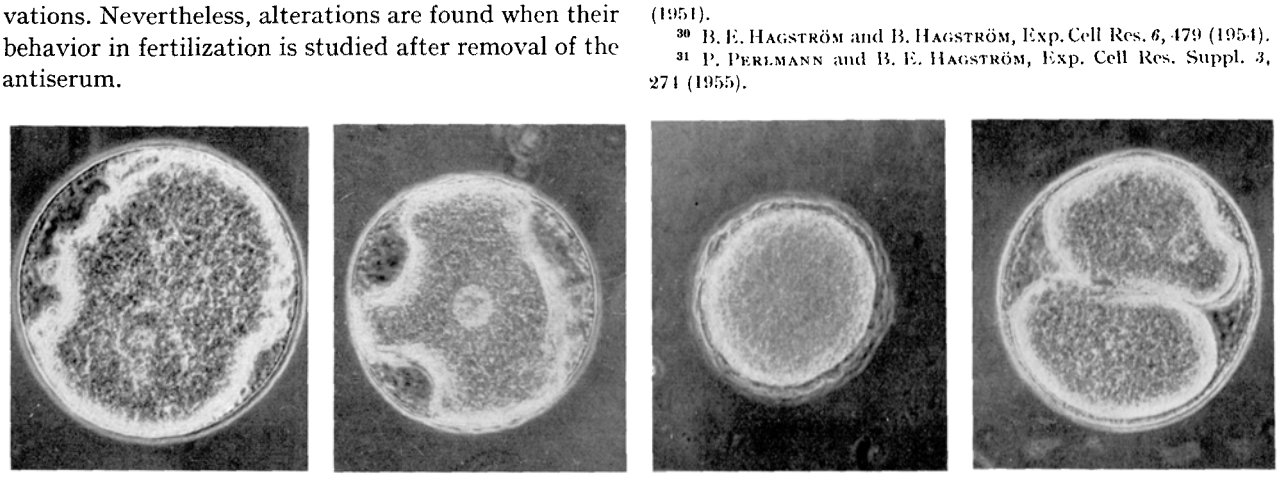
Most characteristical for eggs, fertilized after pretreatment with antiserum, are certain typical damages to the cortex. The fertilization membrane often remains attached to the egg surface and frequently only membrane blisters are formed. The membrane appears precipitated and the dissolution of the cortical granules is more or less inhibited (Figs. 11, 12). This also leads to a high tendency in the eggs for polyspermic fertilization and to severe disturbances of development [27,28].

Treatment of the eggs prior to fertilization with anti-egg serum reduces their fertilizability. One expression of this action is a depression of the 'rate of fertilization'. This rate can be measured by determining the proportion of eggs fertilized after various periods of egg-sperm interaction where fertilization has been interrupted by killing the sperm but not the eggs (Fig. 13) [29,30,27,31].

The Analysis of the Response Reactions.—Extensive series of experiments showed that sera from normal rabbits were inactive. Hence, it could be inferred that the effects described above were due to the action on the eggs of specific antibodies. In addition, comparison of the action of antiserum on the eggs from different sea urchin species revealed a surprising species specificity of the antibodies, as exemplified in Figure 13. Anti-sperm sera could be shown to be inactive also. For details see [26,27,31].

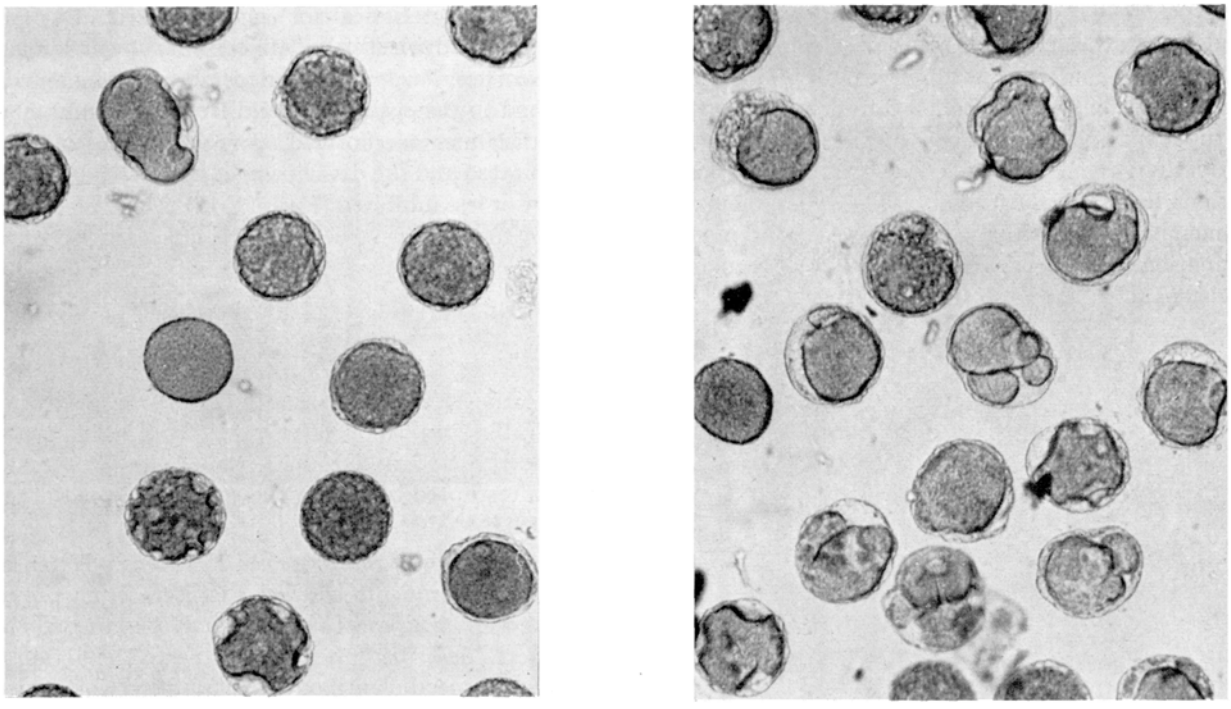
The next aim of the analysis was to achieve a resolution of the complex effects of antiserum into single components. As a first step, the nature of different variables governing the various responses of the eggs was studied. Subsequently, the dose-response relationships, valid under specified experimental conditions, were established for each of the responses described above. The findings from such experiments

[28] P. Perlmann and B. E. Hagström, Exp. Cell Res. *12*, 418 (1957).
[29] Lord Rothschild and M. M. Swann, J. exp. Biol. *28*, 403 (1951).
[30] B. E. Hagström and B. Hagström, Exp. Cell Res. *6*, 479 (1954).
[31] P. Perlmann and B. E. Hagström, Exp. Cell Res. Suppl. *3*, 271 (1955).



a *b* *c* *d*

Fig. 9.— Parthenogenetic activation. Unfertilized eggs of *Paracentrotus lividus*, treated with homologous anti-egg serum. *a*, Egg with incompletely elevated, irregular membrane; *b*, Egg with pronucleus swollen, migrated to the centre of the cell; *c*, Monaster egg, with small irregular membrane blisters; *d*, Slightly abnormal 2-cell stage (Phase contrast).



a *b*

Fig. 10. — Parthenogenetic activation. Unfertilized eggs of *Paracentrotus lividus*, parthenogenetically activated with homologous anti-egg serum. *a*, Photograph taken 2 h after beginning of antiserum treatment; *b*, Photograph from the same experiment, taken 12 h after beginning of serum treatment. Many eggs show beginning cytolysis.

constituted the basis for a comparative study where the activities of a number of differently prepared antisera were tested. The results have been summarized in Figure 14. They indicated, although they did not prove, that the responses were due to the reactions of at least 3–4 separate antibodies, with their corresponding antigens [27].

The validity of these assumptions was finally tested with the aid of *antiserum inhibition techniques* [32,6]. In these, use was made of specific absorption of antibodies prior to testing the antisera for their activities. Thus, when extracts or fractions, obtained from the eggs, are added to the antisera, antibodies are precipitated or

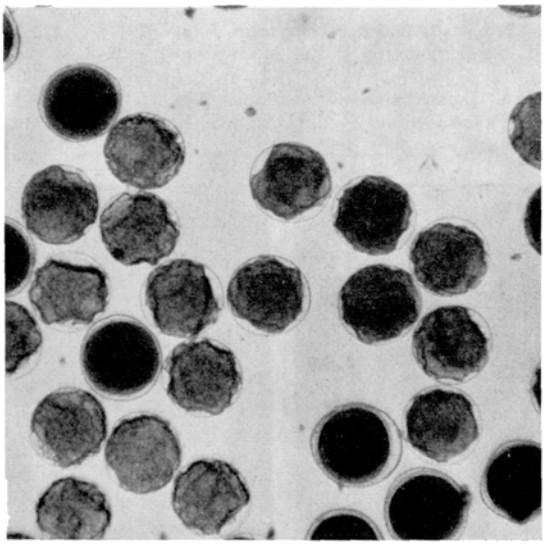


Fig. 11.—Cortical damage. – Fertilized eggs of *Paracentrotus lividus*, pretreated with homologous anti-egg serum. Egg and sperm killed by addition of formalin approximately 10 min after insemination. For explanations see text.

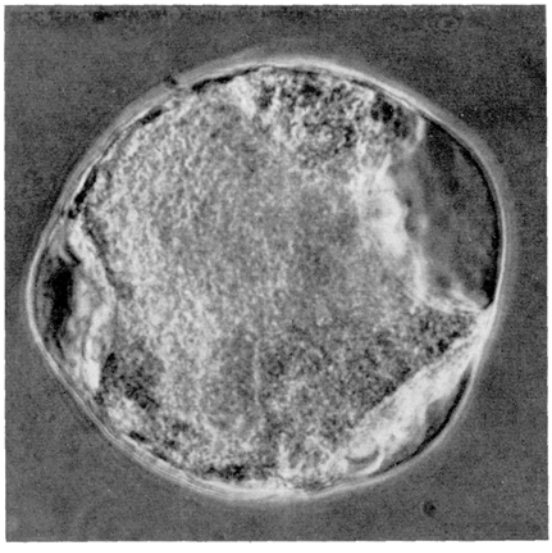


Fig. 12.—Cortical damage. Egg from the same experiment as in Figure 11 (Phase contrast).

[32] K. LANDSTEINER, *The Specificity of Serological Reactions* (Harvard University Press, Cambridge, Mass. 1946).

bound by antigen. When tested on the eggs, various activities of such antisera may be inhibited, depending on which antibodies were eliminated by absorptions. The type of results obtained is exemplified in Figure 15.

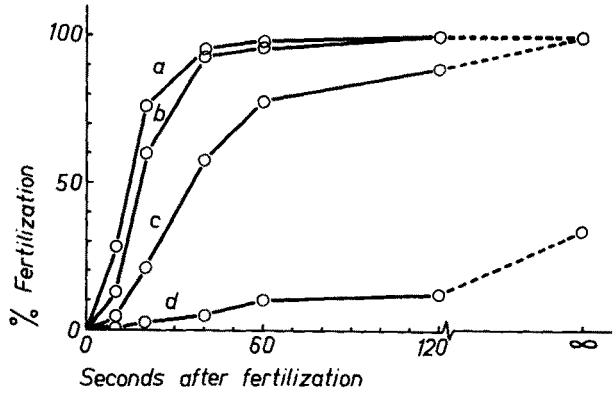


Fig. 13.—Depression of fertilization rate. Eggs of *Paracentrotus lividus*, fertilized after pretreatment with various sera. *Abscissa*, time in seconds of egg-sperm interaction. *Ordinate*, % fertilized eggs. Each symbol represents 200 counted eggs. The various curves correspond to different pretreatments of eggs from the same batch *a*) eggs pretreated with antiserum against eggs from *Arbacia lixula*; *b*) eggs pretreated with serum from normal rabbits; *c*) eggs not pretreated; *d*) eggs pretreated with antiserum against eggs from *Paracentrotus lividus* (From PERLMANN and HAGSTRÖM[31]).

Such inhibition experiments proved in fact that there were four different antigens involved in the response reactions mentioned in this section. In addition, certain conclusions could be drawn concerning the chemical nature of the antigens[33,34].

The antigens which will be discussed in the following are those involved in the response reactions shown in Figure 6. Other responses, e.g. osmotic alterations in the surface of the unfertilized eggs[26], induction of

[33] P. PERLMANN and H. PERLMANN, Exp. Cell Res. *13*, 454 (1957).
[34] P. PERLMANN and H. PERLMANN, Exp. Cell Res. *13*, 475 (1957).

Anti-serum	Activity			
	Jelly precipi-tation	Activation	Fertilization rate depression	Cortical damage
P-9	+	+ + ±	+ + +	+ + +
P-10	±	+ +	+ +	+ + ±
P-11	±	+ + +	+ + ±	+
J-3	+ + +	±	0 ?	0
J-4	+ + +	+ + ±	+	±
Sp-6	0	0	0	0

Fig. 14. Comparison of various activities of a number of anti-Paracentrotus sera. P-9, P-10, P-11, antisera against jelly-free eggs. The mode of preparation of antiserum P-11 was different from that used for preparation of P-9 and P-10; J-3, anti-jelly serum obtained with a purified jelly preparation; J-4, anti-jelly serum obtained with a crude jelly preparation (the precipitin-reactions obtained with these antisera are shown in Figure 5); Sp-6, anti-sperm serum. Each antiserum the serum pool from 5 rabbits. 0 = inactive, ± to + + + = very weak to strongly active (From PERLMANN[27]).

polyspermy and inhibition of cytoplasmic cleavage[28], have not yet been analyzed and will not be treated in this article. The details given below concern the eggs of *Paracentrotus lividus* only. Although eggs from other species respond similarly when exposed to homologous antiserum, certain characteristic differences do exist[26,31]. The antigens of the other species have not yet been analyzed in detail.

Jelly Precipitation; the J-Antigen

With the eggs of *Paracentrotus lividus*, the comparison of the jelly-precipitating potencies of different antisera suggested that the effect was due to the reaction between antibodies and a particular jelly antigen (Fig. 14)[27]. This could be fully confirmed by means of inhibition experiments; thus, absorption of the antisera

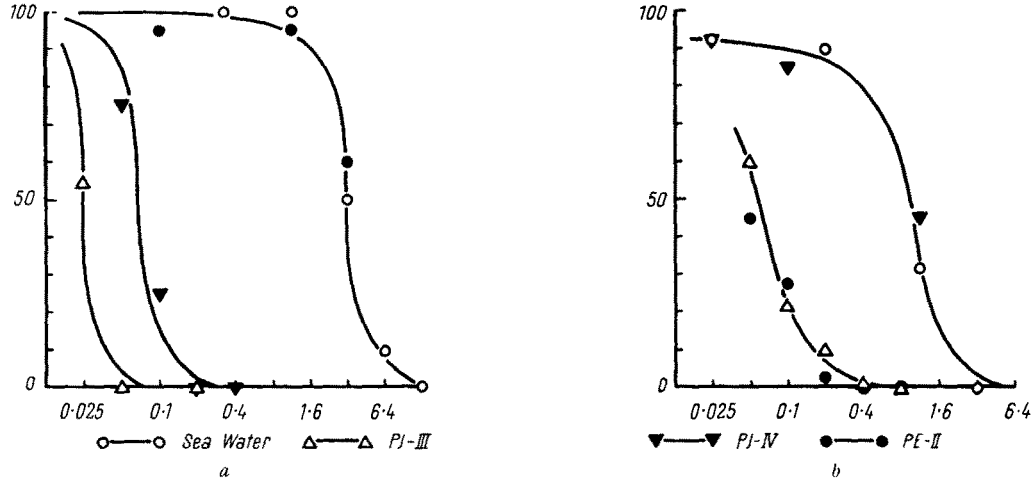


Fig. 15.—Inhibition of jelly precipitation or of activation of Paracentrotus eggs after absorption of antiserum. *Abscissae*, ml of sea water or of various antigen solutions used for absorption of one-ml aliquots of an anti-jelly serum (*a*), or an anti-egg serum (*b*). For testing, 0·8-ml aliquots of the absorbed (or diluted) antisera were mixed with aliquots of 0·2 ml sea water, each containing 500 reactive eggs. *Ordinates*, (*a*) % jelly precipitated or (*b*) % activated eggs. Each symbol represents 200 counted eggs. Antigen solutions used for absorption: *PJ-III*, *PJ-IV*, solutions of 2 jelly preparations, corresponding to ~23,000 or ~7000 eggs/ml, respectively; *PE-II*, soluble part of egg homogenate, made from jelly-free eggs, 32,000 eggs/ml. For further details[33]. (From P. PERLMANN and H. PERLMANN[33]).

with various isolated jelly preparations or with homogenates of jelly-free eggs revealed a high inhibitory capacity of the former preparations whereas the latter were inactive (Fig. 15*a*) [33].

The antigen, which thus must be part of the jelly layer, is called the *J-antigen*. Inhibition experiments showed that it is heat-stable (100°C) between pH 4–8. At pH3·5, it is susceptible to treatment with periodate as well as to heating to 100°C. Treatment with HCl of pH 1 at room temperature reduces the J-activity. The specificity is completely destroyed by 4 h heating to 100°C at pH 1 [33]. Inhibition experiments with a number of single sugars suggest that L-fucose is an essential part of the antigenic determinants (unpublished experiments).

The jelly layer of the sea urchin egg consists of sulfated polysaccharides in combination with protein or peptides[14]. The jelly layer of *Paracentrotus lividus* contains L-fucose as the main sugar and in addition glucose in small and variable quantities [35,36]. The data given above indicate that the J-antigen is an integral part of the main polysaccharide of the jelly layer.

The nature of the jelly precipitation is not understood. The coagulation no doubt represents a common way for the jelly to respond to many kinds of unrelated chemical agents, e.g. basic proteins or substances which can be extracted from the gametes themselves [10,14]. It remains to be established which chemical groupings of the jelly layer are reacting in the various cases.

The response of the eggs is very variable and entirely unresponsive eggs are always found. The reactivity of the eggs seems to be related to their state of maturity and varies during the spawning season [26,27]. Since this variability seems to be correlated to the capacity of the jellies to fix antibodies (unpublished experiments), it might indicate that chemical alterations are taking place in the layers during maturation.

The jelly layer of the sea urchin egg is the bearer of a sperm-agglutinating principle, fertilizin [37], which is species-specific in its action. The agglutination is assumed to be brought about by means of an 'immunological' reaction between the fertilizin of the egg and the corresponding antifertilizin in the sperm [1,8,9,15]. From the data at hand it is not apparent whether or not the J-determinants of the jelly substance are identical with the chemical structures exhibiting fertilizin activity. So far, attempts to inhibit fertilizin-induced sperm agglutination with J-antibodies have been without success [38]. Pretreatment of the eggs with J-antibodies is seemingly without effect on fertilization, even when the antibodies are applied over a wide range of concentrations [27], also [34].

Parthenogenetic Activation; the A-Antigen

In *Paracentrotus lividus* both anti-egg and anti-jelly sera may induce activation [26,27]. However, as shown in Figure 14, the jelly-precipitating and the activating potencies of the antisera were unrelated. The parthenogenetic antibodies could thus be assumed to differ from the jelly-precipitating antibodies. Consistent results were obtained in inhibition experiments; these conclusively demonstrated that the activating antibodies were directed against one or several antigens derived from the cytoplasm of the eggs; however, considerable amounts of these antigens may sometimes occur in isolated jelly substances (Fig. 15*b*) [33].

Further inhibition experiments indicated that the total inhibitory activity of the egg homogenates could be recovered in their soluble fraction. The antigen did not belong to the soluble proteins which are destroyed by the action of proteolytic enzymes. Furthermore, it withstands heating to 100°C both at pH 8 and at pH 3·5. On the other hand, it is partially destroyed by the action of periodate and by heating to 100°C at pH 1 (Fig. 16) [33].

When the soluble part of a boiled homogenate is studied in the electrophoresis apparatus, 3 main components of distinct mobilities are found [33]. These can be separated and tested in inhibition experiments. All A-activity is recovered from the slowest moving fraction, from which it can be further purified by means of precipitation with ethanol. Another way of partial purification is to precipitate the crude extract with HCl at pH 2. The precipitate then contains all the nucleic acid of the extract but only a small fraction of the antigenic activity. About 90% of the latter can instead be recovered from the supernatant from which it can again be purified further by means of ethanol. The methods are summarized in Table I; some qualitative chemical data of A-active preparations, obtained in these ways, are given in Table II.

Table I. – Preparation of A-antigen (*Paracentrotus lividus*)
Unfertilized eggs, freed from jelly layer at pH 5·2, homogenized, treated at 100°C for 4 h (pH 7·8), centrifuged. A-activity in supernatant.

Procedure 1

A-active fraction separated electrophoretically, precipitated with ethanol.

Procedure 2

HCl added → pH 2, centrifuged

Residue	*Supernatant*
10% of A-activity	90% of A-activity, precipitated with ethanol

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Table II.—Chemical properties of A-antigen

Easily soluble in water, insoluble in ethanol. Not susceptible to the action of trypsin or pepsin. Heat stable at pH 3·5–8. Slowly hydrolyzed at pH 1–2 at 100°C. Partially susceptible to IO_4^- (pH 3·5,50°C). Electrophoretic mobility $-1{\cdot}6 \times 10^{-5}$ cm^2 × volt^{-1} × sec^{-1} (pH 7·1, phosphate, $\mu = 0{\cdot}1$).

Some qualitative data on partially purified A-antigen

Glucose +	Hexosamine –	Ribonucleic acid –
Mannose +	Sialic acids –	Sulfate +
Galactose –	Amino acids +	Phosphate –
Fucose –		

The results led to the conclusion that activation, in all antisera, was caused by a particular antigen-antibody reaction. The antigen involved has been called the *A-antigen*. The A-antigenic determinants are of a carbohydrate nature but belong to a molecule different from the J-antigenic molecule. This statement involves no implications as to the nature of the molecule *in toto*. Thus, the polysaccharide may well be combined with peptides and/or with lipids; in addition, in the living cell it might be closely associated with other polysaccharides. The data given in Table II do not yet allow any conclusions as to the composition of the antigen. However, it may be noted that glucose seems to be the only carbohydrate common to both the antigenically active fraction of the egg extract and the isolated jelly layers which are A-active also. This might suggest that glucose, perhaps in combination with sulfate, is an integral constituent of the A-antigenic structures.

The distribution of the A-antigen between the egg cytoplasm and the jelly layer clearly suggests its localization in the cytoplasmic surface of the cell. The existence of substances of this type in the egg surface is well established [39–41]. The antigen seems to be bound in a labile fashion and hence may diffuse readily into the jelly layer [42]. A diffusion, occurring in the living egg, might at least partially explain the considerable variability in the parthenogenetic response towards antiserum of eggs of different origin or state of maturity, and of normal eggs or jelly-less eggs [26,27].

It is of special interest that the eggs can be activated by means of a particular antigen-antibody reaction, different from a variety of other such reactions which take place in or near the surface of the antiserum-treated cells. Although it contrasts with the commonly found inhibitory or cytotoxic effects of antibodies on cells [3], this response is consistent with the functional organization of the egg surface. The rearrangement of acid polysaccharides in the egg surface in connection with egg activation [41], and the activation of proteolytic enzymes [43] may offer analogies to certain events that are known to follow upon the immunological trigger reaction in anaphylaxis and allergy [44–48]. Thus, the stimulating action of the antibodies may be mediated

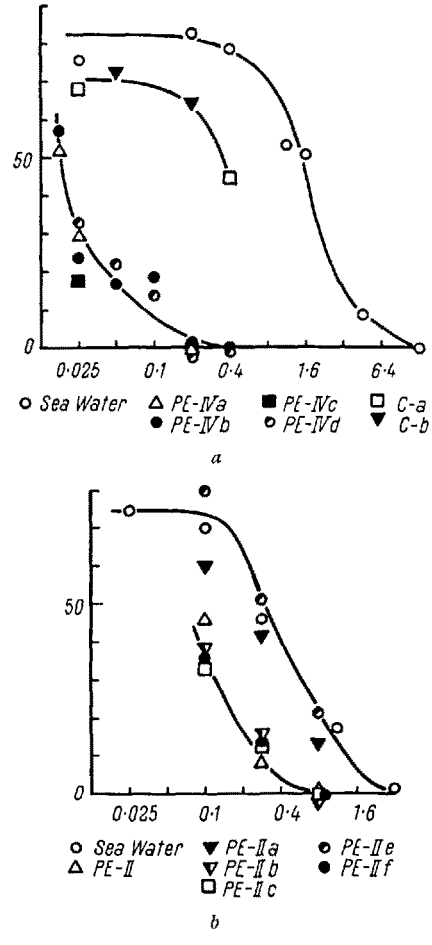

Fig. 16.—Two typical inhibition experiments bearing on the chemical nature of the A-antigen of the Paracentrotus egg. *Abscissae*, ml of sea water or various antigen solutions used for absorption of one-ml aliquots of an anti-egg serum. For testing, 0·8-ml aliquots of the absorbed (or diluted) antisera were mixed with aliquots of 0·2 ml sea water, each containing 500 reactive eggs. *Ordinates*, % activated eggs. Each symbol represents 200 counted eggs. Antigen solutions used for absorptions: (*a*) *PE-IV*, homogenate made from jelly-free eggs; *PE-IVa*, treated with trypsin; *PE-IVb*, corresponding control without trypsin; *PE-IVc*, treated with pepsin; *PE-IVd*, corresponding control without pepsin; *C-a*, *C-b*, controls containing enzymes and enzyme inhibitors but no egg homogenates. (*b*) *PE-II*, homogenate made from jelly-free eggs; *PE-IIa*, treated with periodate, pH 3·5, 50°C, 240 min; *PE-IIb*, heated to 50°C, pH 3·5, 240 min; *PE-IIc*, heated to 100°C, pH 3·5, 240 min; *PE-IIe*, heated to 100°C, pH 1, 240 min; *PE-IIf*, kept at 20°C, pH 1, 240 min. For further details [33]. (From P. Perlmann and H. Perlmann [33].)

through the immunological inactivation of an enzyme inhibitor, viz. the A-antigen, and the subsequent activation of a number of proteolytic enzymes. This

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explanation would be in line with the hypothesis of egg activation developed by RUNNSTRÖM[49, 41, 17].

In the eggs, activated by means of antiserum, the spreading of a submicroscopic 'fertilization wave', believed to induce the *microscopic* cortical reactions[50–54, 1, 17] seems to be inhibited. Most likely, this inhibition is, at least partially, due to the action of unrelated antibodies (cf. below) which affect other antigens in the cortical ground substance or in the cortical granules. Owing to this elimination of the self-propagating mechanism, the stimulating antigen-antibody reactions must probably cover a wide region of the egg surface in order to induce a visible response.

There is little doubt that the A-antigen is an important constituent among the molecules involved in the normal activation of the egg. We may probably assign to it a key role as initiator of activation in fertilization. If so, this assumption does not necessarily imply *that the possible reaction between the A-antigen and the sperm involves the A-active structures, nor that it at all involves an 'immunological' reaction.* The reaction might well be of enzymatic nature.

Cortical Damage; the C-Antigen

As mentioned above, certain characteristic anomalies in the cortical reactions of fertilized eggs are among the most striking features after pretreatment with antiserum (Figs. 11, 12)[27, 28]. No obvious correlation could be detected between the jelly-precipitating or the activating potencies of the antisera on one hand and their cortex-damaging potencies on the other. It was of interest to note that the antisera with weak or non-existent cortex damaging potencies (P-11, J-3, J-4) were devoid also of antibodies reacting with certain soluble proteins in agar-diffusion experiments (Figs. 5 and 14)[27].

Inhibition experiments indicated that the antigen involved in this response is a soluble egg antigen, not detectable in the jellies. The antigenic activity is not destroyed with trypsin. On the other hand, the antigen does not withstand heating at pH 7–8 and is thus chemically entirely different from the J- and A-antigens described above. This antigen, which may be a protein, is called the *C-antigen*[34].

Pretreated and fertilized eggs with cortical damage closely resemble eggs which have been activated by certain of the antisera. It seems reasonable to assume that the pronounced damage of the breakdown of the cortical granules and of membrane formation in both cases may be induced by the antibodies reacting with the C-antigen. In any event, judging from the type of response, a superficial localization of the C-antigen may be postulated. It may possibly be contained in the cortical granules. In any case, it certainly is of importance in determining the course of the cortical reaction. Moreover, it is likely that the increased tendency for polyspermic fertilization induced by certain antisera[28], is due to the reactions of antibodies with the C-antigen.

Fertilization Rate Depression; the F-Antigen

Comparison of the potencies for depressing fertilization rate of various antisera also showed that neither the antibodies against the J-antigen nor those against the A-antigen exert much activity. Moreover, the cortex-damaging and the fertilization-rate-depressing potencies of the anti-egg sera appeared to be unrelated (Fig. 14)[27].

In inhibition experiments, isolated jelly preparations were of low inhibitory capacities whereas equivalent amounts of egg homogenate were strong inhibitors. Hence, the antibodies responsible for fertilization rate depression are also directed against antigens derived from the cytoplasm of the eggs. Absorption with sperm homogenate had no effect (Fig. 17*a*)[34].

The rate-depressing antigens seem to be only partially soluble in water or saline. They are not attacked by trypsin. Heating (100°C, 4 h) reduces the inhibitory capacity of the homogenates without destroying it completely (Fig. 17*b*). The data were interpreted to mean that one ore more additional heat-stable antigens, different from the J- and A-antigens, are probably involved in fertilization rate depression. The antigen(s) has (have) been called the *F-antigen*. The heat labile factor involved in fertilization rate depression may be identical with the C-antigen[34].

The depression of the rate of fertilization suggests a localization of the F-antigen in the cytoplasmic surface also. Since it is heat stable it might well belong to the structures of the cortex assumed to be rich in polysaccharides or mucoproteins[40, 41, 55].

The depression of the rate of fertilization, following the immunological reactions, suggests that the F-antigen might constitute a specific sperm receptor of the egg surface. However, fertilization rate depression is not a simple response and several unrelated mechanisms are likely to be at work[29, 1, 27]. For example, the fertilization rate depression, brought about by the reactions of antibodies with *heat labile* antigens (the C-antigen?), may be due to the inhibition of a cortical mechanism determining the physical or chemical reactivity of the surface. Therefore, in the rate experiments, an attached spermatozoon may sometimes be killed before it succeeds in starting a visible reaction in

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the egg. This mechanism may be quite independent from a possible sperm receptor mechanism, instru-

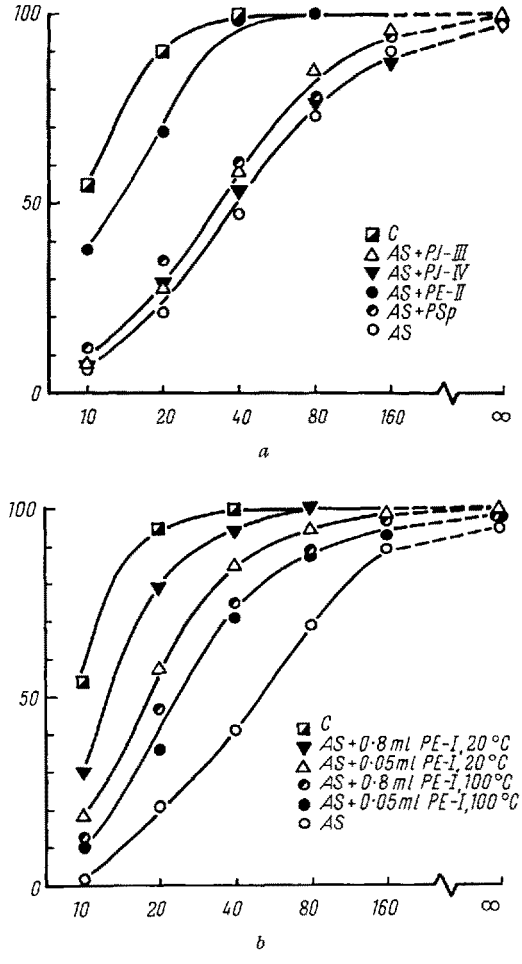

Fig. 17.—Fertilization rate of *Paracentrotus* eggs, pretreated with various absorbed or unabsorbed samples of an anti-egg serum. *Abscissae*, time in seconds of egg – sperm interaction; *Ordinates*, % fertilized eggs. Each symbol represents 200 counted eggs. The various curves correspond to different pretreatments of the eggs as indicated in the Figures. *C* = eggs pretreated with normal rabbit serum; *AS* = antiserum. (*a*) *PJ-III*, *PJ-IV*, jelly preparations, *PE-II*, homogenate from jelly-free eggs, used for absorption of the antiserum. Amounts corresponding to equivalent numbers of eggs were taken for absorption in all cases. *PSp*, sperm homogenate. (The jelly preparation *PJ-III*, which was of a very low inhibitory capacity in this experiment was a strong inhibitor of activation due to a high content of A-antigen.) (*b*) *PE-I*, *20°C*, homogenate from jelly-free eggs, untreated; *PE-I*, *100°C*, aliquot of the same homogenate, heated to 100°C for 240 min at pH 8; 0·05 ml, 0·8 ml, volumes of antigen solutions added to one-ml aliquots of antiserum for absorption. The various curves in this diagram are significantly different from each other. For the statistical treatment of the data of fertilization rate experiments[27]. For further details[34].
(From P. PERLMANN and H. PERLMANN[34].)

mental during the attachment of the sperm and involving other, *heat-stable* factors, such as the F-antigen.

The Specificity of Sperm Attachment

In order to explain the specificity of fertilization, LILLIE introduced the immunological concept by assuming sperm attachment to be mediated by means of antigen-antibody like reactions between complementary molecules. The basis for his assumption was the finding of the sperm agglutinating fertilizin, derived from the eggs[37]. As already mentioned above, fertilizin has later been identified as a component of the jelly layers of the eggs[10,14,15].

Fertilizin has long been believed to have an *improving* effect on the process of sperm attachment and to be indispensable for fertilization[56,8,10]. However, except for certain data indicating an improving action of jelly substance on various activities, e.g. motility or oxygen consumption, of the sperm[57,58,1], there is at present no conclusive evidence in favour of any improving action in fertilization, neither of the jelly layer nor of its fertilizin. On the contrary, there has accumulated an increasing body of evidence indicating general inhibitory effects of dissolved jelly substance as well as of the jelly layer *in situ* on various phases of fertilization or egg activation[59–65]; also [17].

Whatever the biological significance of the 'immunological' fertilizin-antifertilizin reaction, it seems quite evident that it cannot be as predominant in determining the specificity of fertilization as sometimes claimed. Obviously, in the present connection, the immunological concept cannot be taken too literally—a fact which for instance was well recognized by LILLIE[37]. Whereas the reactions between antigens and antibodies are confined to rather restricted areas on the surface of molecules, the specificity of the process of sperm attachment must be assumed to be more complex. It may involve, in its first stages, electrostatic attraction between charged groups, arranged in self-repeating patterns on the surface of the gametes. Bivalent cations may play a rôle in binding together identically charged areas on the surfaces of the gametes[66,67]. Upon closer contact between the molecules, irreversible attachment of a higher degree of specificity may follow, involving various specific patterns of short range forces and steric factors. The final step of egg activation and penetration may require a spatially oriented union between an enzyme, for example in the acrosomal filament of the sperm[16,68,69], and a prosthetic group at the receptor sites of the eggs. Analogous conditions may be found in the fields of bacteriophage attachment and of virus

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hemagglutination [66,70–81]. Whatever the exact mechanism, it appears reasonable to consider the existence of particular requirements of 'complimentariness', involving different molecules, during the various stages of the process. It remains to be established, whether or not the fertilizin-antifertilizin reaction is involved, and, if so, what its rôle and its position in the sequence may be.

The present investigation has given evidence for the occurrence of four structurally different types of macromolecules in the surface layers of the sea urchin egg. At least three of these seem to have particular and important functions in connection with sperm attachment and egg activation. In addition, there are indications of the presence of additional factors, not yet determined but probably available for analysis. Finally, functionally similar but structurally different molecules are present in the eggs of other sea urchins. It may be hoped that further immunochemical studies of the various macromolecules participating in fertilization will permit a direct approach to the problems discussed in this section.

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Zusammenfassung

Im vorliegenden Aufsatz wird über die Ergebnisse immunologischer und immunochemischer Untersuchungen der Oberflächenstrukturen der Eier einiger mediterraner Seeigelarten berichtet. Die Eier werden Antiserum ausgesetzt, das durch Einspritzungen von Seeigel-Gametenmaterial in Kaninchen gewonnen wurde. So erhält man eine Reihe mikroskopisch sichtbarer, artspezifischer Antwortreaktionen. Normales Kaninchenserum ist unwirksam.

Durch das vergleichende Studium der Aktivität verschiedener Antiseren, sowie durch die Antiserum-Hemmtechnik wurden bis jetzt vier voneinander verschiedene Oberflächenantigene bei den Eiern von *Paracentrotus lividus* festgestellt. Mindestens zwei der gefundenen Antigene haben Polysaccharid-Charakter. Die Reaktionen zwischen einem bestimmten Antikörper und dessen Antigen, das A-Antigen genannt wurde, können interessanterweise zur parthenogenetischen Aktivierung unbefruchteter Eier führen. Andere Antigen-Antikörperreaktionen können Befruchtung und Entwicklung der Eier hemmen. Die mögliche Bedeutung der verschiedenen Antigene für das Festsetzen der Spermien an der Oberfläche der Eier sowie für den Beginn der Ei-Aktivierung wird besprochen.

Brèves communications - Kurze Mitteilungen
Brevi comunicazioni - Brief Reports

Les auteurs sont seuls responsables des opinions exprimées dans ces communications. – Für die kurzen Mitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed by their correspondents.

Spektraler Farbintegrator

Die uns umgebenden Farben bestehen alle aus spektral gemischtem Licht, dessen Zusammensetzung mit Hilfe der Spektralanalyse in bekannter Weise ermittelt werden kann. Für Zwecke der Farbenlehre ist aber der umgekehrte Vorgang, die Farbsynthese, von ebenso theoretischer wie praktischer Bedeutung.

Farbintegratoren auf trichromatischer Grundlage, die teils mit Farbfiltern, teils mit spektralen Lichtern arbeiten, sind schon verschiedentlich beschrieben worden. Was aber bisher fehlte, ist ein Instrument, das Farben beliebiger spektraler Zusammensetzung in vorgegebener Weise bis an die theoretischen Grenzen hinan zu erzeugen gestattet.

Ein von uns verwendeter Apparat sei hier kurz beschrieben, da er sich uns trotz noch vorhandener Unvollkommenheiten zur Aufklärung und Lösung vieler Farbprobleme als sehr brauchbar erwiesen hat.

Er geht im Prinzip auf eine schon von NEWTON beschriebene Vorrichtung zurück, wonach ein prismatisch erzeugtes Spektrum auf einer Linse entworfen und von ihr wieder auf einem weissen Schirm zum ursprünglichen farblosen Licht vereinigt wird[1]. Eine analoge Anordnung wurde auch von POHL[2] und von der Firma Zeiss für Vorlesungszwecke empfohlen. Für unsere besonderen Zwecke waren folgende Bedingungen zu erfüllen:

1. Die verwendete Lichtquelle soll möglichst einer der anerkannten Normlichtarten entsprechen.

[1] J. NEWTON, *Optik*, Buch I, Teil II, Prop. I, Lehrsatz I und Prop. V, Lehrsatz 4 (siehe z. B. «Ostwald's Klassiker», Nr. 96, 1. Buch, pp. 76 und 88).

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