ALTERATIONS IN THE PROTEINS OF SEA URCHIN EGG HOMOGENATES TREATED WITH CALCIUM [1]

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The complex of processes involved in the occurrence of a precipitation reaction at the surface of exposed cytoplasm was named the "surface precipitation reaction" by Heilbrunn (1927). A survey of the properties of this reaction in a variety of forms is given by Costello (1932), and a related type of process in muscle fibers is described by Woodward (1948). The most recent treatment of the reaction (henceforth abbreviated SPR), including discussion of its physiological significance, is given in Heilbrunn's (1952) textbook.

The egg of the sea urchin *Arbacia punctulata* provides excellent material for the observation of the SPR. The egg is crushed in sea water, usually between a glass slide and a coverslip. Ordinarily, the egg breaks at one or more points, and the cytoplasm flows out at the site(s) of rupture. The outward movement of the exovates is halted by formation of a precipitation film, and this is usually accompanied by extensive vacuolization within the exovate mass. The Arbacia egg possesses numerous pigment-containing granules (really vacuoles), and these break down in the exovate. The granule lysis and vacuolization reactions are sometimes observed to travel wave-like across the entire cell.

The calcium ions of the sea water are responsible for the initiation of the SPR. If the calcium ions are removed by precipitation with oxalate or by complexing with citrate, the SPR does not occur, and instead, the cytoplasm of crushed eggs is rapidly and uniformly dispersed throughout the volume of medium available to it. This is true of the SPR in all forms studied, from the protozoan Stentor to the egg of the frog (Heilbrunn, 1952).

Heilbrunn (1952) has proposed a theory which attempts to explain such diverse phenomena as stimulation, narcosis, and cell division on the basis of changes in the state of the protoplasmic colloid. Thus, for example, cell division is always preceded by a gelation reaction, the so-called "mitotic gelation."

This physiological gelation process appears to be the analogue of the SPR described above. Indeed, Heilbrunn has proposed that the mitotic gelation is an "internal SPR," differing from the true SPR only in a lesser extent of reaction. Many of the events which accompany the viscosity increase of the cytoplasm in

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living cells do, in fact, have their striking counterparts in the events of the SPR (Heilbrunn, 1928).

Hultin (1950a, 1950b), impressed by the possible important role of calcium in fertilization and in stimulation in general, studied the effects of the addition of Ca^{++} to homogenates of sea urchin eggs. The innovation of his method was the use of unfertilized eggs which had been freed, prior to their homogenization, of practically all of their ionic Ca. When Hultin added Ca in small quantities to such homogenates, several events were observed: a burst of oxygen uptake in great excess of uptake in the controls, the formation of acid, a sharp increase in the viscosity of the homogenate, and the breakdown of certain cytoplasmic inclusions. These events bear a suggestive resemblance to those which take place at fertilization (Runnström, 1951).

The experiment just described is, however, also a model of the SPR. Indeed, Hultin's preparation *is*, in a sense, the SPR, with the modifying condition that the rupture of the cells is temporally separated from the influx of calcium ions. Certainly, if the events enumerated above suggest those at fertilization, they duplicate the events of the SPR with even greater fidelity.

The experiments of Hultin therefore take on great interest in relation to all phenomena in which the "internal SPR" of cytoplasmic "gelation" are supposed to play a role. Now the SPR obviously involves the formation of an insoluble precipitation membrane (Heilbrunn, 1928), and is likely to be causally connected with a drop in the solubility of some cytoplasmic proteins. The formation of a fibrous gel, such as Hultin (1950b) and Gross (1952) describe would be difficult to account for in another way. Hultin was, however, unable to demonstrate any protein solubility changes as a result of addition of Ca.

It is obviously important to discover whether alterations in the solubility of cytoplasmic proteins do or do not in fact occur. Further, if such alterations can be demonstrated, then it is important that the reaction or reactions leading to this effect be analyzed at the molecular level. Such an analysis would serve to throw light upon the as yet obscure mechanism of the SPR and, perhaps, by extension, upon the processes of gelation and solation which are universal manifestations of vital activity at the cellular level.

This report describes experiments whose purposes relate to this analysis.

I wish to express my sincere gratitude to Dr. L. V. Heilbrunn for his assistance and encouragement during the course of this work.

The electron micrographs were made at the Electron Microscope Laboratory, Marine Biological Laboratory, by Mr. Delbert E. Philpott.

Materials and Methods

Eggs were obtained from female *Arbacia punctulata*. Samples from the pooled batches were tested for fertilizability and poor collections were discarded. The animals were usually injected in the oral region with 0.1–0.5 ml. of isotonic KCl. This treatment induced shedding of the ripe eggs but appeared not to affect immature females. The eggs were collected from six to ten females and quickly washed twice by centrifugation through filtered sea water in order to remove debris. A wash in sea water at pH 5.0 now served to loosen or dissolve

the jelly coats, and these were carried away in the subsequent washings in Ca-free media. Washing by centrifugation and decantation five to eight times in Ca-Mg-free artificial sea water was followed by two washes in 0.35 *M* sodium citrate.

The eggs were now extremely fragile and were easily homogenized after centrifugal collection. Prior to this step, however, the eggs were cooled to 2° C. or below. The homogenization was carried out in a pre-cooled Potter-Elvejhem type of glass homogenizer. A few strokes of the tight-fitting pestle were sufficient. The process described resulted in homogenates of uniform consistency, with few if any intact cells.

As pointed out by Harris (1943), procedures of this type result in the rupture of a certain small percentage of the pigment vacuoles, although most of these bodies are resistant enough to withstand breakage in the course of homogenization. The homogenate was usually prepared with one part of packed eggs and one to ten parts of homogenization medium, this last being isotonic KCl, KCl with buffer (veronal-acetate-HCl), or Ca-Mg-free artificial sea water. The homogenates were strongly self-buffering at a pH of 6.5–6.9.

The details of the several experimental techniques are given in the appropriate sections of the experimental part of this report.

Experiments and Results

1. *Ionic specificity*

The first problem which arises in connection with the striking changes caused by the addition of Ca to the homogenates is that of ionic specificity. Since Hultin (1950b) was able to mimic several of the Ca effects by means of strongly hypertonic salt solutions, the possibility arises that the effects of Ca might be attributed simply to the increased ionic strength of its "isotonic" solutions as compared with that of isotonic solutions of uni-univalent salts. Thus, an isotonic solution of $CaCl_2$ (0.32 *M*) has an ionic strength of 0.96, given by the relation

$$\mu = 1/2 \sum_i c_i z_i^2$$

where μ is called the ionic strength, and the computation consists of summing the products, for each ionic species, of c, the concentration in moles, and z^2, the ionic charge [squared], and dividing the sum over all species by two. By comparison, an isotonic solution of KCl (0.48 *M*) has an ionic strength of 0.48. Since ionic strength, and not molarity, is the value of interest in kinetic salt effects, the above possibility must be investigated.

An aliquot of a homogenate was treated with Ca and a control aliquot was treated with a solution of KCl of identical ionic strength. Thus, 0.1 ml. of 0.32 *M* $CaCl_2$ was added to ten ml. of homogenate and 0.1 ml. of 0.96 *M* KCl was added to the control sample (10 ml.).

The control homogenate retained its normal appearance, whereas the material in the experimental tube underwent, within fifteen minutes, a marked precipitation reaction, as a result of which the viscosity of the homogenate (as a whole) rose. The Ca-treated homogenate formed fiber-like aggregates which adhered to the sides of the test-tube. The granule lysis reaction was evident, and the homogenate

underwent a color change from orange-red to a deep wine-red. As will be shown below, the color change could be prevented by strong additional buffering of the homogenate at pH 7.0.

2. Solubility changes

Hultin (1950b) was unable to observe any alterations in the protein solubility of his homogenates as a result of Ca-treatment. He assumed that such changes do occur, but that the eventual loss of protein from the soluble fraction of the homogenate was balanced and even over-compensated by the release of trichloroacetic acid-insoluble N from the cytoplasmic inclusions which lyse in the presence of Ca.

There is, however, a simple method by which the difficulties caused by granule lysis can be overcome without removing the granules during the reaction period (this latter step constituting a partial fractionation of the homogenate which is not desired at this stage of the investigation). Harris (1943) showed that the pigment granules of the sea urchin egg behave osmotically. Now it is these granules, or vacuoles, which contribute the major quantity of trichloroacetic acid- (henceforth "TCA") insoluble N to the homogenate upon their lysis. Thus, if the granules could be osmotically ruptured in *controls as well* as in Ca-treated homogenates, the phenomenon of granule lysis should have its effect cancelled, and solubility changes in the initially soluble cytoplasmic proteins alone should be measurable.

It is possible to produce osmotic lysis of over 85% of the pigment granules by the addition of water to the homogenate *after* the treatment under investigation. In this way, the effects of Ca^{++} upon the soluble proteins of the homogenate can be studied.

The same basic experimental design was used in all of the solubility experiments. Homogenates were treated with the agent suspected of effecting a solubility change in the proteins. Controls were treated with uni-univalent salt solutions with ionic strengths equal to those obtaining in the experimentals. The tubes containing the homogenates were incubated, either at 2° C. or at 21° C., for various periods. At the end of the incubation period, the homogenates were treated with twice their volume of ice-cold distilled water. This brought about the desired lysis of remaining pigment granules. The homogenates were now diluted with (usually) ten volumes of ice-cold distilled water or *M* KCl, buffered in some cases to pH 7.0. Extraction was carried out in the icebox at 1° C. for one to twenty-four hours. At these low temperatures and relatively great dilutions, the aggregation reactions initiated by Ca were brought to a practical halt.

At the end of the extraction period, the tubes were spun in a Servall angle-head centrifuge at 17,000 to 20,000 g. The clear supernatants were analyzed for their total protein content by one of several methods. The experiments were thus set up to reveal differences in solubility or, more precisely, in sedimentability, the latter being a function of the relative states of aggregation of the soluble or suspended proteins.

For the comparative results required, a quantitative modification of the biuret test, slightly modified from Fine (1935), was found adequate. The results were reproducible, and the slight turbidity which tends to develop at the higher protein

concentrations can often be avoided by suitable dilution and temperature control. Only measurements obtained upon visually non-turbid systems were admitted.

Most of the experiments were repeated with analysis by spectrophotometry. Aside from offering a means of estimating total protein, the absorption spectra of the water extracts provide information concerning the relative distributions of protein and nucleic acid in experimentals and controls.

When the experiment described was performed, there was always a measurable precipitation of protein or protein containing material from the homogenates which had been treated with Ca. At least, at every given relative centrifugal force, more protein was sedimented in the experimentals than was in the controls. When the homogenates were relatively concentrated, that is, when the dilution with homogenization medium was less than five times, the reaction was very rapid at 21° C., although not "instantaneous" in the way that ionic precipitation reactions often are at room temperature.

Depending upon the conditions obtaining, from zero to ninety per cent of the total protein extractable from the whole homogenates by water could be precipitated. Actually, the extraction medium is a very dilute salt solution, since the homogenate itself contains considerable salt.

For reasonable reaction velocities at 21° C., concentrations of Ca^{++} in the range of 0.001 to 0.01 were required. The significance of these levels will be discussed below.

The results suggest that the rate, but not the final extent of solubility loss is dependent upon the concentration of Ca; but this effect could not be noted if dilutions were too great, since the over-all velocity of precipitation is very sensitive to dilution of the homogenate. Indeed, homogenates made with one part of eggs to more than ten parts of medium often failed to give a measureable rate of precipitation in the presence of 0.01 *M* $CaCl_2$, although other Ca effects, such as granule lysis, were observed.

In many experiments where the reaction was permitted to proceed only a short time after treatment with Ca^{++} and a wide range of Ca concentrations was added, the following phenomenon was observed. Plots of solubility loss as a function of Ca^{++} added were often not simple, concave-downward curves, but rather, showed a maximum at about 0.5–1.0 millimols/ liter, followed by a local minimum and finally by steadily increasing values asymptotic with approximately 90% precipitation. The amount of protein lost from the homogenate at the low concentration maxima, when these occurred, was of the order of 20–30% of the total protein extractable with water from the controls.

This is demonstrated by the data in Table I, which shows the results of two typical experiments carried out in successive summers at Woods Hole. Both homogenate preparations were made from 1 : 6 homogenates, *i.e.,* one part of packed eggs homogenized in five parts of isotonic KCl. The considerable variation from one homogenate preparation to another is illustrated by the two sets of data, as are the general characteristics of the relation between solubility loss (or increased sedimentability) and the concentration of added Ca^{++}.

Spectrophotometric methods corroborated the results of the chemical determinations. The water extracts of experimental and control homogenates were centrifuged at 17,000 g for 10 minutes to one hour in the cold, thus brought to water-clarity, and then diluted and read directly in the Beckman spectrophotometer.

TABLE I

Loss in the water solubility of homogenate proteins in the presence of varying amounts of calcium ions. Two separate experiments

Experiment number	Concentration of Ca in mM/liter	Per cent loss in solubility
1A	0.00	0.00
1B	0.57	38.8
1C	5.70	33.4
1D	57.0	72.2
1E	113	81.5
1F	170	94.4
2A	0.00	0.00
2B	1.00	19.0
2C	5.00	14.8
2D	10.0	37.0
2E	100	91.6
2F	500	90.5

Complete absorption spectra in the wave-length region 220 to 300 millimicrons were made for the experimentals and for the controls. The optical density at wave-length 280 millimicrons is a measure of the protein (and aromatic amino acid) content of the sample. The effects of the presence of nucleic acid and of scattering do not introduce a serious error in comparative measurements such as were made here.

In every case, a loss in protein from the Ca-treated homogenate occurred, and was indicated by a reduced optical density at 280 mμ. The absorption spectra had interesting features aside from the changes in density at 280. Figure 1 illustrates the result of a typical experiment. Here, a 1 : 5 homogenate was treated with 0.03 *M* $CaCl_2$ and the control was treated with a solution of KCl of equal ionic strength. After five minutes of incubation at 21° C., each tube was diluted with five volumes of ice-cold distilled water. The extraction was continued in the ice-box for four hours. At the end of this period, both tubes were centrifuged at 17,000 g for ten minutes. The clear supernatants were removed and further diluted with water buffered to pH 8.5 with bicarbonate. Absorption spectra were made in the ultraviolet region.

The upper curve (half circles) in the figure represents the absorption spectrum of the control extract, while the lower curve (solid circles) is that of the experimental. In both cases, the spectrum is that of nucleic acid plus protein, with a peak at 260 millimicrons and an appreciable absorption at 280 millimicrons. Obviously, an over-all loss of water-soluble material has occurred in the experimental system. In addition, the shapes of the curves suggest that the composition of the precipitated material differs slightly from that of the supernatant as a whole, as represented by the upper curve.

The ratio of the molar extinction at 280 mμ to that at 260 mμ has a characteristic magnitude for most proteins and a different, but equally characteristic, magnitude for nucleic acids. Thus, for PNA, this value is in the neighborhood of 0.5, whereas for protein alone it is more likely to have a value near 1.75.[3]

[3] This is not the case for proteins of unusual amino acid composition, such as, for example, protamine.

Warburg and Christian (1942) used these facts as the basis of a method of estimating the relative quantities of protein and nucleic acid in a mixture of the two.

Optical density, the quantity measured in these experiments, is given by the relation

$$D = kcd$$

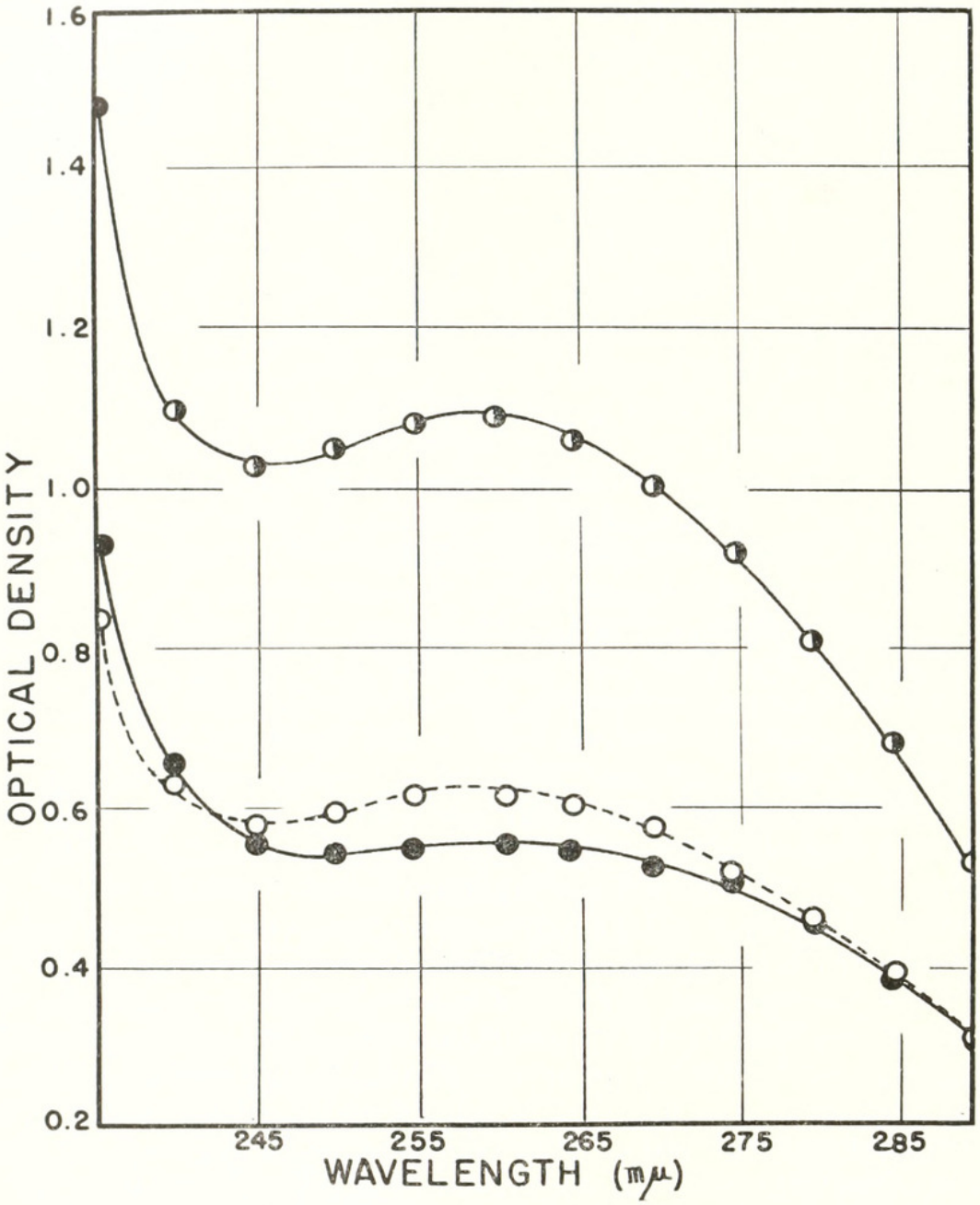

FIGURE 1. Ultraviolet absorption spectra of water extracts of sea urchin egg homogenates. Half circles: control. Full circles: experimental, treated with Ca^{++}. Open circles: calculated curve for experimental. See text.

where D is the optical density, k is a specific extinction coefficient, c is the concentration of absorbing substance, and d is the length of the light path through the sample. The extinction ratio mentioned above is readily obtained from the measured "D" values. For a single sample, the optical density will differ at the two wave-lengths, but the concentration of chromophore and the light path

do not change and are therefore constants, so that

$$\frac{D_{280}}{D_{260}} = \frac{k_{280}}{k_{260}},$$

which is the desired ratio.

The value of this ratio is 0.74 for the upper (control) curve and 0.83 for the experimental curve in Figure 1. This is a significant difference, and indicates that the lower curve represents a chromophore which is relatively poorer in nucleic acid than is the control. But the spectra have been recorded for water extracts, and thus the insoluble, aggregated material in the experimental homogenate must be *richer* in nucleic acid than that in the control.

It is possible, by preparations of several dilutions of the control, to obtain mean values of k (independent of concentration) at the several wave-lengths. These values are multiplied by $d(= 0.996$ cm.) and by "c," which is now that fraction of the absorption (at 280 millimicrons) of the control shown by the experimental. This is equivalent to assuming that the differences between the control and the experimental absorption spectra can be accounted for on the basis of the changes indicated by the 280 mμ absorptions. The product $(k \cdot c \cdot d)$ gives a series of theoretical values for D, and these values are plotted for purposes of comparison with the experimental absorption spectrum. Such a calculated curve is shown by the open circles in Figure 1.

Although, due to the large absorption by nucleic acids even at 280 millimicrons, this type of manipulation can give no quantitative data on the distribution of nucleic acid and protein in the system, it does qualitatively indicate that the experimental water extract is relatively poorer in a component absorbing strongly at 260 mμ than the control extract.

Finally, a preliminary localization of the reaction was attempted in the following way:

A homogenate was centrifuged in the cold at 2000 g for 20 minutes. This deposited a layer of red granules, and no red pigment was left in the supernatant fraction. Treatment of these supernatants was the same as that for whole homogenates.

In every case, the addition of calcium to this supernatant layer brings about a solubility loss on the part of protein components. Figure 2 gives the spectrophotometric analysis of one such experiment, "c" represents the water extract of the control, and "x" that of the Ca-treated supernatant.

Plainly, the material which becomes insoluble in the whole homogenate comes, at least in part, from the fraction devoid of the largest granules.

3. Acid formation

The appearance of acid upon fertilization of the egg of *Paracentrotus lividus* was first reported by Runnström (1933), and this was confirmed for *Psammechinus miliaris* by Borei (1933) and by Laser and Rothschild (1939). These observations led Hultin (1950b) to test for the appearance of acid upon the addition of Ca to his homogenates. Hultin was successful in demonstrating this effect.

The acid formation has considerable significance for the SPR, and accordingly an attempt was made to measure it in our own material.

A homogenate was prepared in the usual way, and then frozen in dry-ice and acetone and dried *in vacuo*. Later, this material was reconstituted with ice-cold distilled water. This material was self-buffered at pH 6.55, as measured with the glass electrode. When the homogenate was brought to 21° C. and treated with $CaCl_2$ (final concentration = 0.005 *M*) the material changed color from brown to deep red within a few minutes. The pH of the system fell from 6.55 to 5.50

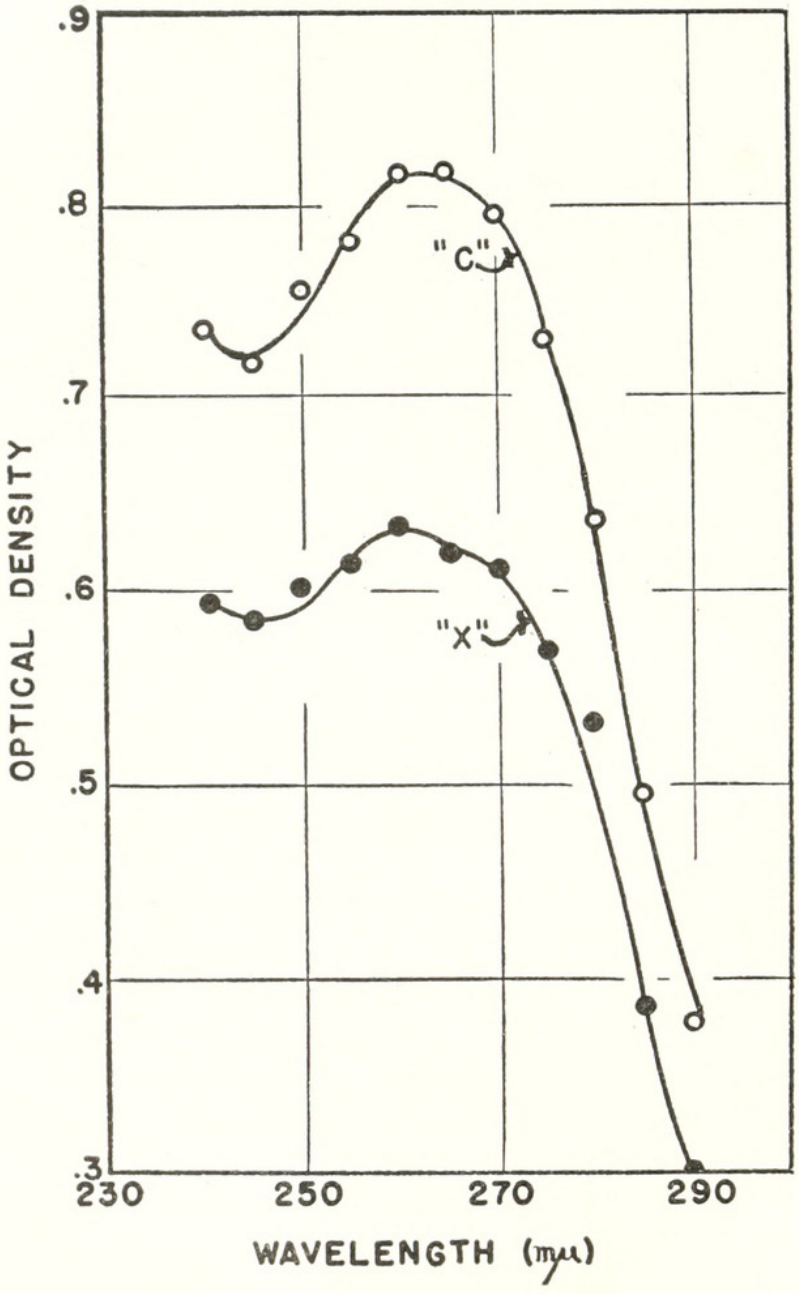

FIGURE 2. Ultraviolet absorption spectra of water extracts of homogenates free of pigment granules. "c" = control. "x" = Ca^{++} — treated experimental.

within 90 seconds. It is easily calculated that, even with buffering neglected, this large release of H^+ cannot be accounted for on the basis of the slightly acid pH of the added $CaCl_2$.

The data indicate that even in the presence of the considerable buffer capacity of the homogenate proteins, etc., the hydrogen ion concentration increased more

than ten times within the first 1.5 minutes after the addition of Ca. The acid formation must thus be of considerable magnitude. In the light of these observations, it is important to ask whether the change in pH is not the factor responsible for the precipitation of protein by Ca.

That this is not the case is easily demonstrated in experiments in which the homogenates are strongly buffered at pH 7. In these systems, the color change does not take place and, of course, the pH remains close to 7, but the loss of protein solubility under the influence of Ca is unaffected.

The matter of color change is of interest, because in the SPR as observed in crushed eggs, the breakdown of the pigment granules is accompanied by a pronounced reddening of the protoplasm of the exovate and often of the whole cell, as the wave of granule lysis sweeps across it. This change in the pigment (echinochrome) released from the vacuoles is particularly striking when eggs are crushed in media somewhat richer in Ca than is sea water. This effect is undoubtedly due to the change in pH of the regions of the cell effected by the SPR.

Two homogenates were prepared and treated in the usual way, except that one was strongly buffered at pH 7.15 with tris-(hydroxymethyl)-aminomethane while the other was unbuffered except by its own ampholytes. Both were treated with the same quantity of Ca^{++} (0.005 M) and incubated for a few minutes. The precipitation reaction took place to approximately the same extent in each. Both tubes were centrifuged to sediment the solids, leaving faintly turbid supernatants. These were diluted somewhat and their pH values measured once again with the glass electrode. Absorption spectra of the two preparations were made in the visible region of the spectrum.

The results of the spectrophotometric measurements are plotted in Figure 3. Clearly, the system whose pH was 6.5 was red, while the one with pH = 7.15 was yellow-orange. This shift in absorption is, as closely as it is possible to judge, the same type as that which occurs when the SPR is observed in cells. These color changes are, of course, due to the presence of the pigment echinochrome, which is a pH indicator, apparently even when in combination with its protein ligand in the homogenate or in the egg.

4. Release of non-protein nitrogen

Woodward (1949) reported experiments in which various fractions of Arbacia egg granules were assayed for proteolytic activity. He found low but significant proteolytic activity, increasing from 35 to 50 per cent upon addition of cyanide, in the "granular" fraction of the eggs. Ca-activation of the protease(s) was not demonstrated.

Gross (1952) reported in a note that the whole homogenate of Arbacia eggs contained a Ca-activated protease, with a pH optimum near 6.6. Lundblad (1952), in the course of his extensive investigations upon the proteolytic activity of sea urchin gametes, reported that the weak proteolytic activity of extracts and homogenates of unfertilized eggs was increased by Ca.

Because of the possibility of a relation between proteolysis and coagulation, and perhaps precipitation, of proteins in the homogenates, it seemed worthwhile to attempt a further step in the analysis. Lundblad's assays of protease activity were made by a viscosimetric method. In the light of many investigations such

as those of Örström (1941), demonstrating changes in the non-protein nitrogen (NPN) of eggs after fertilization, it seemed further indicated that one should determine whether NPN increased directly after the addition of Ca to the homogenates. The method used by Lorand (1952) in his studies on fibrinogen was adapted for use in the homogenate system.

Homogenates were prepared in the usual way. Calcium was added in amounts sufficient to give a reasonable rate of precipitation. At intervals, one of the several

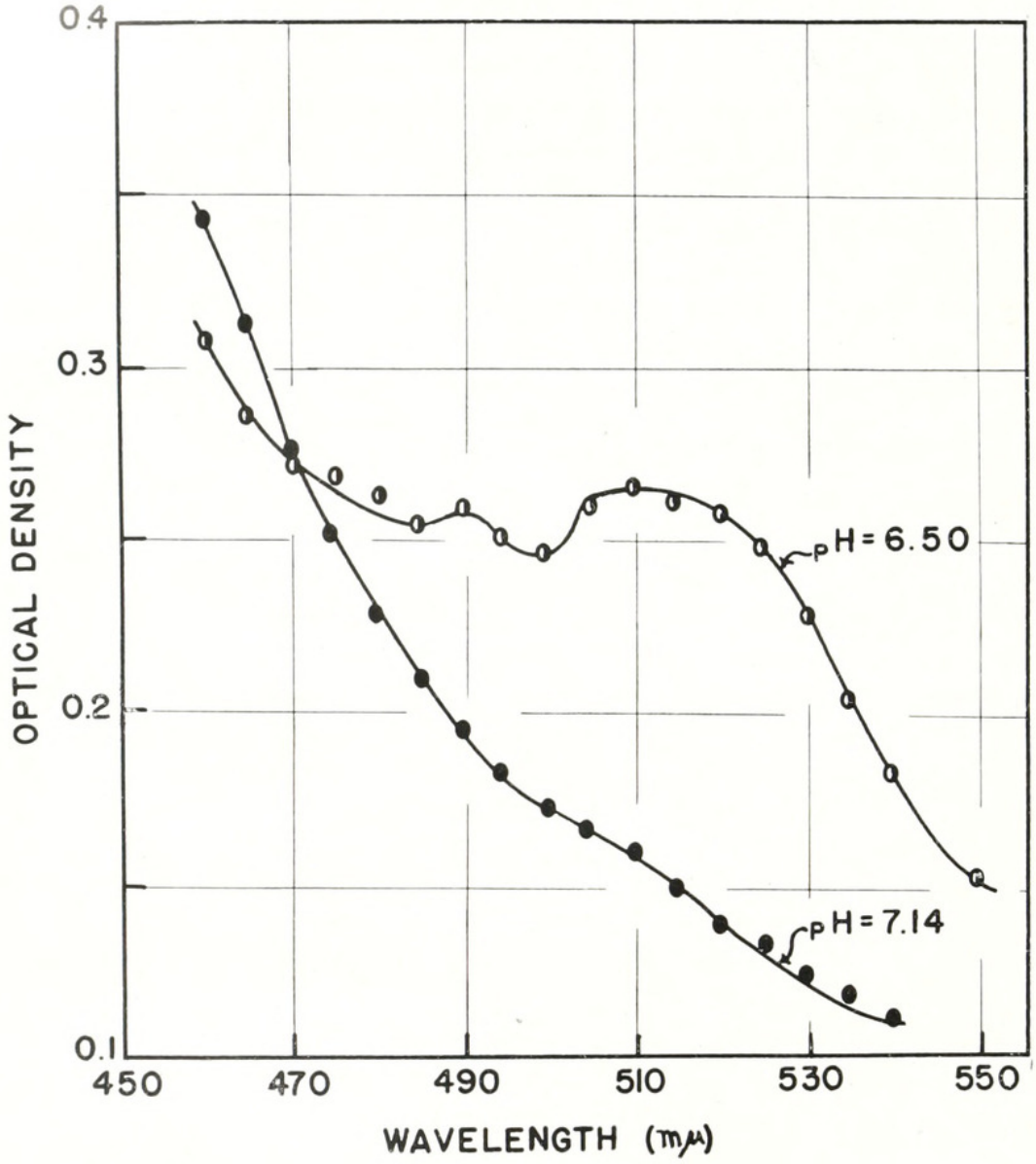

FIGURE 3. Visible light absorption spectra of Ca^{++} — treated homogenate extracts. Half circles: unbuffered. pH = 6.50. Full circles: buffered at pH 7.14.

tubes employed in the experiment was treated with two to five volumes of cold distilled water. An equal volume of 2% monochloroacetic acid was added. After several minutes of gentle shaking, the proteins were precipitated with trichloroacetic acid in a final concentration of 5%. The precipitation was permitted to proceed for 24 hours in the icebox. Finally, the flocculent precipitate was sharply centrifuged down and the clear supernatants collected. The NPN of these super-

natants was determined after digestion in boiling H_2SO_4 and "Superoxol" by direct nesslerization.

Small and very variable increases in NPN were observed to follow the addition of Ca to the homogenates. When measurements were made within the first hour after Ca treatment, the increments were usually of the order of 2–3 per cent, or barely within the limits of precision of the method. With increasing time, however, more NPN appeared. The controls showed little or no protease activity in the absence of Ca when NPN release was the criterion of activity. When the experiments were continued for several hours, however, the controls also showed increments in NPN at the end of the experiment (as compared with a primary control determination at zero time). These increments were always smaller than those observed in the homogenates which contained Ca.

Thus, for example, in an experiment which ran for six hours, the experimental homogenate, which was treated with 0.01 *M* $CaCl_2$, contained 17.3 mg.% NPN (the value for the final diluted assay sample). The control, run along with the experimental, contained 15.6 mg.%, while the primary control, which had been precipitated with TCA six hours previously, contained 14.6 mg.%. These values are the averages of triplicate determinations.

A typical result for an incubation period of 24 hours (at 1° C.) is the following:

Material: Ca-free homogenate, lyophilized and reconstituted.
Treatment: Experimental—0.02 *M* Ca^{++}
Control—KCl of ionic strength equal to that of added Ca solution.
Result: Control: 31.0 micrograms of NPN/ml.
Experimental: 52.2 micrograms of NPN/ml.

5. Electrophoretic experiments

Although electrophoretic examinations of sea urchin egg proteins and protein fractions have been reported previously (Monroy, 1950; Monroy and Monroy Oddo, 1951), earlier work dealt with frozen and dried material. Since the publication of the critical experiments of Lindvall and Carsjö (1951), it has become important to re-examine these results in the light of possible artifacts produced by the lyophilization of fertilized and unfertilized eggs which retain their internal as well as external calcium.

Electrophoresis of extracts of homogenates such as are used in the present work promises to be a useful tool in the further analysis of the SPR-type reactions. The method employed in the experiments was as follows:

Two aliquots of homogenate were obtained. One was calcified, and the other, the control, was treated in the usual way with KCl. After a few minutes incubation, there was the usual treatment with distilled water, followed by extraction in the cold with distilled water. The extracts were finally centrifuged for twenty minutes at 17,000 to 20,000 g. Only clear or very faintly turbid supernatants were accepted for electrophoretic analysis. These were placed in 1-cm. diameter dialysis tubing and dialyzed in the icebox (1° C.) for 48 hours against several changes of 500 ml. of 0.1 N $NaHCO_3$ at pH 8.5 (21° C.). The thoroughly dialyzed colloids were then submitted to electrophoresis in the Perkin-Elmer Tiselius-type instrument, using the 2-ml. cell. The cylindrical lens system was

used for the photographic records of the runs. Photographs were made at definite intervals with the current temporarily turned off. For analysis of these data, the negatives were placed in a photographic enlarger and projected, and the enlarged patterns were traced on cross-section paper. The pattern shown in Figure 4 was prepared in this way, and then inked for reproduction.

The choice of bicarbonate buffer calls, perhaps, for justification. This system was used by Monroy and Monroy Oddo (1951) in their recent experiments upon the protein fractions of fertilized and unfertilized sea urchin material. Their patterns seemed in general to show satisfactory separation. To facilitate com-

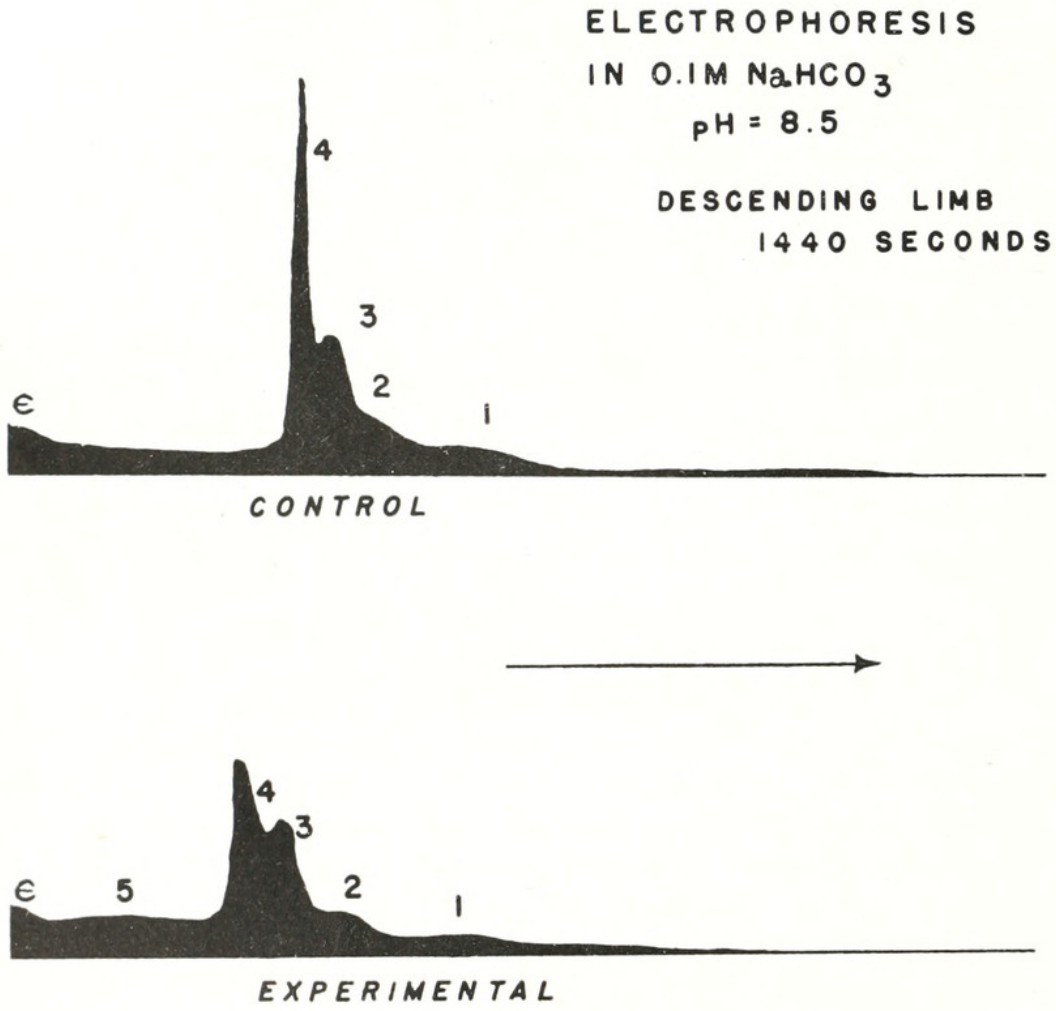

FIGURE 4. Electrophoresis of water extracts of homogenates. Control, untreated. Experimenal, treatment with Ca^{++}.

parison, bicarbonate was used in the present experiments. The separation (resolution) was not as good as could be desired, although sufficient for the comparative data needed. Migration was quite rapid.

Figure 4 shows a typical result. The pattern marked "control" is that of the water extract of a control homogenate. The "experimental" pattern was given by the water extract of a Ca-treated homogenate. Both patterns were photographed at the same interval relative to the commencement of the run. The descending limbs are shown. The field strength (potential gradient) was 6.5 volts/cm. It is apparent that the migration was rapid, and that acceptable resolution was achieved after only 1440 seconds.

In general, three boundaries were observed in all experiments (marked "2," "3," and "4"), with a suggestion of a fourth ("1") in some. Better resolution of these boundaries has already been achieved in experiments with buffers at a lower pH (to be reported elsewhere). In two experiments, a suggestion of a very low, rapidly spreading boundary (marked "5" in Fig. 4) was given by the experimental extracts. In all of the experiments, all boundaries other than the slowest and largest (marked "4") seemed unaffected by the precipitation reaction which had taken place in the experimental. The loss in area of boundary "4," as illustrated in the experiment described, may possibly be ascribed to a selective interaction of the component represented by the boundary with calcium. It remains possible that losses occurred in the smaller boundaries and are not detectable. Nevertheless, the major effect of the calcium ions seems to be upon the largest component.

6. Electron microscopy

If the precipitation reaction under investigation does, indeed, involve one or more specific fractions of the cell's macromolecular or particulate population, then this should be demonstrable by direct visualization of the insoluble products of the reaction. The electron microscope makes such visualization theoretically possible.

Dilute homogenates were prepared. Ca was added to the experimentals, and the controls were treated with KCl of equal ionic strength. Small drops from the experimental and control tubes were immediately withdrawn and placed on collodion films deposited on copper grids.

When the "SPR" in the experimentals had proceeded for the proper interval of time, the reactions were stopped by flooding the mounts with distilled water and removing the large drops formed with filter paper. This process was repeated several times, with increasing intervals between flooding and withdrawal of the distilled water. This process served to 1) dissolve away the salts, 2) lyse the large pigment granules and wash away their ghosts, and 3) remove all water-soluble parts of the preparation. In addition, the mechanical disturbance occasioned by the withdrawal of the drops removed all but the most tenaciously sticky materials from the collodion.

The samples were either permitted to dry immediately, or they were first fixed with 0.2% phosphotungstic acid for two minutes and then washed again and dried. Finally, all preparations were shadow-cast with chromium. The electron micrographs were made with an RCA model EMU microscope.

Fixed and unfixed preparations gave essentially the same results, except that the materials fixed with phosphotungstic acid showed particles which were slightly more discrete and less flattened. Some slight syneresis was observed in these preparations. The discreteness and lesser degree of flattening are probably due to a certain rigidity imparted to the proteins by the fixative action of the acid, rather than by its electron stain properties. The photographs presented in this report were all made from fixed preparations.

Figure 5 is typical of the results obtained with the control preparations. Whether fixed or unfixed, these preparations showed only a thin layer of material blanketing the collodion substrate. The washing procedure appears, in these preparations, to have removed not only the water-soluble materials, but in addition

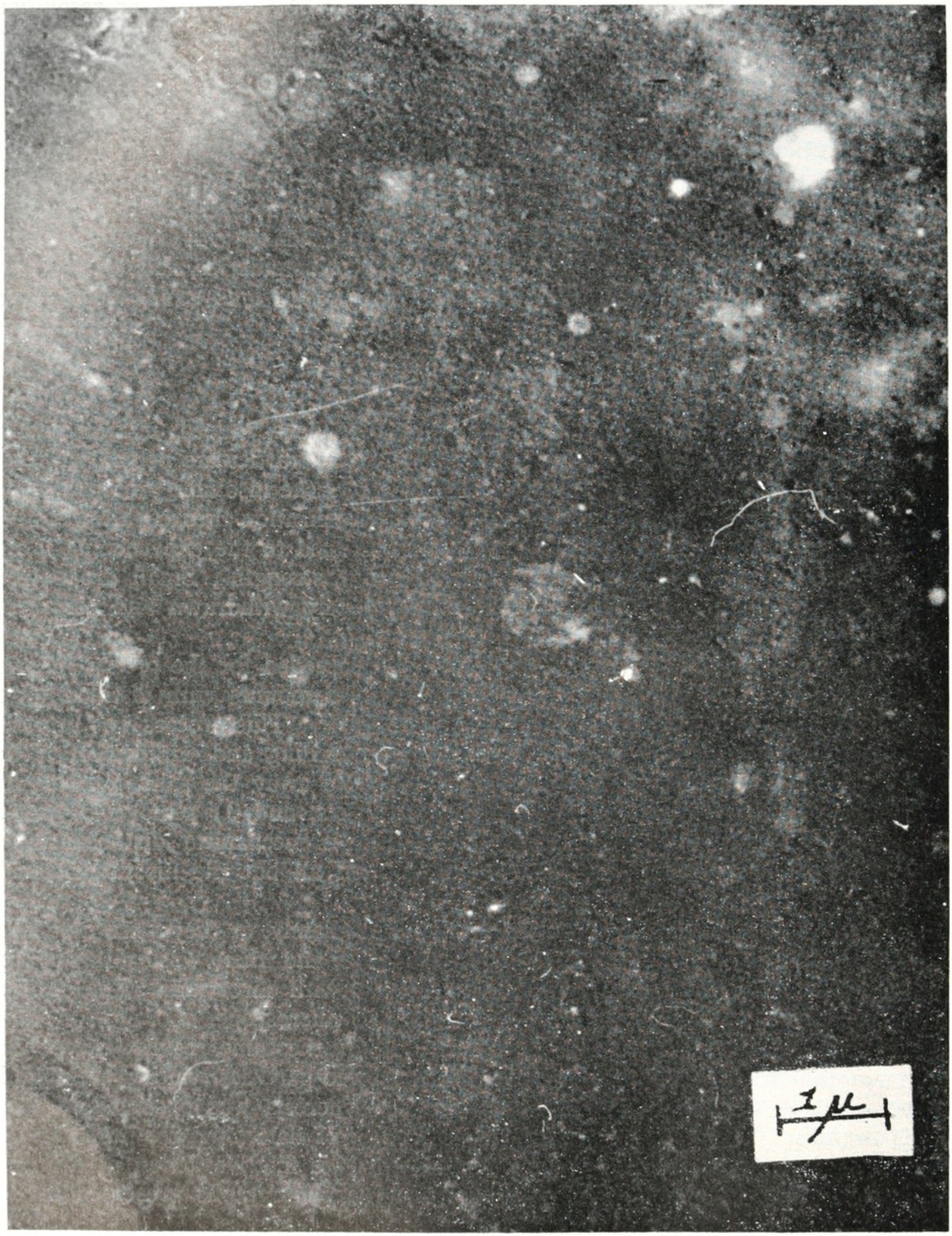

FIGURE 5. Electron micrograph of control preparation. See text. Magnification: 15,000 ×. Collodion surface with some low-molecular materials.

most of the water-insoluble materials. This is a result of the mechanical disturbance effected by the repeated withdrawal of the water droplets. Such a lack of larger structures in the control preparations is indeed fortunate, for such materials as do appear as a rule in the experimental preparations and are absent from the controls must in some way have had their solubility in water or their adherence to collodion sharply altered by the treatment with Ca. The specimen in Figure 5 is essentially clean collodion with perhaps some deposit of relatively low-molecular material. The magnification is 15,000 diameters.

Figure 6 is a typical experimental specimen, seen at a magnification of 28,000 diameters. Plainly, the result of the Ca-treatment has been the formation of a flocculent precipitate, insoluble in water, and involving particles other than the large pigment granules, which have been lysed and washed away by the preparation method. At the magnification shown, it is not possible to resolve the fine-structure of the microflocs to any appreciable extent; however, the edges of the floc shown, as well as the very small aggregates on the collodion, suggest that the basic aggregating unit is small and approximately spherical. Although there is a suggestion of linear aggregation on the figure, there is in general no pronounced order in the flocs as observed. They appear always, at this magnification, to be of the close-packed, polyfunctional type.

When observed at the highest magnifications, such as that in Figure 7 (110,000 ×), the thinnest microflocs reveal their fine-structure. The "linear" aggregates seen only poorly at the lower magnifications dominate these pictures as long, ropy strands of material, made up of (highly flattened) spheres in linear array. Since high surface pressures are exerted upon the specimens with the air-drying technique used in these preparations, it is likely that the discs were originally spheres. The aggregation reaction may thus consist in part of a difunctional type of polymerization at the earlier stages and then an irregular and extensive interchain packing superimposed.

It is probable that the extreme irregularity of the final packing in the microflocs observed is a result of 1) the drastic treatment of the material during the preparation of the specimens, and 2) syneresis due to the fixative. Of course, since the particles in question do not stick (probably because they are not aggregated) in the control, the foregoing considerations do not apply to controls.

Several representative plates at the high magnification (electronic magnification = 13,250 ×) were subjected to microscopic examination and measurement of the images of the small particles making up the aggregates. These particles, which it should be remembered do not remain on the control mounts, are distributed in diameter about a mean value of 380 Å. The distribution is skewed with the mode displaced somewhat toward the smaller diameters, and the variance is rather large (S.D. = 80 Å.).

The large variance, although not unusual in electron microscopic preparations of cell fractions, indicates, probably, rather extensive deformation of the original particles.

Discussion

The objectives of these investigations were two-fold: first, the clarification of the question of protein solubility changes as a result of the "test-tube SPR" and

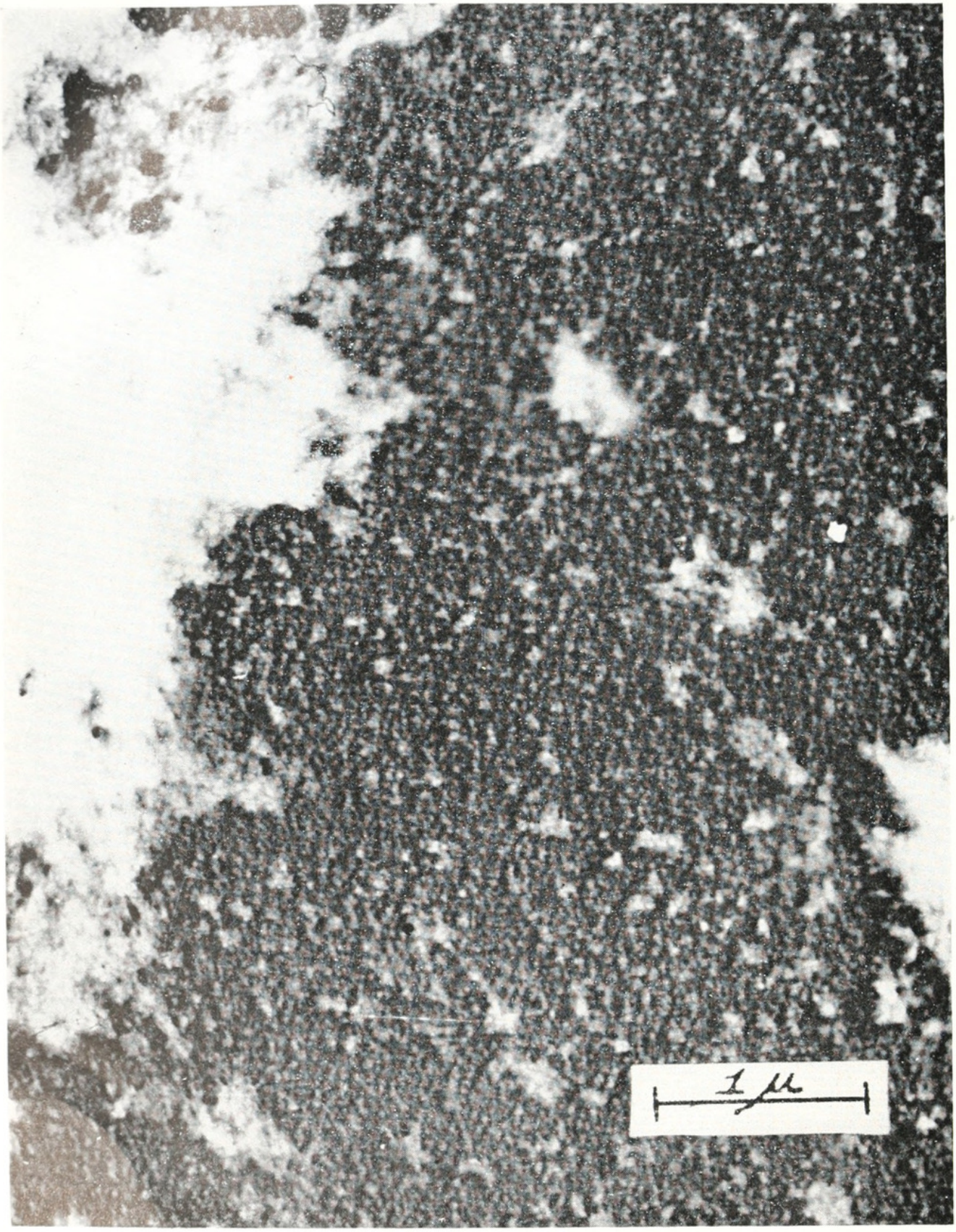

FIGURE 6. Electron micrograph of experimental preparation. See text. Magnification: 28,000 ×.

second, the construction of a reaction mechanism for the over-all phenomenon. By extension, such a mechanism would be of great utility in interpreting the molecular basis of colloidal alterations in the cytoplasm of living cells.

The addition of calcium ions *has* been found to reduce the solubility of some proteinaceous constituent of the homogenate, or at least, the sedimentability of this constituent is increased, which can be interpreted as an increase in aggregation.

But the second objective remains remote. One has had, so far, to be content with the acquisition of a spectrum of *possible* reactions involved in the macroscopic event called the SPR. Thus the experiments reported make no attempt to test interrelations of reactions, but rather to establish that certain reactions do occur as a result of the initiation of a "test-tube SPR" by Ca.

The fact that a local level or optimum for precipitation exists in the homogenate with regard to concentrations of Ca added at the level of 0.001 to 0.01 *M* may be related to the following facts.

1) The "physiological" rates of reaction (with half-time of the order of seconds to minutes) at 21° C. are the ones observed at these concentration levels.

2) The free Ca of unfertilized eggs is of the order of 0.0005 *M* (Mazia, 1937). Also, the concentration below which an SPR (in whole eggs) will *not* take place is very close to this, *viz.*, 0.0003 *M*. When the *free* Ca concentration of the cytoplasm is increased beyond this general level (5×10^{-4} *M*), the sea urchin egg experiences a protoplasmic gelation, or clotting. Mazia (1937) demonstrated that the fertilization of sea urchin eggs results in the liberation of *free* (ultrafilterable) calcium in the egg. Furthermore, the concentration of free Ca in the egg was shown to increase by 0.001 *M* upon fertilization, placing the total free Ca of the fertilized egg at 0.0015 *M*.

It may be significant that this level of Ca concentration, which we may term "physiological" for the system studied, is at one end of the range indicated as required for reasonably rapid rates of reaction in the *in vitro* system. Considering the dilution of reactants which takes place in the preparation of an homogenate, the correspondence of these concentration levels is surprisingly good.

The fact that the precipitated material contains nucleic acid may explain why such large proportions of the total cellular protein can ultimately (at very high Ca concentrations) be aggregated, and still appear to comprise, through most of the concentration range, a single fraction or group of fractions closely related. First, it is safe to assume that most of the nucleic acid being precipitated is PNA. Villee *et al.* (1949) have shown that the DNA of the uncleaved Arbacia egg corresponds to only about 6% of the total nucleic acid, and according to Abrams (1951) this figure is probably too high. The small quantity of DNA present in the homogenate could then hardly account for the large proportion of the total nucleic acid which can be precipitated by small quantities of Ca. Finally, if the affected fraction contains PNA, it may be one of the PNA-rich particle fractions of the cytoplasm, in which case it could conceivably represent a large proportion of the total protein of the egg.

The electrophoretic experiments suggest that the fraction(s) undergoing change under the influence of Ca are a part of one of the largest macromolecular or small-particulate fractions of the cell. The electron microscope pictures show that the particle in question is (after reaction with Ca) a large macromolecule or a very

small complex cytoplasmic particle and that it is probably globular before polymerization.

It is specified that the particle has these characteristics *after* treatment with Ca, because a careful inspection of high magnification photographs, such as that in Figure 7, reveals a fine-structure within the particles themselves, at the level of 40–70 Å. Probably, this represents the fine structure of the particles, but there is no evidence that the 380 Å particle is not *itself* an aggregation product resulting from the addition of Ca. The latter is, however, rather unlikely for many reasons, among which the results of the electrophoretic experiments and the probable large decrease in entropy in such a specific aggregation are prominent.

Thus, the experimental evidence seems to indicate a process in which Ca brings about the aggregation of certain specific particles of the cytoplasm into an insoluble coagulum. The specificity of this reaction, as well as the quality of its insoluble product, fit well with what is known of the SPR and of its normal analogue, the cytoplasmic sol-gel transformation.

The other events of the test-tube SPR also have their analogues in the SPR of the whole cell, and, interestingly, in the events of fertilization and stimulation. Whether the acid formation observed in the system under investigation here is related to that which occurs at fertilization (Runnström, 1933) is, of course, not decided by these experiments. Nevertheless, there is a strong suggestion that this might be the case. The color changes appearing in the test-tube reaction have their direct parallel in the SPR, and suggest that injury can produce large local increases in the hydrogen ion concentration of protoplasm. This is, of course, a fact generally recognized from other types of experiments (*e.g.*, Chambers and Pollock, 1927). The proteolysis measured in the *in vitro* system has its analogue in fertilization (Lundblad, 1950) and, *in vivo*, probably in the form of a release of NPN (Örström, 1941).

It is perhaps significant for the evaluation of the "colloid chemical" theory of stimulation of Heilbrunn (1952) that the *in vitro* system here studied has similarities to the clotting of blood in two heretofore undemonstrated areas. Not only do both reactions involve initiation or activation by Ca ions, but both involve polyfunctional polymerizations of macromolecular monmers, and both involve some step in which a proteolytic enzyme is activated and small quantities of NPN are released. This must not be taken to indicate that the precipitation reaction in the homogenate is necessarily mediated by a proteolytic enzyme. Such an enzyme is indeed present, but its participation in the reaction leading to aggregation in the homogenate must be demonstrated by methods not reported here. Such methods, particularly the use of alternating low and high temperatures, and the addition of clotting inhibitors are currently in use in this laboratory. It is also interesting, in this connection, that the fat solvent anesthetics such as ether and alcohol inhibit the *in vitro* reactions between the homogenates and calcium. These results will be more fully reported in another publication.

It should be noted that the formation of true fibers, such as those of fibrin (Hawn and Porter, 1947), is not observed in the SPR-type reaction. Plainly, the fibrin-clot type of structure could not, and does not occur within a living cell. It is for this reason that it is erroneous to speak of cytoplasmic gelation or of "protoplasmic clotting." The word "gel" defines a system of infinite viscosity,

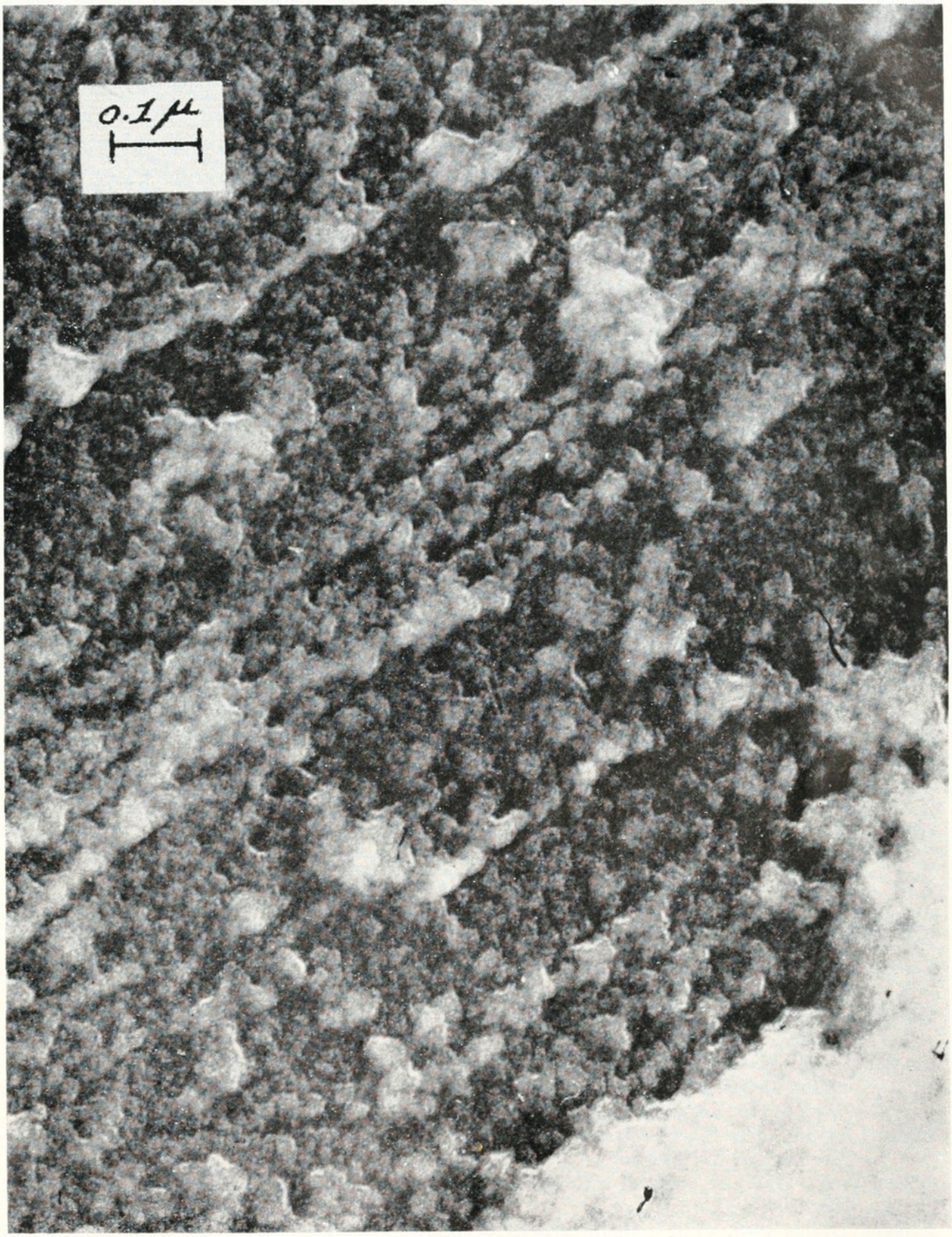

FIGURE 7. Electron micrograph of experimental preparation. See text. Magnification: 110,000 ×.

which is the case with a normal fibrin clot. But in a living cell, the cytoplasmic viscosity increases only by a factor of three to six in the usual sol-gel transformation. One presumes that in the SPR, these changes would increase in magnitude, and could indeed lead to a gel.

From these considerations, one may conclude that an aggregation process that might be expected to occur in a living cell, or even in the SPR, should certainly not lead to the formation of fibers of great length. Rather, the expectation is a reversible aggregation of small units into networks of dimensions easily accommodated within the confines of a single cell. The nature of the aggregation process observed in the homogenate system is thus, at least teleologically, justified.

Summary

1. The addition of Ca^{++} to homogenates of Arbacia eggs which have been previously made calcium-free results in the loss in solubility of certain protein constituents of the homogenate.

2. The precipitated material is rich in nucleic acid, and comes, at least in part, from the fraction non-sedimentable at 2000 g.

3. The addition of Ca^{++} to homogenates activates a proteolytic enzyme and leads to the release of small amounts of additional non-protein nitrogen.

4. None of these reactions can be ascribed to the high ionic strength of solutions of calcium salts as compared with that of isosmotic solutions of uni-univalent salts.

5. The fraction undergoing solubility loss appears to be electrophoretically homogeneous, since it is represented by the largest and slowest-migrating boundary in the water extract of the homogenate.

6. Electron microscope observations reveal that the aggregating material consists of spherical or discoidal particles of (flattened) diameter 380 Å. The aggregation pattern observed is that of microflocs.

7. The possible significance of these observations for the mechanism of the surface precipitation reaction and for sol-gel changes in the cell interior is discussed.

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