IMMUNO-ELECTRON MICROSCOPE ANALYSIS OF THE SURFACE LAYERS OF THE UNFERTILISED SEA URCHIN EGG

II. Localisation of Surface Antigens

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ABSTRACT

The immunological properties of the surface layers of *Paracentrotus lividus* eggs have been studied further by using ferritin-labelled antibody to localise specific antigenic sites. In order to detect a wider spectrum of antigenic determinants, several antisera against egg and jelly substance have been employed in combination with absorption procedures using lyophilised antigen. This use of absorbed antisera was made feasible by adding ferritin label in a second antiserum layer of ferritin–anti-γ-globulin. Eggs were treated with antibody for short periods to detect antigenic sites without incurring structural changes (shown in previous paper) resulting from long antibody treatment. Unspecific ferritin uptake, found in pinocytotic vesicles and yolk granules, is considered in relation to yolk formation. The jelly layer, found to be immunologically heterogeneous, included one component interacting with antijelly γ-globulin and one with antiegg γ-globulin. The vitelline membrane proved to be rich in egg antigens (heat-stable and heat-labile). The role of this layer in specificity of fertilisation, parthenogenetic activation, and the possibility of being analogous to a basement membrane are discussed. Few antigenic sites were found on the plasma membrane with antiegg γ-globulin. This γ-globulin resulted in some specific labelling of cortical granules and its action is considered in relation to the permeability properties of the egg.

INTRODUCTION

The presence of a number of surface antigens in the sea urchin egg has been demonstrated and it has been shown that these antigens have a close link with specific changes taking place in different parts of the egg surface (1–5). It was therefore considered desirable to localise these antigens within the egg ultrastructure. The use of specific antibody labelled with the electron-opaque ferritin molecule (6) was deemed to be the most suitable approach to this.

The ferritin-labelled antibody technique of detecting specific antigens is now well proved, having been used for cell surface studies in virus (7–10), phage (11), bacteria (12), protozoa (13, 14), tumour cells (15–18), and sea urchin gametes (19, 20). Intracellular antigens have been studied less, owing to difficulty in making the cell membrane permeable to the large ferritin–γ-globulin conjugate without damaging the ultrastructure, but use has been made of complement-plus-

antiserum treatment (15, 17) or freezing and thawing of the material before applying the labelled antiserum (9, 18). Osmium tetroxide fixed (13), formol-fixed (9, 12, 18), and unfixed (8, 11, 15, 16, 17, 19) material have all been treated with the ferritin-antibody conjugate; material from animals injected with specific antibody has been treated with a ferritin conjugate of an anti-γ-globulin serum (21); and an indirect or two-layer method of labelling with a ferritin–anti-γ-globulin conjugate, analogous to that of fluorescence microscopy, has also been used (19, 20). Other methods of making the antibody molecule sufficiently dense to be detected with the electron microscope have been used by coupling antisera to uranium (22) or to mercury (23, 24), but ferritin labelling appears to be easier to detect and is preferable in most cases. Unmodified antibody can also be visualised for electron microscopy (5, 13, 25–27).

Experiments have been made on living sea urchin eggs prepared for electron microscopy with a view to correlating immunochemical (1) and ultrastructural studies (5). In particular, use has been made of two layers of antisera in order to facilitate the analysis of a greater number and variety of antibodies. In most experiments, γ-globulin from anti–sea urchin sera was first applied unabsorbed or after absorption with lyophilised preparations from gametes, and the ferritin anti-rabbit–γ-globulin serum was added subsequently. It has also been possible to experiment with different concentrations of anti–sea urchin γ-globulin when using two layers.

MATERIALS AND METHODS

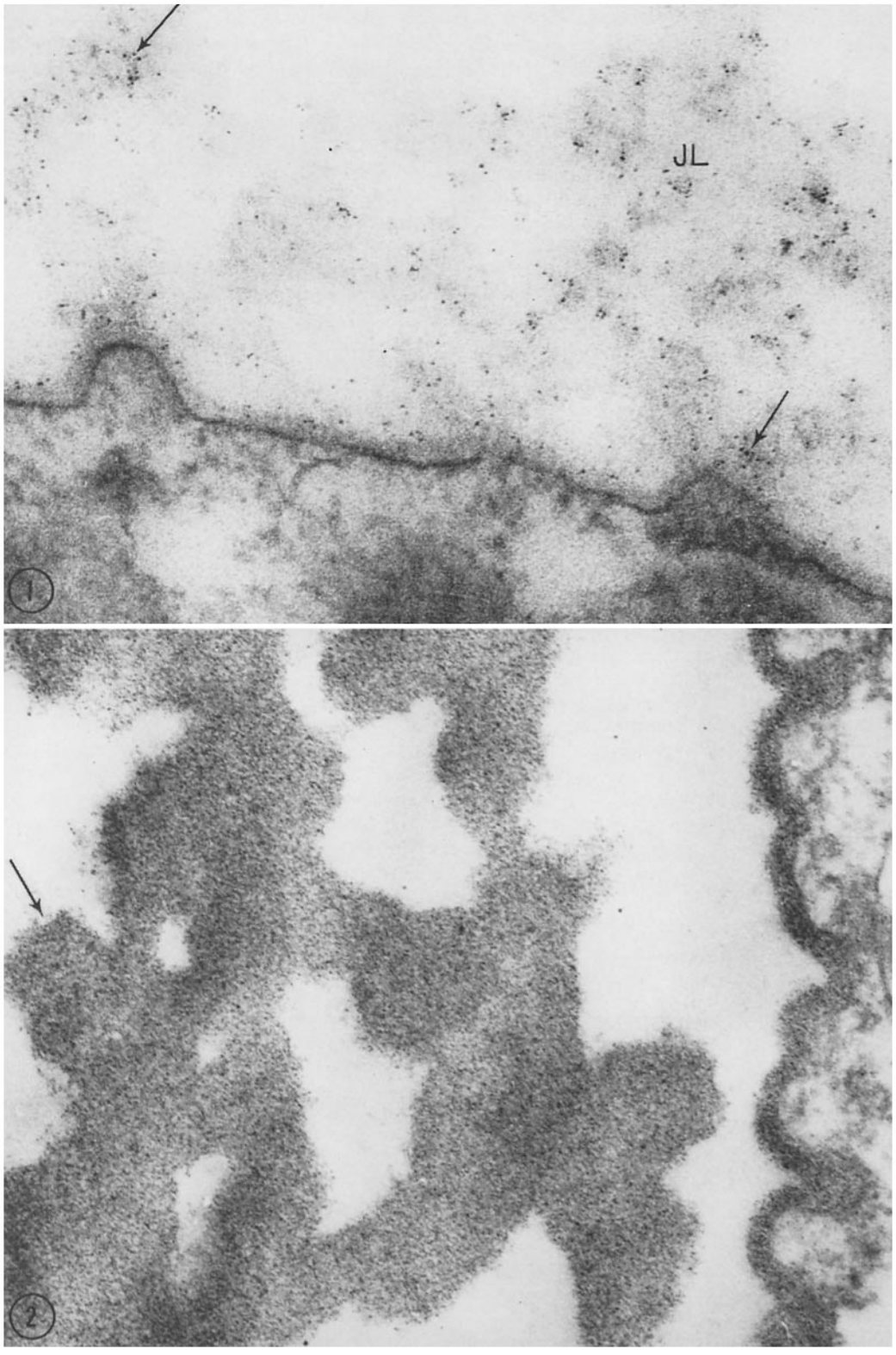
The same rabbit antisera against *P. lividus* eggs or jelly substance described in the previous paper (5) as well as antisperm serum (19) were used, and the experiments were again performed with chromatographically isolated γ-globulin (28). In addition, the isolated γ-globulin of a sheep anti-rabbit γ-globulin serum was used for indirect staining. This antiserum was obtained by injecting a sheep 3 times intramuscularly with a total of 600 mg of isolated rabbit γ-globulin (prepared by continuous flow electrophoresis and column chromatography (29) and incorporated in Freund's adjuvant).

Some of the ferritin-globulin conjugates were prepared after Singer's original technique using meta-xylylene diisocyanate (XC) as the intermediate coupling molecule (6). The majority of the γ-globulin fractions were conjugated to ferritin, according to the second method of Singer and Schick (30), with toluene 2,4-diisocyanate (TC) as the intermediate coupler, in order to obtain exclusively covalent linkage between the two protein molecules. These latter conjugates were also further purified of free unconjugated γ-globulin by ultracentrifugation in a Spinco Swinging Bucket rotor No. 39 at 36,000 RPM for 1½ to 2 hours (30), and of free ferritin and any remaining free γ-globulin by continuous flow paper-electrophoresis (31) on a Spinco Model CP electrophoresis apparatus, using barbiturate buffer (pH 8.6 and i = 0.02) and a current of 55 to 75 mamp. The optical density of the fractions thus obtained was determined at 280 mμ and those samples within the central peak containing the ferritin-globulin conjugate alone were concentrated with polyvinyl pyrrolidone (32) to about ½ of the original volume, stored frozen in small phials and, before use, dialysed against sea water.

Absorption of the γ-globulin preparations for specific removal of antibody was performed as described in the previous paper (5). The efficiency of absorption was checked in the light microscope (1, 3) and in the electron microscope by addition of ferritin conjugate to eggs treated with absorbed γ-globulin. The antigens used for absorption were lyophilised

FIGURE 1 An egg treated with a ferritin-XC-conjugate of an antijelly γ-globulin (one-layer method). The jelly layer (*JL*) is precipitated to some extent and the ferritin label (arrows) of the antijelly globulin is present throughout it. Outside the cell membrane there is a layer of precipitated jelly which is ferritin-labelled on the outer surface. There are a number of small surface papillae. × 100,000.

FIGURE 2 Treatment first with a concentrated solution of antijelly γ-globulin followed, after washing with sea water, by a ferritin–TC–anti-γ-globulin conjugate (F/G, two-layer method). The jelly layer is very densely, but heterogeneously, precipitated in a distinctive pattern. The dense jelly substance containing the ferritin label (arrow) is also present along the cell surface which has more and thicker papillae than in untreated eggs. The electron-translucent areas between the egg surface and the periphery of the precipitated jelly coat contain no detectable jelly substance and no ferritin. × 80,000.


JL
1
2

preparations of isolated jelly substance, homogenate of jelly-free eggs, homogenate of heated (100°C, 2 hours) jelly-free eggs, and the soluble fraction of a homogenate of heated jelly-free eggs. The latter preparation was obtained by centrifugation and was electrophoretically heterogeneous, as described earlier (3).

Fresh unfertilised eggs were taken from *P. lividus* as previously (33). In experiments where jelly-free eggs were needed, 100 ml of eggs in sea water were treated with 2.3 ml 0.1 M HCl giving a pH of 5.2. This was immediately followed by 1.5 ml 0.1 M NaOH, and the eggs were then thoroughly washed (34). A small number of living eggs (approximately 500 in 0.2 ml of sea water) were treated, without fixation, with a ferritin-globulin conjugate (*one-layer method*), or with an unconjugated γ-globulin, followed, after washing with sea water, by a ferritin conjugate of the sheep anti-rabbit γ-globulin (*two-layer method*, 18, 19). For details of treatment see reference 5 and previous publications (2–4, 19). However, unless otherwise stated, the eggs were treated with γ-globulin or ferritin conjugate *for only 15 to 30 minutes at room temperature.* For simplicity the ferritin-anti-rabbit-γ-globulin conjugate will be referred to as F/G. After antiserum treatment the eggs were well washed, fixed in 2 per cent osmium tetroxide, and embedded in Epon 812 (35). In order to make the ferritin molecules more conspicuous, some sections were treated with 2 per cent hydrogen peroxide for 30 minutes to remove reduced osmium tetroxide (36). No other staining was performed. (For further details see reference 5).

RESULTS

The Jelly Layer

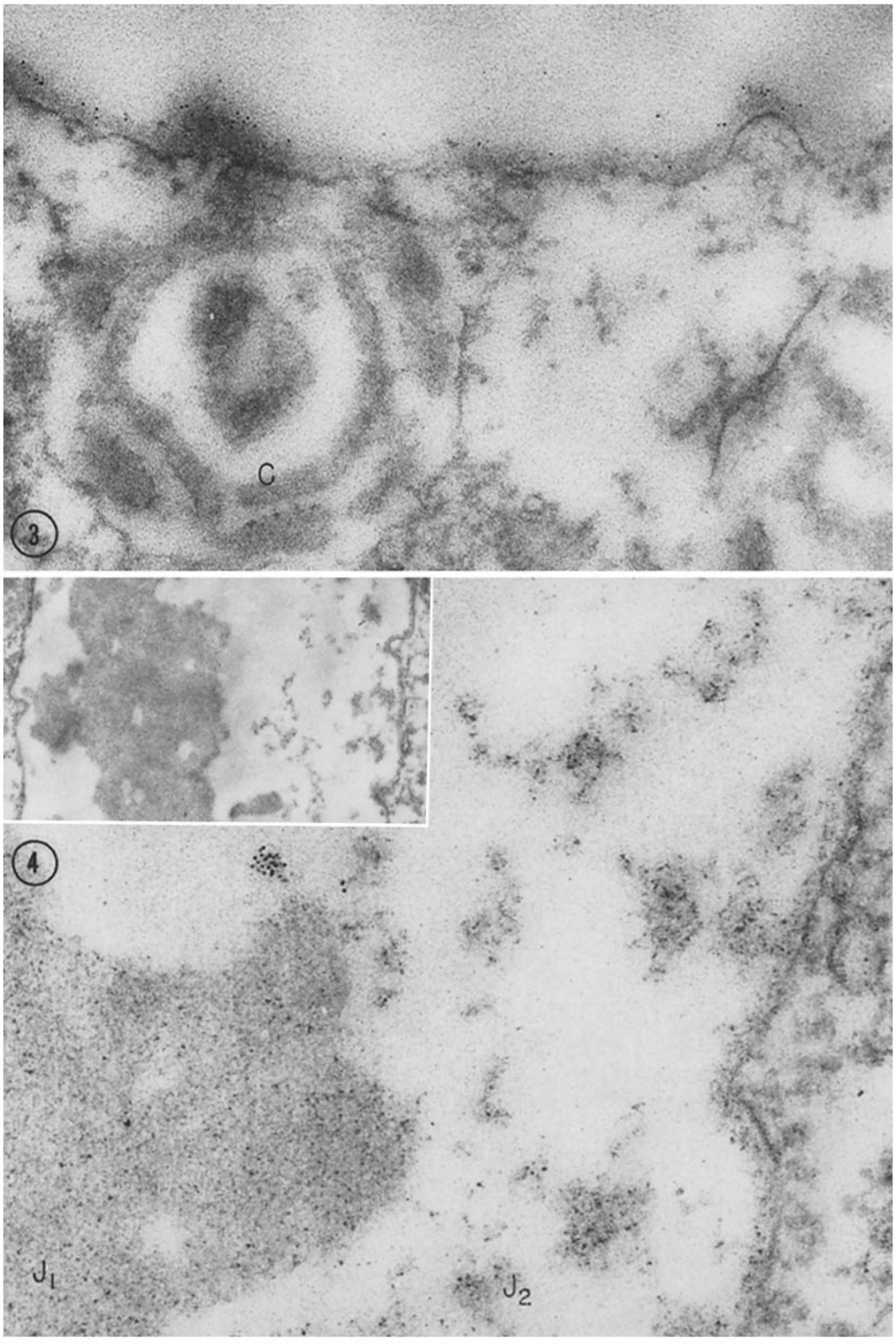
Even with the light microscope it could be seen that the ferritin conjugate of the antijelly γ-globulin caused the jelly layer to precipitate significantly although the reactions were weaker than with a solution of unmodified antijelly globulin in corresponding concentration. With the one layer method the ferritin label of the antijelly γ-globulin conjugate could be seen distributed throughout the jelly coat surrounding the egg (Fig. 1). The visibly precipitated jelly layer (not more than 1 μ wide) was slightly electron-opaque and was heterogeneous, the denser clumps of jelly substance being more heavily labelled with ferritin. Ferritin was not closer than 150 to 200 A to the plasma membrane and it was located on the outer surface of an electron-opaque layer adjacent to the egg surface.

When eggs were treated in the two layer method with a 2.5 per cent solution of the antijelly γ-globulin, only a narrow band of jelly (0.3 μ wide) was visible with the ferritin label sparsely distributed in it. The effects of the antijelly γ-globulin in a 80 per cent solution (5) were very pronounced however (Fig. 2). The precipitation occurred in a distinctive pattern, there being a strongly precipitated layer on the periphery of the jelly coat and close to the egg surface, with an intermediary zone which was electron-translucent except for small clumps of densely precipitated jelly substance. The ferritin label was thickly distributed in the precipitated jelly, but was absent from the electron-translucent regions of the jelly coat.

When eggs from which the jelly layer had been chemically removed were treated with the antijelly γ-globulin conjugate (Fig. 3), some ferritin molecules were present close to the egg surface. These were few in number compared with those in the jelly eggs however. The ferritin was not associated with the plasma membrane, but with the outer surface of some adjacent material which might have been jelly substance remaining in spite of the acid treatment.

FIGURE 3 An egg with the jelly layer chemically removed subsequently treated with ferritin–antijelly γ-globulin conjugate (as was used in Fig. 1) (one-layer method). Some ferritin markers are seen on the outer surface of a layer exterior to the plasma membrane. Cortical granule *c*. × 76,000.

FIGURE 4 Eggs which have been treated with antijelly γ-globulin absorbed with a lyophilised homogenate of total jelly-free eggs, and secondly with F/G (absorbed, two-layer method). The jelly layers of the two eggs have reacted differently. One (*J1*) is densely precipitated while the other (*J2*) has reacted less strongly. Ferritin is present in the jelly substance and also in some jelly adhering to the outer surface of the vitelline membrane on the egg surface. Inset, low magnification showing the relation of the precipitated jelly layers to the two eggs. × 110,000; Inset, × 22,000.


C
3
4
J1
J2

In order to ensure that only antigens of the jelly substance were being localised, the antijelly γ-globulin was absorbed with lyophilised jelly-free eggs so that the globulin solution would be free of any antibody against egg substances (4). After the F/G had been added (Fig. 4), ferritin labelling throughout the precipitated jelly was seen which was similar to that observed after treatment with unabsorbed antijelly γ-globulin.

In many experiments, agglutination of the jelly layers of adjacent egg was evident (see also reference 4). In Fig. 4, agglutination can be seen where the jelly layer of the one egg was much more strongly precipitated than that of the second egg. There is, however, a definite merging of the two jelly substances.

The antiegg γ-globulins produced no consistent jelly precipitation although ferritin was found in the region of the jelly layer. However, as will be shown in the next section, this was due to the reaction of egg substances in the jelly layer. Normal γ-globulin, antisperm γ-globulin, free ferritin, and antijelly γ-globulin absorbed with lyophilised jelly substance (4, 5) (Fig. 16) resulted in no jelly precipitation and no labelling of the jelly substance.

The Egg Surface

The egg surface, that is, the plasma membrane and the vitelline membrane, is immunologically different from the jelly layer. As has already been shown (3, 5), in favourable conditions antiegg antibodies evoke large surface alterations, often leading to parthenogenetic activation. In the experiments described here, where a ferritin label was employed to localise specific antigens, short-term γ-globulin treatment was generally used so that such large-scale structural changes would not be incurred.

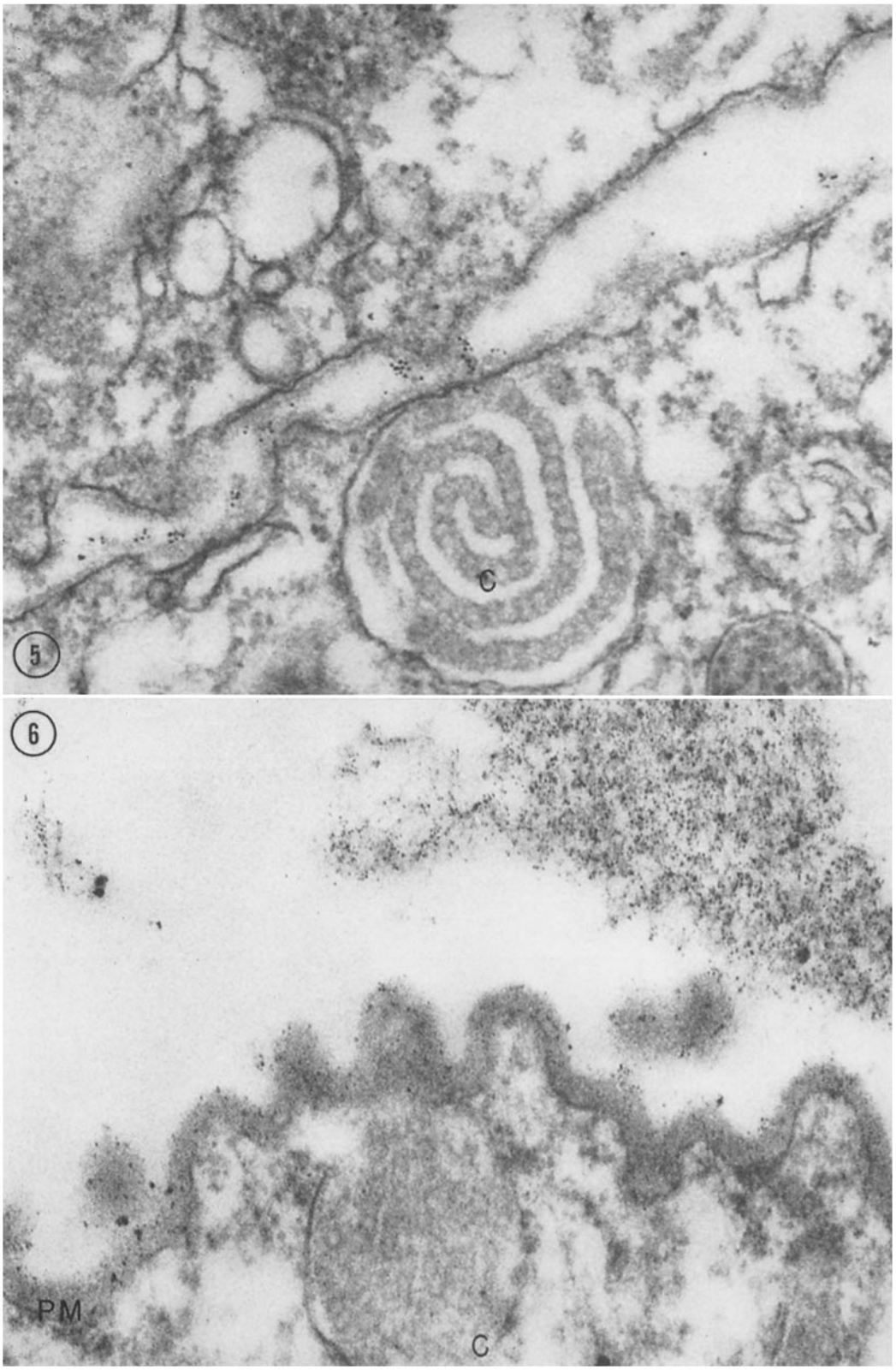
The ferritin conjugate of the antiegg γ-globulin (Fig. 5) was localised at intervals along the egg surface, there appearing to be more ferritin on the papillae where there was also a thicker layer of dense material. With the unmodified antiegg γ-globulin in 80 per cent concentration (5) and the two-layer method, a relatively wide (0.05 to 0.075 μ) dense layer outside the plasma membrane was stained thickly with the ferritin label. Sometimes an apparently isolated spot of similarly labelled material was observed a little removed outside this layer. Both the wide layer and isolated patches of this dense material presumably originated from the egg itself, either from within the egg or from the plasma membrane or the vitelline membrane, since specific ferritin labelling with the antiegg sera was found (Fig. 6). This material appeared to remain within the jelly layer, but is definitely distinct from it both in its structure, which is fibrous instead of granular (5), and in its immunological specificity, since it is specifically labelled by the antiegg, but not by the antijelly, γ-globulin.

When this unmodified antiegg γ-globulin was absorbed with lyophilised jelly-free eggs and the ferritin label was subsequently added with the anti-γ-globulin conjugate, there was almost no ferritin visible (Fig. 17), which demonstrates not only the specificity of the labelling but also the effectiveness of the absorption procedure.

Previous experiments have shown that different egg responses to antibodies are associated with different heat-stable and heat-labile antigens, respectively, all probably located in the egg surface (1–4). In the preceding paper the parthenogenetic activity of antibodies against heat-

FIGURE 5 Eggs treated with the ferritin-XC conjugate of an antiserum against a homogenate of jellyless eggs (one-layer method). The surfaces of the two eggs are in close proximity and the papillae almost interdigitate. The ferritin label is present outside the plasma membrane, and groups of ferritin molecules appear to occur more frequently around the papillae. The cortical granule (*C*) shows the distinctive structure present in *P. lividus*. × 80,000.

FIGURE 6 An 80 per cent solution of the same γ-globulin as used in Fig. 5, employing the two-layer method (anti-total egg γ-globulin followed by F/G). There are many thick surface papillae, and adjacent to the plasma membrane (*PM*) is a dense layer which contains ferritin label throughout. Outside the cell surface is fibrous material (similar to that seen in Fig. 11 of the previous paper) which is densely labelled with ferritin. The cortical granule (*C*) contains a little ferritin. × 80,000.


C
5
6
PM
C

stable egg components was also demonstrated electron microscopically (5). Eggs treated with a ferritin conjugate of an antiserum against heat-stable egg antigens (one-layer method) had small surface papillae (Fig. 7). The vitelline membrane was thin and sometimes appeared distinct from the plasma membrane, being slightly separated from it in places. The majority of the ferritin label was again on the vitelline membrane rather than on the plasma membrane. In eggs first treated with the unmodified (and more active) γ-globulin in 80 per cent concentration and then F/G, ferritin was distributed in a way similar to that seen with anti-total egg globulin, namely in the thick (0.15 μ) layer outside the plasma membrane and in a slightly electron-opaque, heterogeneous layer of varying width in the region of the jelly coat (Fig. 8).

When the γ-globulin from the antiserum against heated eggs was absorbed with lyophilised homogenate of jelly-free eggs or heated jelly-free eggs, ferritin label was rarely found after addition of F/G (Figs. 18 and 23). Even after prolonged incubation with the eggs, the absorbed γ-globulin did not produce any of the structural changes typical of the unabsorbed γ-globulin.

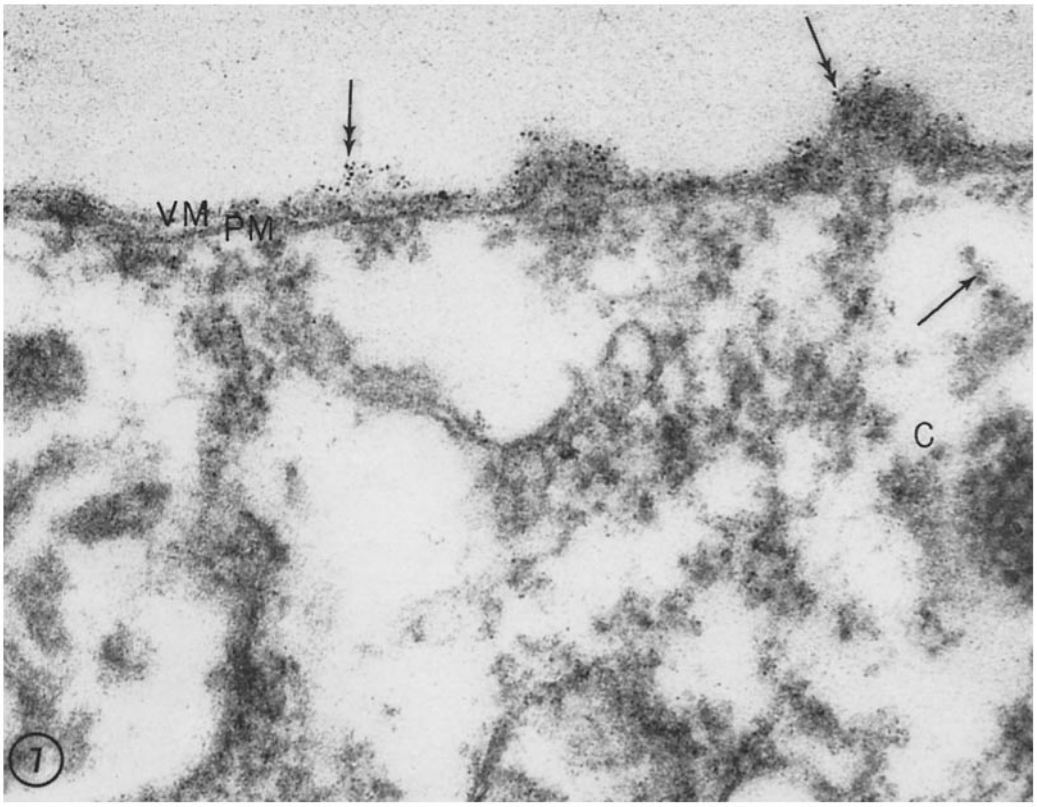
Thus, these experiments indicated that some or all of the ferritin-labelled egg antigens appearing mainly in the vitelline membrane or outside it were heat-stable. In fact, previous light microscope work has shown that only the heat-stable antigens were associated with parthenogenetic activation whereas heat-labile antigens were not (1, 3). Instead, evidence has been brought forward that one or several heat-labile antigens were involved in an antibody-induced cortical damage (C antigen, *cf.* references 1, 4). Moreover, some results also suggested that the activation antigen(s) (A antigen) was easily water-soluble but that there were additional heat-stable antigens of different immunological specificity which were insoluble and whose reaction with antibodies rendered a decrease in egg fertilisability (F antigen, *cf.* references 1, 4).

It was of interest to establish whether the method of ferritin labelling in combination with antiserum absorption would allow a distinction in the localisation of these 3 different categories of surface antigens (heat-stable soluble and insoluble, heat-labile). In a first series of experiments the γ-globulin from antiserum against total jellyless eggs was absorbed with a lyophilised heat-stable and soluble egg-fraction (see Materials and Methods). Absorption with this fraction could be assumed to remove antibodies against heat-stable soluble antigens, leaving only those reacting with the *heat-labile* antigens and the *heat-stable insoluble* antigens. The efficiency of the absorption was also tested in the light microscope by measuring the activating potency of this antiserum after absorption with graded amounts of this soluble fraction (3). As can be seen from Fig. 9, approximately 1 ml of this preparation per ml of antiserum was enough to abolish completely all visible parthenogenetic activity. This figure also shows that the responsible antigen(s) most likely were of a carbohydrate nature (*cf.* references 1, 3).

The amount of absorbant used for electron microscopy was about 10 times larger than the highest amount employed in the experiment of

FIGURE 7 An egg after the addition of a ferritin-XC conjugate from an antiserum against heated (100°C, 2 hours) jellyless eggs (one-layer method). At the cell surface the ferritin molecules (double-barbed arrow) are present mostly on the vitelline membrane (*VM*), but rarely on the plasma membrane (*PM*). There appears to be more ferritin label in the region of the surface papillae. Ferritin molecules (arrow) can also be seen intracellularly in association with the cortical granule (*C*) both on its bounding membrane and in the lamellae. × 90,000.

FIGURE 8 The two-layer method in which treatment with an 80 per cent solution of the unmodified γ-globulin against homogenated jellyless heated eggs was followed by F/G. A relatively uniform ferritin-labelled dense layer covers the plasma membrane which has formed many thick papillae. The substance in the jelly region is similar to the dense layer adjacent to the plasma membrane and is apparently diffusing into the jelly layer. The presence of the ferritin molecules in this experiment distinguishes this diffuse substance from the jelly substance labelled by the antijelly globulin. The cortical granules (*C*) are not intact. × 90,000.


VM
PM
C
7

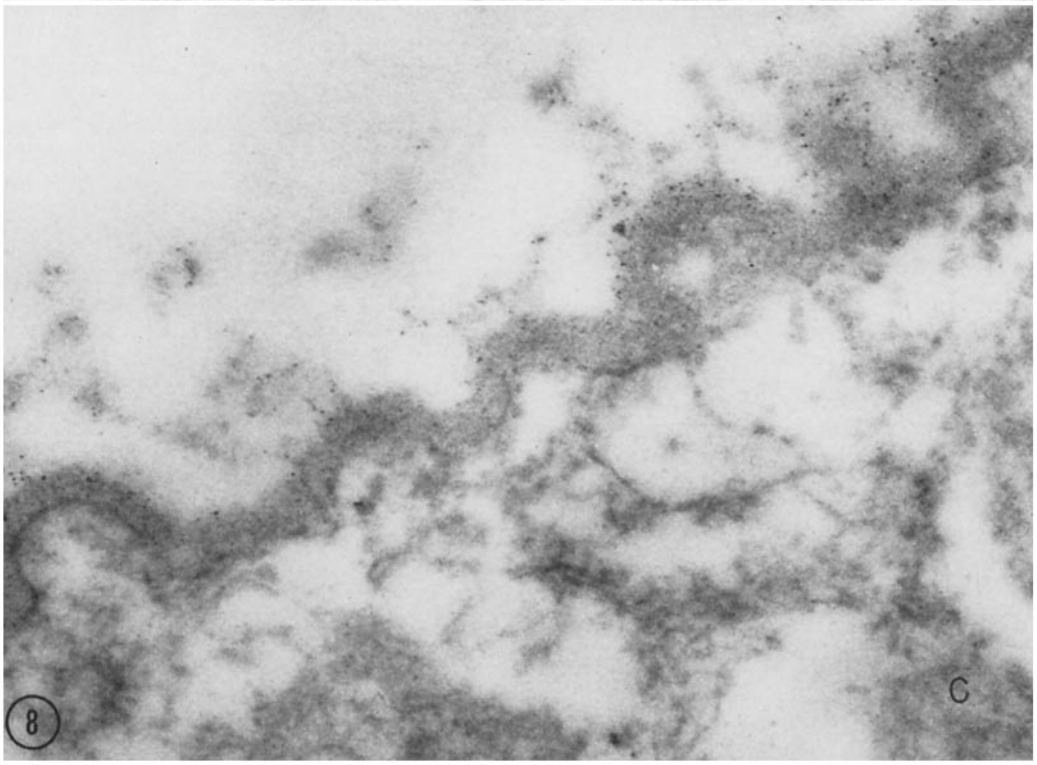

C
8

Fig. 9. After treatment with such an absorbed γ-globulin solution (Figs. 11 and 14), the egg surface was noticeably smooth and without papillae, and a distinct layer or membrane (approximately 35 A thick), which was presumably the vitelline membrane, was raised for considerable distances, only coming in contact with the plasma

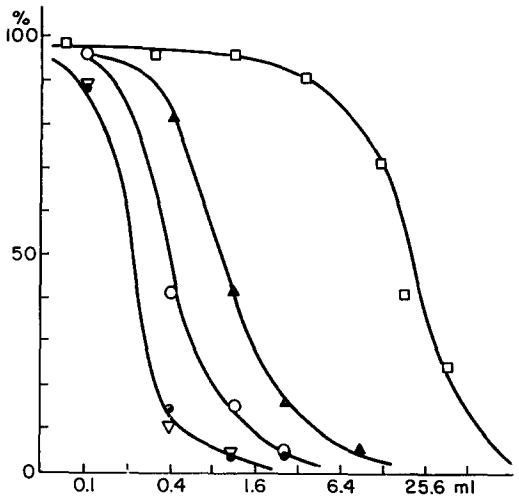


FIGURE 9 Inhibition of activation of *Paracentrotus* eggs after absorption of γ-globulin from antiserum against total jelly-free eggs, *Abscissa:* ml of sea water or antigen solutions added to 1.0 ml aliquots of γ-globulin solution. For testing, 0.8 ml of the diluted or absorbed serum was mixed with 0.2 ml of sea water containing 500 reactive eggs. *Ordinate:* per cent activated eggs. Each symbol represents 200 counted eggs. Explanation of symbols: *squares,* sea water; *open triangles,* absorption with the heat-stable (100°C) soluble fraction of a homogenate of jelly free *P. lividus* eggs (0.25 mg/ml); *solid circles,* aliquot of the same solution, treated with HCl (pH 2) for two hours at room temperature and finally dialysed against sea water; *open circles,* the same but heated at pH 2 for 45 minutes; *solid triangles,* heated at pH 2 for 120 minutes.

membrane at certain places. Ferritin was seen at irregular intervals along the elevated layer (Fig. 14) and also in places where this layer was thickened by having similar material adhering to it (Fig. 11).

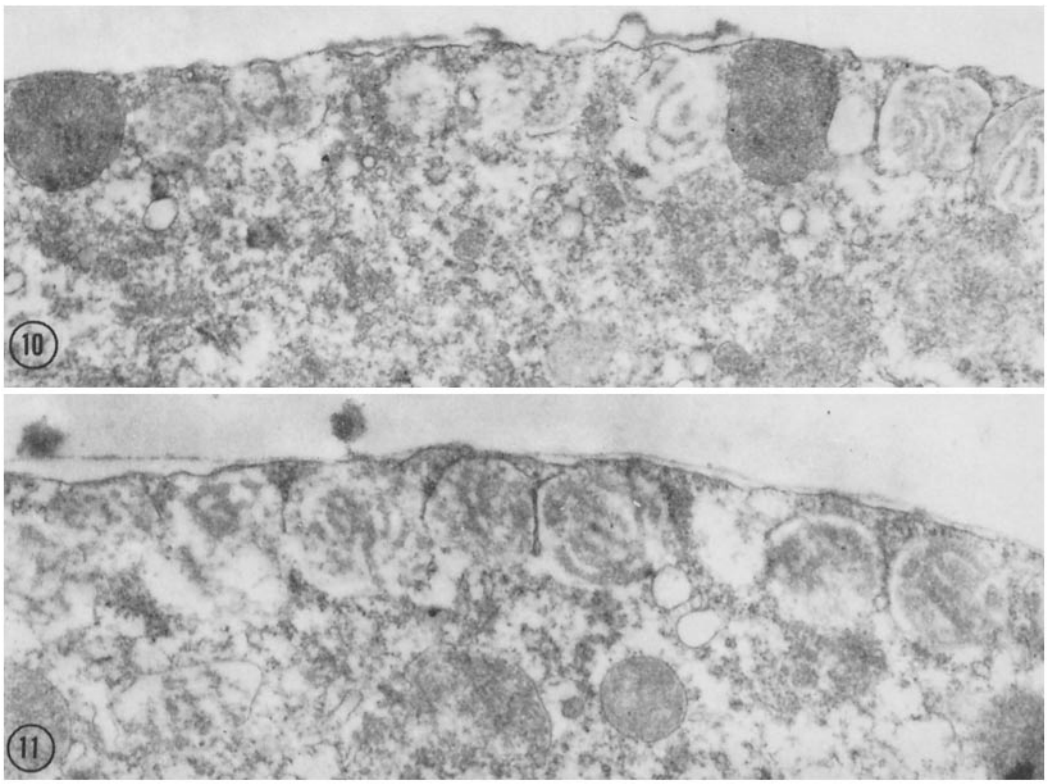
In a second series of experiments the localisation of the heat-labile surface antigens was examined separately. The γ-globulin from the antiserum against total egg homogenate was therefore absorbed with a lyophilised heated egg homogenate in order to exclude antibody against both soluble and insoluble heat-stable antigens and leave only those reacting with the *heat-labile* antigens (Figs. 10, 12, and 13). Eggs treated with this absorbed γ-globulin had an appearance distinctly different from that of those described above (compare Figs. 10 and 11). Dense material occurred only outside certain portions of the egg surface, but, when present, it was in a thick irregular layer. The ferritin label was found in these dense regions (Figs. 12 and 13) and in places on the vitelline membrane where it was very slightly separated from the plasma membrane.

In order to localise only the *heat-stable insoluble* antigens, the γ-globulin from the antiserum against heated eggs was absorbed with the soluble fraction of heated eggs. That this γ-globulin, when unabsorbed, gives rise to parthenogenetic activation and surface alterations was established light microscopically and has also been shown in the electron micrographs of the preceding paper (5). The eggs treated with the absorbed preparation showed very little alteration (Fig. 15). The vitelline and plasma membranes remained united, and there were few papillae. A few groups of ferritin occurred along the surface on the vitelline membrane, but there was much less ferritin labelling than in the other experiments. In some preparations there were a few regions of electron-opaque material, labelled very densely with ferritin, which were found some distance from the egg and had no apparent connection with it.

Finally, a number of additional control experiments will be described. Free ferritin could be detected in small amounts on the vitelline membrane after 15 minutes' treatment with a concentrated solution (about 60 mg/ml), but this seemed to decrease after longer intervals (*e.g.* 2 hours). The ferritin conjugate of antisperm γ-globulin in contrast to its effect on sperm or fertilised eggs (data to be published), could only be detected in a few places on the vitelline membrane of unfertilised eggs (Fig. 7 in reference 19). The normal γ-globulin–ferritin conjugate was also found to a small extent on the egg surface (particularly with the unpurified conjugates), but it occurred in groups chiefly where papillae and pinocytotic vesicles appeared to be forming (Fig. 22; also Fig. 5 in reference 19). When normal γ-globulin was used with the two-layer method, many eggs were devoid of surface ferritin while others had a small amount (Fig. 6 in reference 19). If, on the other hand, the eggs were kept in concentrated (80 per

cent) normal γ-globulin for 5 hours (instead of 15 to 30 minutes) there was a considerable amount of ferritin on the vitelline membrane (Fig. 19) although less than when the specific antiegg γ-globulins were used for even a short period. There were no signs of activation on the egg surface however.

than those with the unpurified ones. The controls of the absorption experiments showed the least unspecific ferritin. The yolk granules could frequently be seen almost in contact with the plasma membrane (Figs. 12 and 13) and often appeared elongated and between the cortical granules at the egg surface (Fig. 23). Normally the yolk



FIGURES 10 and 11 Low magnification micrographs of eggs treated with the antiegg γ-globulin (anti-total eggs) absorbed with lyophilised preparations of total heated jelly-free eggs (Fig. 10) and the soluble fraction of heated jelly-free eggs (Fig. 11), followed by F/G. The difference in the material outside the plasma membrane can be clearly seen. In Fig. 10, regions of dense material are along only a small proportion of the egg surface, whereas in Fig. 11 a layer (the vitelline membrane) is slightly elevated from the plasma membrane over all the egg surface. × 22,000.

The Cell Interior

In almost all eggs examined ferritin was found intracellularly in the yolk granules owing to an immunologically unspecific uptake of ferritin or ferritin conjugate by the yolk (Figs. 24 and 25). The label was found in the control experiments, even those made with free ferritin alone (Fig. 25). Eggs from the experiments with the purified conjugates appeared to contain less unspecific ferritin granules are spherical and are not generally present among the cortical granules. The bounding membrane of the yolk granules, which contains smaller (yolk) particles, was frequently broken thus releasing yolk and ferritin. Ferritin-containing vesicles were also observed (Figs. 20, 21).

A little ferritin was found in the cortical granules, in both the bounding membrane and the lamellar structure, in eggs treated with antiegg

and antiheated egg, γ-globulin, and with the antiegg γ-globulin absorbed with the total heat-stable or heat-stable soluble egg preparations. Control experiments with normal γ-globulin, free ferritin, and absorbed sera showed no specific labelling of the cortical granules.

DISCUSSION

Specificity of the Labelling

In this study of the surface layers of the sea urchin egg, most of the experiments involved the use of the indirect or two-layer method of labelling. As discussed earlier, this method has certain experimental advantages (19, 20). Obviously, the introduction of the second γ-globulin layer or the additional manipulation of the material did not obscure the results.

The localisation of the antigenic sites with labelled antibody is presumably rather imprecise in Ångström units since both the γ-globulin (37) and the ferritin (38) are rather large molecules. The addition of an extra γ-globulin, in the two-layer method, between the antigen and the visible label will inevitably reduce the precision further. How great the error in localising the specific antigen is cannot be determined since it is not known at what point on the γ-globulin molecule the ferritin is coupled.

The validity of the absorption technique for electron microscopy has also been doubted (39). In the present study, however, the absorption experiments provided a valuable tool, as in previous light microscope studies, for the distinction of different antigens (*e.g.* antijelly γ-globulin absorbed with lyophilised jelly substance gave neither jelly precipitation nor ferritin labelling, whereas both phenomena were observed after absorption with egg homogenates (*cf.*, also references 1, 3, 4)). Whether the few ferritin molecules observed in some of the controls indicate incomplete absorption, the presence of antibody against some antigen not present in the absorbant, or the unspecific adsorption of the anti-γ-globulin–ferritin conjugate to the egg cannot be ascertained. In these experiments, there was so little ferritin to be found, however, that it could not possibly obscure the results.

The control experiments with free ferritin showed that it is important to purify the conjugates in order to eliminate any unspecific surface adsorption or pinocytosis of ferritin which would obscure the results. The removal of unconjugated γ-globulin will prevent blocking of the antigens by unlabelled antibodies, thus allowing the maximum number of sites to be detected. It will also prevent unlabelled antibody from producing excessive structural alterations in the cells.

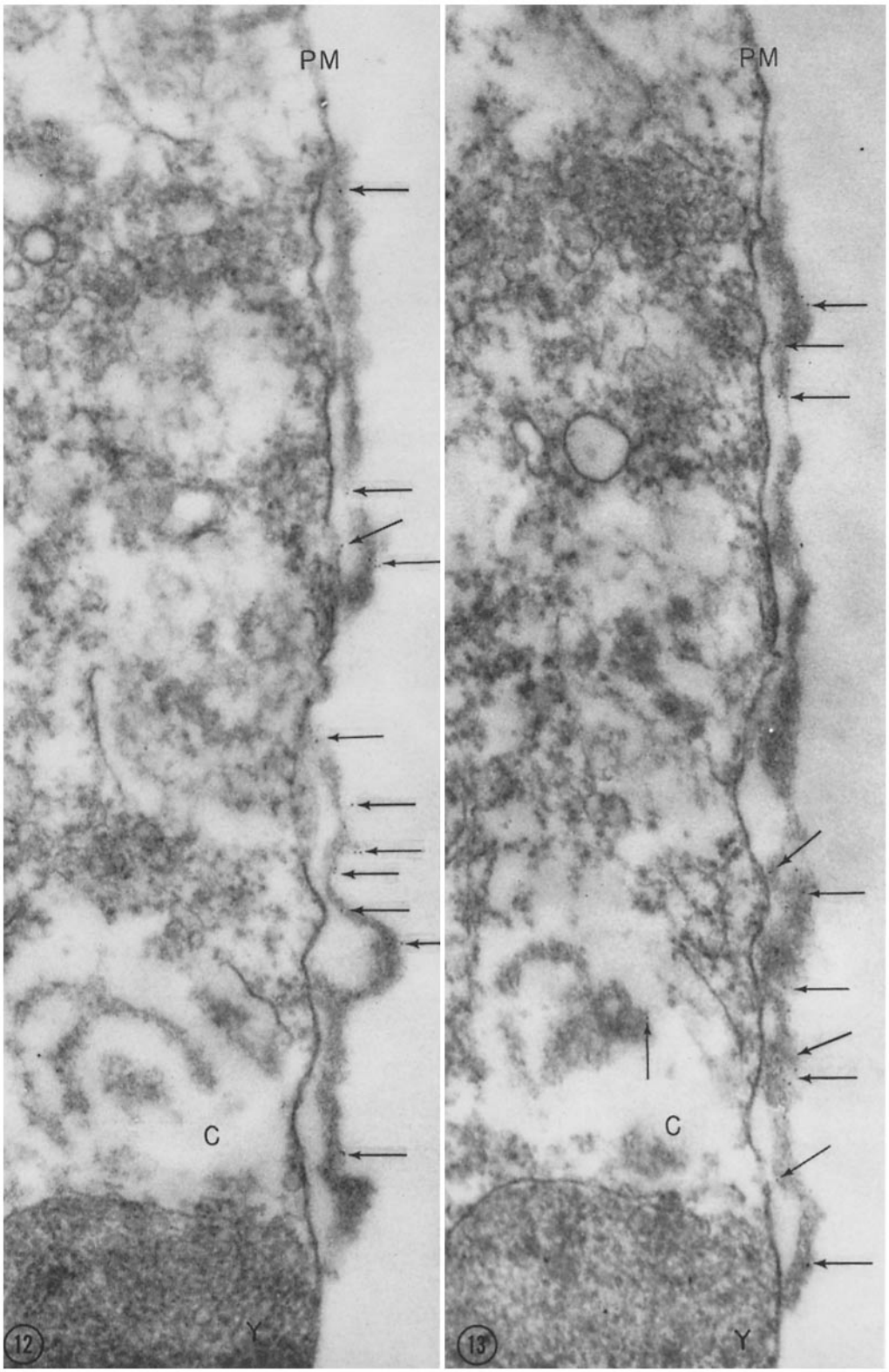
Pinocytosis

A discussion of the specificity of the technique cannot be dissociated from the problems of surface adsorption and pinocytosis when unfixed material is incubated with the immune conjugate. Although fixation prevents pinocytotic uptake of the ferritin conjugate, it is not always desirable to use fixed material, especially when considering factors, such as structural or physiological changes, other than the pure localisation of the antigen.

The probable first step in pinocytosis of soluble proteins is the formation of bonds between the protein and cell membrane (40, 41), but fluorescence microscope observations show that most of these bonds are easily broken, for example, by washing (40). In isolated Ehrlich ascites tumour cells incubated with I^{131}-serum albumin, however, a small residue was apparently irreversibly bound (42). In the present experiments with sea urchin eggs it appears that there was surface adsorption of the normal γ-globulin which also was not completely removed by the subsequent washing and embedding procedures.

Since more ferritin uptake has been observed with the immune than with the non-immune conjugate in unfixed Krebs ascites tumour cells (16), it has been suggested that the fixation of

FIGURES 12 and 13 Serial sections of an egg treated with antiegg γ-globulin (anti-total egg) absorbed with a lyophilised preparation of total heated jelly-free eggs (see Fig. 10). The ferritin label was added indirectly (F/G) in order to detect heat-labile antigens. The plasma membrane (*PM*) shows little change with very few surface papillae or ferritin molecules. The ferritin label (arrows) is present in the electron-opaque material outside the plasma membrane, as well as in some cortical granules (*C*). The yolk granule (*Y*) is in close contact with the plasma membrane. × 70,000.


PM
C
Y
12
PM
C
Y
13

immune γ-globulin to the cell surface gives a greater possibility for pinocytosis of the conjugate. In the present experiments, no significant difference in pinocytosed ferritin was observed in the cells after treatment with immune and non-immune globulin, respectively. However, these experiments were carried out in sea water (pH 7.8). This is a favourable medium for reducing the amount of protein-membrane binding due to molecular charge, since at low pH the protein binding to the cell surface, and consequently the amount of protein uptake, is increased (43). The importance of pH has also been demonstrated by fluorescence microscopy where non-specific staining is caused by protein-protein interaction between basic proteins of the cytoplasm and the labelled serum proteins carrying more negative charges due to the conjugation procedure (44).

In the sea urchin egg the amount of ferritin uptake by the yolk granules is much greater than by the pinocytotic vesicles, but ferritin is often found in circular or concentric arrangements in both these structures. Similar arrays can also be seen in micrographs of vesicles (possibly lysosomes) in unfixed Krebs ascites tumour cells treated with immune ferritin conjugate, but there the ferritin seems to be associated with membranes (see reference 16).

It is feasible that the ferritin uptake by the yolk granules is of some developmental and physiological importance, and that uptake of protein by the yolk granules (which can be seen close to the egg surface here) contributes towards their formation. Experiments on saturniid moth oocytes (45) which take up proteins from the blood have shown that the blood proteins are present in the yolk sphere and that they probably reach the oocyte by an intracellular route and are transformed into yolk spheres by a pinocytosis-like process. The behaviour of the sea urchin yolk granules towards ferritin and γ-globulin suggests that they may also be built up from extracellular proteins. Apparently, even in the mature egg the yolk granules continue this engulfment or pinocytosis when exposed to certain relatively concentrated protein solutions. The membrane bounding the granules is often seen to be broken, either owing to fixation (19) or for some metabolic or physiological reason (46, 47), thus releasing yolk both before and after the uptake of ferritin. Studies of oocytes of other species point to the intracellular synthesis of yolk proteins however (48, 49).

The Surface Layers

The Jelly Layer

Immunologically, the jelly layer can be divided into at least two components: one which interacts with and is precipitated by (5) the antijelly γ-globulin, and another which is susceptible to the anti-egg γ-globulin and may not be a permanent component of the jelly layer. After treatment with low concentrations of antijelly γ-globulin there are ferritin-labelled, electron-opaque aggregates of jelly substance throughout the layer, while after high concentrations there appear large, densely precipitated zones (notably on the inner and outer boundaries) with the ferritin label distributed more or less uniformly throughout, and other parts which appear devoid of jelly substance. These electron-translucent zones could either lack jelly substance completely or they could contain jelly which, because it has different properties, does not become precipitated or

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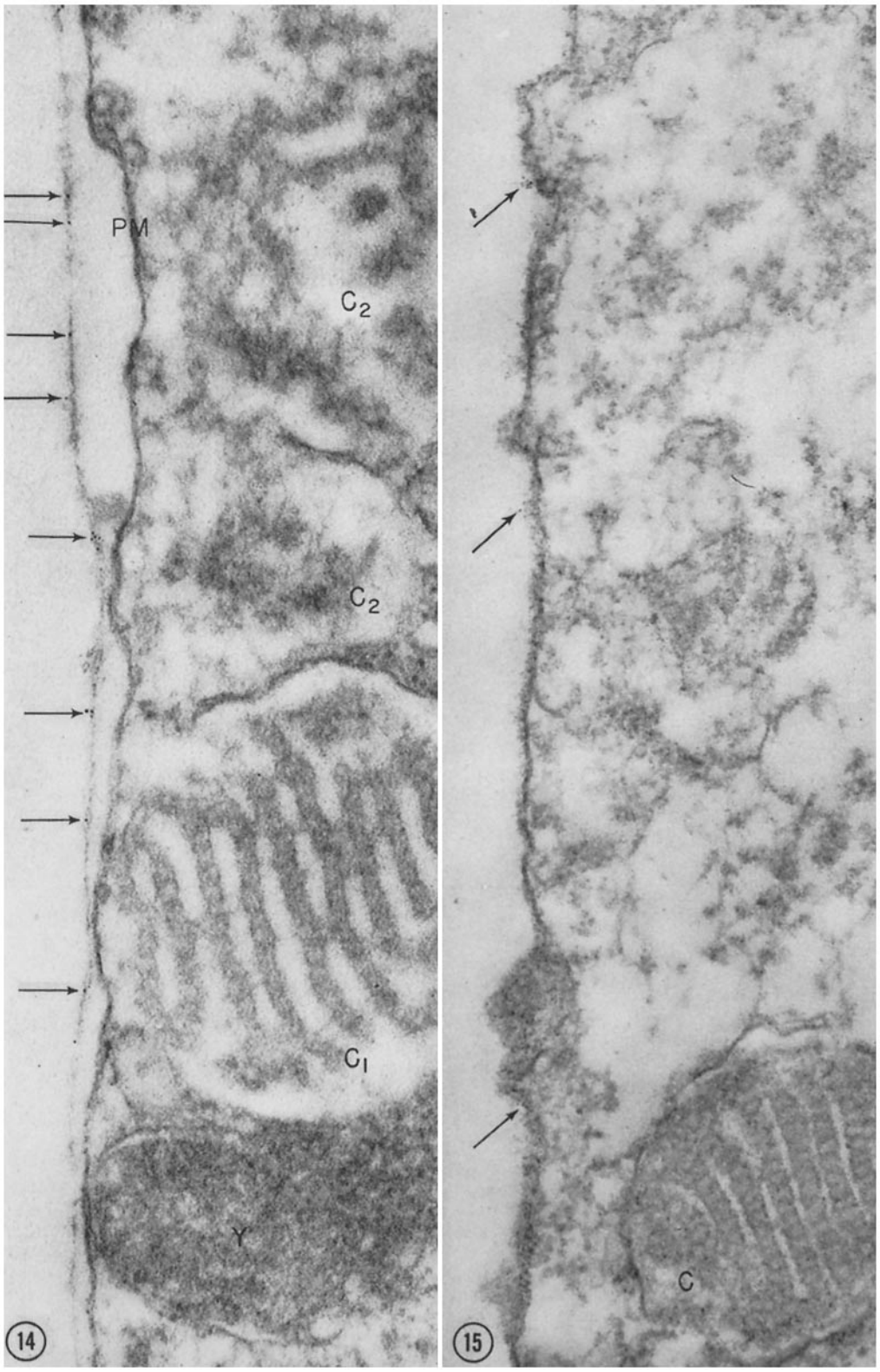
FIGURE 14 An egg treated first with antiegg γ-globulin absorbed with the lyophilised preparation of the soluble fraction of heated eggs, and secondly with F/G (see Fig. 11) in order to detect the heat-labile antigens and the heat-stable insoluble antigens. Outside the plasma membrane (*PM*) there is an elevated layer on which ferritin molecules can be seen (arrows). No surface papillae are seen. A cortical granule with the typical structure (C_1) and other less intact ones (C_2), and a yolk granule (Y) are close to the cell surface. The section was treated with 3 per cent hydrogen peroxide for 5 minutes in order to visualise the ferritin. × 75,000.

FIGURE 15 Eggs treated with γ-globulin from an antiserum against heated jelly-free eggs absorbed with a lyophilised preparation of the soluble fraction of heated eggs in order to localise heat-stable insoluble antigens. After treatment with this absorbed antiserum the eggs were washed and F/G was added. Ferritin can be seen occasionally along the egg surface (arrows). Cortical granule, (*C*). × 75,000.


PM
C2
C2
C1
Y
14
C
15

specifically labelled by the antijelly γ-globulin. The precipitable jelly substance often appears to be adhering to the outer surface of the vitelline membrane, but it has no association with the plasma membrane. There was no evidence for the presence in the jelly layer of micelles said to be fertilizin, *i.e.* jelly substance, bearing extensions of the plasma membrane (50).

The second component was found in the jelly layer only after treatment with γ-globulin from antiserum against total as well as heated homogenates of jellyless eggs. This electron-opaque material, which appears as if it had diffused from the egg and was subsequently retained by the jelly coat, must be derived from the egg, as it is specifically labelled by the antibodies against egg antigens. It not only is immunologically different from the jelly antigen seen after antijelly γ-globulin treatment, but it is also structurally different (5). One cannot tell whether it is normally present outside the egg and only made visible by the anti-egg antibodies, or whether it is produced by the eggs in response to the antibodies. Its absence in jelly-free eggs could either be due to its removal with the jelly substance, or to the lack of supporting medium to retain it once the jelly coat is removed.

The present electron microscope findings are compatible with previous light microscopical and immunochemical results which suggested that isolated jelly substance may contain varying amounts of the heat-stable egg antigen(s) involved in the activation reaction (A antigens) (3). Since the antiserum against heat-stable eggs was of a good parthenogenetic potency (5), the A antigen can be assumed to be among those egg antigens visualised with ferritin in the jelly layer. This also implies that the binding of the A antigen, or at least part of it, is rather labile and that it is easily released from the egg surface into the jelly layer. Variable ratios of different sugars, or of carbohydrate to nitrogen, as found in isolated jelly substances (3, 34, 51) would also favour this explanation (*cf.*, however, reference 52). It would also be compatible with the fact that jelly-free eggs are easier to activate (as well as to fertilise) by homologous antiserum treatment (2), since, if such large amounts of this diffusible antigen are present, the antibody molecules will have to combine with antigen before excess antibody is able to reach the actual cytoplasmic egg surface and to produce there the alterations involved in egg activation.

Although the egg antigens in the jelly seem to be mainly heat-stable, some heat-labile egg antigens may also be released since there is a difference between eggs treated with γ-globulin from anti-total and antiheated egg serum. In the former group there are localised regions of dense fibrous material adhering to the egg surface, whereas in the latter there is a layer of less dense amorphous material. However, since both the heat-stable and the heat-labile components probably are immunologically heterogeneous, and since the concentra-

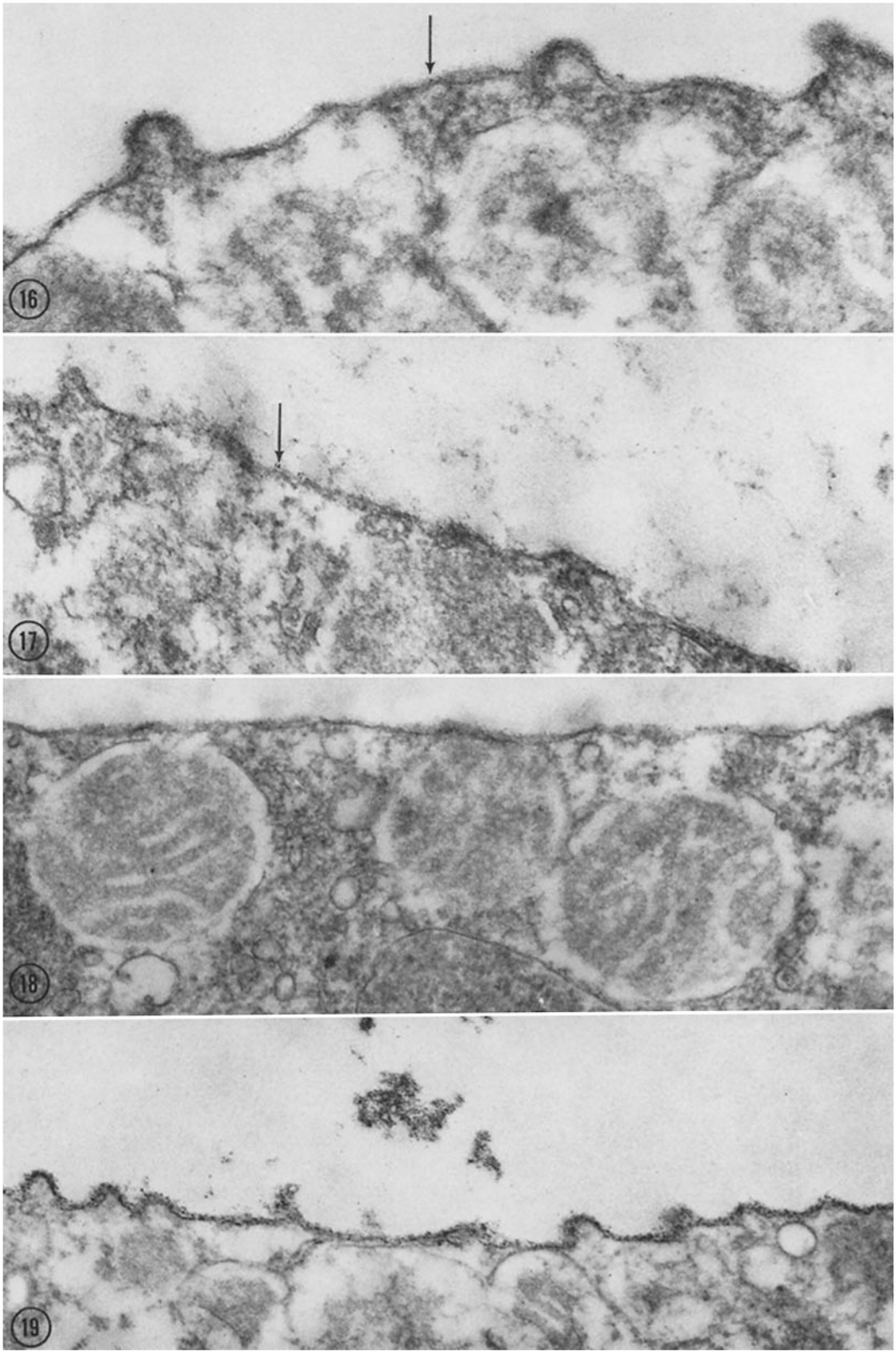
FIGURES 16 to 18 A series of control experiments in which treatment of the eggs with the absorbed antiserum described was followed by the F/G conjugate (two-layer method). These experiments act as a control both of the specificity of the antiserum and of the completeness of the absorption.

Fig. 16, Antijelly γ-globulin absorbed with lyophilised jelly substance. An occasional ferritin marker (arrow) can be detected on the cell surface, and there are a few small papillae. Compare with absorption with the egg preparation in Fig. 4. × 80,000.

Fig. 17, Anti-total egg serum absorbed with a lyophilised preparation of total jelly-free eggs. A few molecules of the ferritin label still remain (arrow), and amorphous material is also present outside the cell surface. × 62,000.

Fig. 18, γ-globulin from an antiserum against heated jelly-free eggs, absorbed with lyophilised total jelly-free eggs. Ferritin is not present on the cell surface. × 55,000.

FIGURE 19 An egg which was treated for 5 hours with 80 per cent solution of normal γ-globulin, washed with sea water, and treated with F/G. A relatively large amount of the ferritin conjugate remains on the surface, probably due to adsorption of the normal γ-globulin, and this is not removed by the usual amount of washing. There is no evidence of parthenogenetic activation however. × 48,000.


16
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18
19

tion of different antibodies may vary considerably in these two sera, the explanation of these differences will have to await further experiments.

The Vitelline Membrane

The vitelline membrane appears to be much more sensitive to the antiegg γ-globulins than the plasma membrane, in respect to both ferritin labelling and detectable structural alterations (5). The vitelline membrane is normally narrow and closely applied to the plasma membrane and it can only occasionally be distinguished by a slight separation at places on the egg surface. In eggs of *Limnea stagnalis* there is a distinct separation of these two membranes, however (53).

A large part of the antigens situated in the vitelline membrane must be heat-stable since both antiegg and antiheated-egg γ-globulin result in strong ferritin labelling. After treatment with both of these γ-globulin preparations, one can also see a dense layer adjacent to the plasma membrane. This layer may represent a thickening of the vitelline membrane or the deposition, secretion, or diffusion of some antigenic substance associated with it. In some treated eggs where this dense layer is not formed (either because of smaller γ-globulin concentrations or lower sensitivity of the eggs) the vitelline membrane can usually be distinguished from the plasma membrane, however (Fig. 7).

After treatment with the antiegg-γ-globulin which had been absorbed in order to detect (*a*) heat-labile antigens and (*b*) heat-stable insoluble antigens, the vitelline membrane became somewhat elevated and was separated from the plasma membrane over most of the egg surface (Figs. 11 and 14). The plasma membrane and cortical granules were virtually unaltered and the surface

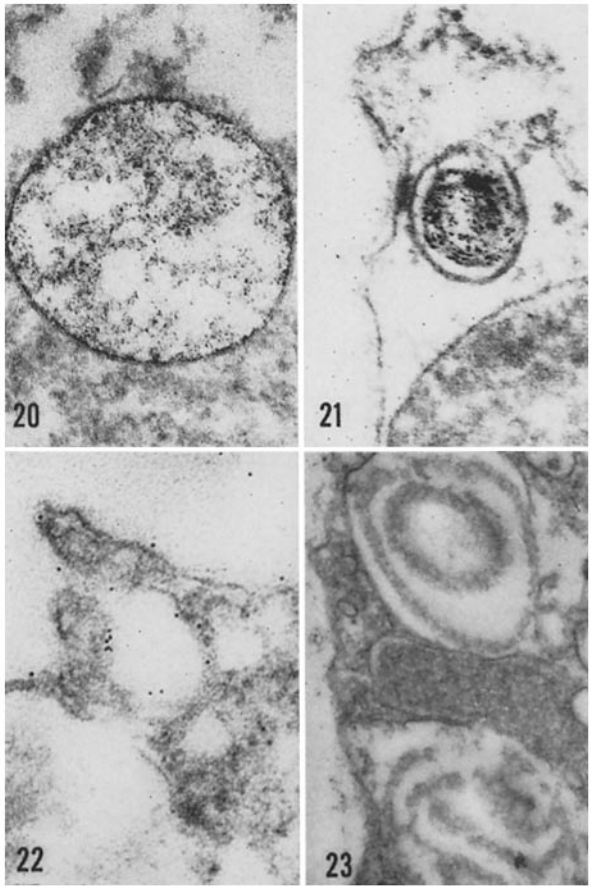

FIGURE 20 Treatment with the antijelly serum followed by F/G (XC) which had not been purified of the unconjugated components. The vesicle containing ferritin was found 1 μ below the egg surface immediately under the layer of cortical granules. × 70,000.

FIGURE 21 An egg after treatment with the unpurified ferritin-XC conjugate of the anti-heated eggs' γ-globulin. Close to the egg surface is a vesicle in which the pinocytosed ferritin molecules are in a concentric arrangement. × 80,000.

FIGURE 22 The egg surface when treated with the ferritin-XC conjugate of normal γ-globulin which had not been purified of free ferritin and free γ-globulin. Two elongated surface papillae are forming a pinocytotic vesicle to enclose part of the vitelline membrane on which many ferritin molecules have been adsorbed. × 80,000.

FIGURE 23 The surface of an egg treated with γ-globulin from an antiserum against heated jelly-free eggs, absorbed with lyophilised heated eggs, and then treated with F/G. The micrograph shows a yolk granule, between two cortical granules, which appears to have migrated towards the plasma membrane presumably to enable it to pinocytose γ-globulin and/or ferritin adsorbed to the egg surface. No ferritin label is visible on the surface. × 40,000.

of these eggs strongly resembled the transitional zone seen shortly (approximately 40 seconds) after sperm addition (54), there being separation of the two membranes but no breakdown of cortical granules. The separation of these two surface membranes could be either a specific reaction related to activation (and fertilisation), or a result of antiserum treatment leading to breakage of heat-labile, none of them involved in parthenogenetic activation. When the γ-globulin from the same antiserum was absorbed to detect the heat-labile components alone, the ferritin label was found only in limited regions in a discontinuous layer which also was thicker but less uniform (Figs. 10, 12, and 13) than that occurring when antibodies against both types of antigens were

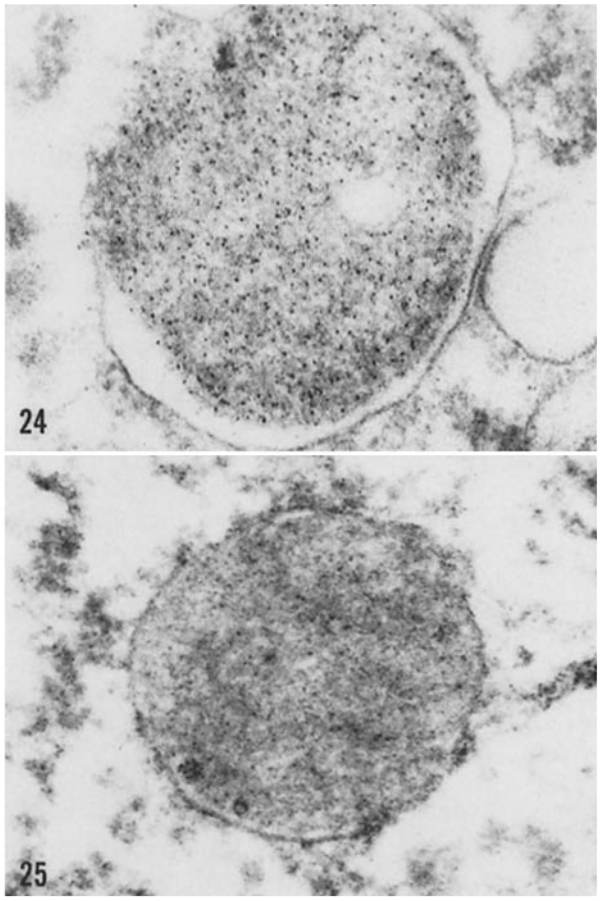

FIGURE 24 A yolk granule approximately 0.5 μ below the cell surface of an egg which had been treated first with the anti-total egg serum and then with unpurified F/G (XC). Ferritin is present throughout the actual yolk but is absent from areas within the yolk granule which do not contain the small yolk particles. × 80,000.

FIGURE 25 An area 0.6 μ below the periphery of an egg which had been treated with a concentrated solution of free ferritin for 15 minutes. The yolk granule contains ferritin molecules which are in a circular array at two places (lower left). × 88,000.

linkages holding the two membranes together (*cf.* reference 55).

In view of previous findings (1) and of the results obtained in this study, the activation antigen (A antigen) may be assumed to occur among the strongly ferritin-labelled antigens appearing in the vitelline membrane. The ferritin-labelled antigens remaining visible in the vitelline membrane after the above-mentioned absorption of the γ-globulins would seem to be immunologically distinct antigens, some insoluble and heat-stable and some present (Figs. 11 and 14). A definite vitelline membrane slightly separated from the plasma membrane could be distinguished only in regions where the dense layer was absent. However, the labelled material probably has a close association with the vitelline membrane and may even be derived from it. Whether this antigen is identical with the C antigen(s) leading to cortical damage when reacting with antibody for longer periods can not be decided from these experiments.

Heat-stable insoluble antigens alone could be

detected by absorption of the γ-globulin against heat-stable egg homogenates (Fig. 15). The different appearance of the cell surface and the weaker labelling in this case as compared to Figs. 11 and 14 may be due to a lower concentration of the relevant antibodies in this γ-globulin preparation.

The vitelline membrane plays an important role in fertilisation. The higher incidence of cross-fertilisation in eggs from which the vitelline membrane has been removed (56, 57) suggests that an important site of species specificity resides in this layer. It is likely that the antigens visualised in this membrane by means of ferritin-labelling also constitute specific sperm receptor sites. This concerns not only the heat-stable soluble antigens, including the A antigen, but also those which are insoluble and those which are heat-labile. That the latter antigens may participate in the fertilisation process is strongly suggested by previous studies which showed that antisera, although freed from antibodies against heat-stable soluble antigens by means of absorption, still reduced the fertilisability of the eggs (1, 4). The heat-stable insoluble antigen(s) involved in this reaction were called F antigen(s). Hence both the F antigen and the heat-labile C antigen (see above) could function as sperm receptors in the vitelline membrane. The depression of fertilisation rate after treatment with antiserum could then be due to the sites of sperm attachment being blocked or inactivated by the antibody molecules. Alternatively, since the fertilisation rate depression by antiserum was obtained under experimental conditions where the antibody/egg ratios were much lower than in the present study (100 to 200 times more eggs per milliliter of antiserum), there could be physiological or structural alteration in the egg surface, secondary to the antigen-antibody reaction, and resulting in reduced fertilisability. The γ-globulin–induced separation of the vitelline membrane from the plasma membrane, shown in Figs. 11 and 14, would seem to point in this direction.

Thus, the present results are compatible with the view that the reactive groups involved in fertilisation are part of the vitelline membrane. They are in contrast to a frequently given scheme of fertilisation according to the fertilizin theory (50), however, where the specific receptors in the egg are placed on the plasma membrane. In order to make this scheme feasible it is postulated that microvilli of the plasma membrane protrude through the vitelline membrane. At fertilisation, the tip of a microvillus, bearing fertilizin, would come in contact first with the sperm. Although the morphological aspects of this scheme may be valid for the surface layer of *Mytilus* eggs, where the vitelline coat is penetrated by such microvilli (58), no similar structural organisation has been observed in the sea urchin egg. Therefore, the sperm must encounter the vitelline membrane first, and it can be assumed that the specificity of sperm-egg attachment is determined by this layer. Ultrastructural studies on sperm penetration in *Saccoglossus* (59) also show very clearly that the sperm first comes in contact with the granular outer layer of the vitelline membrane and has to traverse the entire width of the vitelline membrane before encountering the plasma membrane.

Little is known about the structural properties and origin of the vitelline membrane. An analogy between the vitelline membrane and the basement membrane has many attractive features. The vitelline membrane of the sea urchin egg resembles a basement membrane in that it is a structureless layer of varying width (60), is less opaque than the plasma membrane (61), and is sometimes separated from the plasma membrane and sometimes adjacent to it (62). There are some reports of the basement membrane's having an inner structure though (63–65). If, as has been demonstrated recently in epithelial cells (65), the basement membrane is formed by cell secretion and not by condensation of ground substance, then, by analogy, the vitelline membrane might be formed by an egg secretion. Any analogy between the basement and vitelline membranes is important in considering the localisation of antigens on the plasma membrane or within the cell, since the basement membrane acts as a filtration barrier which does not normally allow the penetration of ferritin (66, 67).

The Plasma Membrane

The plasma membrane showed very little ferritin labelling with the γ-globulins used here. This may have been due to a genuine lack of antigens in this layer or to the inability of the conjugate molecule to penetrate the vitelline membrane (see preceding paragraph). The first possibility seems to be more likely since eggs from which the vitelline membrane had been removed with trypsin prior to addition of the ferritin conjugate showed no more labelling of the plasma membrane (unpublished observations). All the egg antigens tested

were apparently absent or rarely found in the plasma membrane, with the possible exception of heat-stable insoluble antigens which cannot be excluded. The only structural response of the plasma membrane to γ-globulin treatment was the formation of surface papillae.

The Egg Cortex

The experiments reported here were designed for the study of the surface layers, and little conclusive information has been obtained about intracellular antigens. The main site of specific ferritin labelling with the antiegg γ-globulin was in the cortical granules, both on the bounding membrane and in the lamellae, but not in the cortical hemispheres. Ferritin was also seen occasionally immediately below the plasma membrane.

The presence of ferritin label within the cortical granules is relevant to the properties of the plasma membrane which is generally impermeable to the conjugate molecule. It seems possible, from the structural changes occurring when the cortical granule open up at fertilisation (54, 68), that these granules are in reality outside the cell permeability barrier, and that the cortical granule bounding membrane is therefore homologous with the plasma membrane. The cortical granules are still within the vitelline membrane however and this, particularly if it is analogous with a basement membrane, is probably impermeable to the conjugate. This may be the reason why so little ferritin is found within the cortical granules.

It is not known whether or not the ferritin found within the cortical granules also marks the sites of the heat-labile C antigen as defined earlier (4). It can be expected that after interacting with antibody the contents of the cortical granules from unfertilised eggs may not be properly released, or, if they are extruded, they may be incapable of participating in the formation of the activation or fertilisation membrane. Hence, as has been seen previously (1, 5), eggs which are fertilised after antiserum treatment, or eggs activated immunologically, form a series of membrane blisters of different sizes instead of a completely elevated membrane. Further studies are needed to establish the immunological identity and precise localisation of the antigens participating in these reactions.

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REFERENCES

1. PERLMANN, P., *Experientia*, 1959, **15**, 41.
2. PERLMANN, P., *Exp. Cell Research*, 1957, **13**, 365.
3. PERLMANN, P., and PERLMANN, H., *Exp. Cell Research*, 1957, **13**, 454.
4. PERLMANN, P., and PERLMANN, H., *Exp. Cell Research*, 1957, **13**, 475.
5. BAXANDALL, J., PERLMANN, P., and AFZELIUS, B. A., *J. Cell Biol.*, 1964, **23**, 609.
6. SINGER, S. J., *Nature*, 1959, **183**, 1523.
7. RIFKIND, R. A., HSU, K. C., MORGAN, C., SEEGAL, B. C., KNOX, A. W., and ROSE, H. M., *Nature*, 1960, **187**, 1094.
8. MORGAN, C., HSU, K. C., RIFKIND, R. A., KNOX, A. W., and ROSE, H. M., *J. Exp. Med.*, 1961, **114**, 825.
9. MORGAN, C., HSU, K. C., RIFKIND, R. A., KNOX, A. W., and ROSE, H. M., *J. Exp. Med.*, 1961, **114**, 833.
10. MORGAN, C., HSU, K. C., and ROSE, H. M., *J. Exp. Med.*, 1962, **116**, 553.
11. LEE, S., *Exp. Cell Research*, 1960, **21**, 249.
12. SMITH, C. W., METZGER, J. F., ZACKS, S. I., KASE, A., *Proc. Soc. Exp. Biol. and Med.*, 1960, **104**, 336.
13. MOTT, M. R., *J. Roy. Micr. Soc.*, 1963, **81**, 159.
14. LUDVIK, J., SIIM, J. C., and BIRCH-ANDERSEN, A., *J. Ultrastruct. Research*, 1963, **9**, 399.
15. GOLDBERG, B., and GREEN, H., *J. Exp. Med.*, 1959, **109**, 505.
16. EASTON, J. M., GOLDBERG, B., GREEN, H., *J. Cell Biol.*, 1962, **12**, 437.
17. EASTON, J. M., GOLDBERG, B., and GREEN, H., *J. Exp. Med.*, 1962, **115**, 275.
18. RIFKIND, R. A., OSSERMAN, E. F., HSU, K. C., and MORGAN, C., *J. Exp. Med.*, 1962, **116**, 423.
19. BAXANDALL, J., PERLMANN, P., and AFZELIUS, B. A., *J. Cell Biol.*, 1962, **14**, 144.
20. BAXANDALL, J., PERLMANN, P., and AFZELIUS, B. A., *J. Roy. Micr. Soc.*, 1963, **81**, 155.
21. ANDRES, G. A., MORGAN, C., HSU, K. C., RIFKIND, R. A., and SEEGAL, B. C., *J. Exp. Med.*, 1962, **115**, 929.

22. STEINBERGER, L. A., DONATI, E. J., and WILSON, C. E., *Fed. Proc.*, 1962, **21,** 143.
23. PEPE, F. A., *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 515.
24. PEPE, F. A., and FINCK, H., *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 521.
25. PEPE, F. A., FINCK, H., and HOLTZER, H., *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 533.
26. ANDERSON, T. F., *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor) New York, Academic Press, Inc., 1962, **1,** 251.
27. ALMEIDA, J., CINADER, B., and HOWATSON, A., *J. Exp. Med.*, 1963, **118,** 327.
28. SOBER, H. A., and PETERSON, E. A., *Fed. Proc.*, 1958, **17,** 1116.
29. FAHEY, J. L., and HORBETT, A. P., *J. Biol. Chem.*, 1959, **234,** 2645.
30. SINGER, S. J., and SCHICK, A. F., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 519.
31. BOREK, F., and SILVERSTEIN, A. M., *J. Immunol.*, 1961, **87,** 555.
32. KOHN, J., *Nature*, 1959, **183,** 1055.
33. HARDING, C. V., and HARDING, D., *Exp. Cell Research*, 1952, **3,** 475.
34. VASSEUR, E., *Acta Chem. Scand.*, 1948, **2,** 900.
35. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
36. MARINOZZI, V., and GAUTIER, A., *Compt. rend Acad. Sc.*, 1961, **253,** 1180.
37. HALL, C. E., NISONOFF, A., and SLAYTER, M. S., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 407.
38. FARRANT, J. L., *Biochim. et Biophysica Acta*, 1954, **13,** 569.
39. SMITH, C. W., METZGER, J. F., and HOGMAN, M. D., *Am. J. Clin. Path.*, 1962, **38,** 26.
40. MARSHALL, J. M., SCHUMAKER, V. N., and BRANDT, P. W., *Ann. New York Acad. Sc.*, 1959, **78,** 515.
41. BENNETT, H. S., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4 suppl., 99.
42. RYSER, H., AUB, J. C., and CAULFIELD, J. B., *J. Cell Biol.*, 1962, **15,** 437.
43. BRANDT, P. W., and PAPPAS, G. D., *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 675.
44. LOUIS, C. J., *Brit. J. Cancer*, 1958, **12,** 537.
45. TELFER, W. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 747.
46. WOLPERT, L., and MERCER, E. H., *Exp. Cell Research*, 1963, **30,** 280.
47. ELBERS, P. F., and BLUEMINK, J. G., *Exp. Cell Research*, 1960, **21,** 619.
48. WARD, R. T., *J. Cell Biol.*, 1962, **14,** 309.
49. BEAMS, H. W., and KESSEL, R. G., *J. Cell Biol.*, 1963, **18,** 621.
50. TYLER, A., *Am. Zoologist*, 1963, **3,** 109.
51. VASSEUR, E., and IMMERS, J., *Arkiv Kemi*, 1950, **1,** 39.
52. MINGANTI, A., and VASSEUR, E., *Acta Embryol. et Morphol. Exp.*, 1959, **2,** 195.
53. ELBERS, P. F., Doctoral thesis, Utrecht 1959—Over de Beginoorzaak van het Li-effect in de Morphogenese.
54. WOLPERT, L., and MERCER, E. H., *Exp. Cell Research*, 1961, **22,** 45.
55. ISAKA, S., and AIKAWA, T., *Exp. Cell Research*, 1963, **30,** 139.
56. HULTIN, T., *Arkiv Zool.*, 1948, **40A,** No. 12.
57. BOHUS-JENSEN, A., *Exp. Cell Research*, 1953, **5,** 325.
58. DAN, J. C., *Biol. Bull.*, 1962, **123,** 531.
59. COLWIN, A. L., and COLWIN, L. H., *J. Cell Biol.*, 1963, **19,** 477.
60. RHODIN, J., and DALHAMN, T., *Z. Zellforsch. u. mikr. Anat.*, 1956, **44,** 345.
61. ZETTERQVIST, H., Doctoral Thesis, Stockholm 1956. The Ultrastructural Organization of the Columnar Absorbing Cells of the Mouse Jejunum.
62. RHODIN, J., *Physiol. Rev.*, 1962, **42,** suppl. 5, 48.
63. BARGMANN, W., KNOOP, A., and SCHIEBLER, T. H., *Z. Zellforsch. u. mikr. Anat.*, 1955, **42,** 386.
64. JAKUS, M. A., *Am. J. Ophthalmol.*, 1954, **38,** 40.
65. PIERCE, G. B., MIDGLEY, A. R., and SRI RAM, J., *J. Exp. Med.*, 1963, **117,** 339.
66. FARQUHAR, M. G., and PALADE, G. E., *J. Exp. Med.*, 1961, **114,** 699.
67. BRANDT, P. W., *Circulation*, 1962, **26,** 1075.
68. ENDO, Y., *Exp. Cell Research*, 1961, **25,** 383.