STUDIES ON AMPHIBIAN YOLK

2. The Isolation of Yolk Platelets from the Eggs of *Rana pipiens*

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ABSTRACT

Previous methods which employed simple sucrose or salt solutions to isolate yolk platelets have failed to preserve their superficial layer, and the preparations obtained generally exhibit some contamination when observed with the light or electron microscope. When yolk platelets are suspended in a sucrose-polyvinylpyrrolidinone medium, however, they remain relatively intact and their superficial layer is not lost to the medium. A method, which takes advantage of this fact, is described for the isolation of frog (*Rana pipiens*) yolk platelets which are free from nuclear contamination and practically free from cytoplasmic contamination. After such platelets are treated with distilled water, the superficial layer is no longer seen and a new dense and granular matrix is frequently found surrounding the crystalline main body. The significance of this and other observations concerning the effects of calcium and ethylenediamine tetraacetate are discussed.

INTRODUCTION

Several interesting features of the formation, structure, and breakdown of amphibian yolk platelets and their role in embryological processes have been indicated by recent electron microscopic studies (for literature, see 10). Much of this work, however, is admittedly fragmentary and speculative, and more information is needed concerning the components of the amphibian yolk platelet. A prerequisite for this information is a method for isolating yolk platelets in appreciable amounts, intact and relatively free from contamination. In previous methods designed for this purpose (3, 4, 15, 17), ovarian tissue has generally been used as a starting material, together with a simple sucrose or salt solution as the isolating and suspending medium for differential centrifugation. These methods, in our experience, have invariably yielded yolk platelets which were completely devoid of their outer superficial layer and which, when ovary or postgastrula embryos were used as a starting material, were frequently contaminated with what appeared in the electron microscope to be extracted nuclei (presumably from follicle or embryonic cells). It was believed that such preparations were undesirable, especially since one of our interests was to decide whether or not yolk platelets contain a large proportion of the nucleic acid in the egg as claimed by some authors (1, 6, 15, 18, 19). Furthermore, since it has been shown that the periphery of yolk platelets *in situ* stains in a qualitatively different manner from the interior (14, 17) and that the superficial layer of the yolk platelet frequently disappears during the initial stages of differentiation (9, 11, 26), it was considered important to preserve this structure during the isolation procedure so that its chemistry might be more accurately indicated by further studies.

Recently, Ohno, Karasaki, and Takata (14) have centrifuged urodele embryo homogenates

prepared in 0.25 M sucrose–1 per cent polyvinylpyrrolidinone (PVP) in a discontinuous gradient ranging up to 0.25 M sucrose–12 per cent PVP and have obtained by this method yolk platelets which more adequately met the above criteria of purity and intactness. On the basis of this observation, this paper documents with the electron microscope a method of preparing a 50 per cent yield of pure and relatively intact yolk platelets.

METHODS

Female *Rana pipiens* frogs were obtained from Wisconsin (Steinhilber and Company, Oshkosh) and were stored at 10°C for periods ranging up to 4 weeks before use. Ovulation was induced by injecting about 33 to 50 per cent more than the usual number of pituitaries needed for a normal ovulatory response, according to the season (20). When the injected frogs had been left for 15 hours at room temperature (24°C), 1000 to 2000 eggs could be washed from the body cavity. The use of "body cavity" eggs avoids contamination from follicular elements and from jelly material, which interferes with homogenization at the concentrations used. All water used was glass distilled.

Isolation of Yolk Platelets in the Absence of PVP

Platelets were isolated according to the methods of Panijel (15), Flickinger (4), and Ringle and Gross (17). The amount of pigment and nuclear contamination in the resulting preparations was determined with the light microscope, and was greatest when Panijel's method was used and least in the Ringle and Gross preparations. The method of Ringle and Gross was therefore chosen as a model for further observations with the electron microscope on platelets prepared in solutions of low salt concentration. Also, we tried to isolate yolk platelets in sucrose solutions lacking PVP by the method employing sucrose-PVP as described below. In these cases, PVP was either replaced by 2 mM $CaCl_2$ or 2 mM ethylenediamine tetraacetic acid, disodium (EDTA) or simply eliminated, and centrifugation of the less viscous solutions was performed at 1000 RPM for 15 minutes (F_{max} = 245 g).

Isolation of Yolk Platelets Employing Sucrose-PVP

All operations were performed in a cold room (3 to 5°C). Body cavity eggs were washed several times in cold 10 per cent amphibian Ringer's solution. Foreign matter, blood clots, and "blotched" eggs were removed, since such eggs often did not homogenize thoroughly and contaminated the final pellet. After being counted, the eggs were washed with cold 0.25 M sucrose–5 per cent (w/v) PVP (PVP solutions were all neutralized with NaOH).

The necessary number of gradient tubes (250 eggs per tube) was prepared by placing 15 ml 1.0 M sucrose–5 per cent PVP in each round-bottomed Lusteroid tube (50-ml size no. 638 for the International Head no. 856). Conical tubes were avoided since their use gave a more contaminated product caused by increased wall effects.

The washed eggs were gently hand-homogenized in 0.25 M sucrose–5 per cent PVP with a moderately tight-fitting Teflon pestle in a smooth-surfaced glass tube (16) (ground glass homogenizers or those which are too tight should be avoided) until thorough dispersion was obtained as judged visually (usually 1 minute). The egg concentration was maintained at 250 ± 25 eggs per 20 ml homogenate. Above this concentration the contamination was greatly increased, whereas below this concentration the operation was more laborious. Twenty milliliters of the homogenate were layered over 15 ml 1.0 M sucrose–5 per cent PVP in such a manner that neither a sharp interface nor a mixing of more than a third of the way down the lower layer was produced. The homogenate was most conveniently poured with a 20-ml beaker. The filled tubes were placed in 50-ml bronze shields (no. 320) with rubber cushions (No. 571) and these in turn were supported by the No. 253 horizontal yoke in the PR-2 International centrifuge. They were then centrifuged at 1500 RPM for 20 minutes (F_{max} = 550 g). This particular sequence of operations was performed rapidly, otherwise aggregation and contamination occurred. This procedure gave a well packed yellow pellet of yolk platelets (with a small semicircle of contaminating pigment at the top), a slightly yellow and turbid lower solution layer (mostly small platelets), and a black upper solution layer.

The supernatant solutions were decanted and the walls of the tube washed down with a squeeze bottle containing 0.25 M sucrose–5 per cent PVP. The tubes were swirled, decanted again, and allowed to drain for several minutes. The pellets were then taken up with the aid of a glass stirring rod into 0.25 M sucrose–5 per cent PVP (the final volume was the same as that used for the original homogenate), homogenized, layered, and centrifuged as before.

After the second centrifugation, each tube contained a small bright yellow pellet with no pigment or particulate contamination. At this stage, the supernatant was simply decanted and the inverted tubes were allowed to drain (without washing) for 5 minutes on an absorbent surface. Such drained pellets provided the basis for a "wet pellet weight," a

TABLE I

Summation of Observations on Isolated Yolk Platelets

Isolation method	Starting material	Fixation time	Embedding medium	Contamination		Membrane present	Superficial layer present	Crystalline structure		Denatured material	Aggregation
				Nucleus	Cytoplasm			Lamellar breakdown	Cracking		
		min.									
In situ	Ovary	30–60	Epon 812			+++	+++	−	−	−	
	Ovary	30–60	Methacrylate			+/++	++	−	−	−	
	Blastula	30–60	Epon 812			+++	+++	−	−	−	
	Blastula	30–60	Methacrylate			+/++	++	−	−	−	
Ringle and Gross	Ovary	30		+	+	−	±	++	+	++	±
Sucrose	Ovary	60		+/++	++	−	+	+	+	++	+
Sucrose-$CaCl_2$	Ovary	30	Epon 812	+++	+++	−	+	−	+	++	+++
Sucrose-EDTA	Ovary	30		±	+	−	−	+++	+++	++	−
	BCE*	30		−	±	−	−	+++	+++	++	−
Sucrose–2% PVP	Ovary	30	Epon 812	±	+	±	++/+++	−	+	−	±
	Ovary	30	Methacrylate	±	+	±	+	±	+	−	±
Sucrose–5% PVP	Ovary	60		±	+	±	++	−	−	−	±
	BCE*	30		−	+	±	++/+++	−	±	−	±
	BCE*	60	Epon 812	−	+	±	++	−	−	−	±
Sucrose–5% PVP →distilled H_2O	BCE*	60		−	±	−	−	+	++	+++	+

* Body cavity eggs (after ovulation).

value which necessarily included any sucrose–PVP medium adhering to the inside of the tube.

Fixation and Embedding

Isolated pieces of early blastula ectoderm, fragments of matured oocytes or body cavity eggs, and yolk platelet pellets isolated by the above methods were fixed, embedded, sectioned, and stained as previously described (10).

The choice of fixation time (30 to 60 minutes) or embedding medium (Epon or methacrylate) apparently was not critical for the preservation of yolk platelet structure *in situ*. Isolated platelets, however, were very sensitive to these two factors. As the fixation time increased from 30 to 60 minutes, the superficial layer of isolated platelets (in sucrose-PVP) became more electron-opaque and diminished in volume, but at the same time the main body did not display many cracks due to embedding or polymerization damage. Methacrylate as an embedding medium promoted both the disappearance of the superficial layer and cracking of the main body, regardless of fixation time. We have therefore concluded that isolated platelets can best be preserved by a 40-minute fixation time coupled with Epon embedding.

OBSERVATIONS AND DISCUSSION

Table I summarizes on a three-point scale some observations on numerous pictures taken of yolk platelets both within the cell and after twenty-three different attempts at isolation, and may be referred to during the discussion. Although descriptively inadequate, it is felt that *this table better presents general impressions gained by the authors than could selected pictures alone.*

Also for reference, Fig. 1 shows the appearance of a number of yolk platelets within an early blastula cell of *Rana pipiens*. This figure was chosen to demonstrate the typical variations in the morphology of the yolk platelet. We have found the appearance of yolk platelets to be essentially the same in the late oocyte, ovulated egg, and early embryo, whereas in the early oocyte (24) and later stages of embryogenesis (11) a different morphology typically exists. Each platelet (Fig. 2) contains a superficial layer of fine particles or fibrils, a central main body displaying a crystalline periodicity, and a single membrane enclosing the entire structure (10).

Isolation of Yolk Platelets in the Absence of PVP

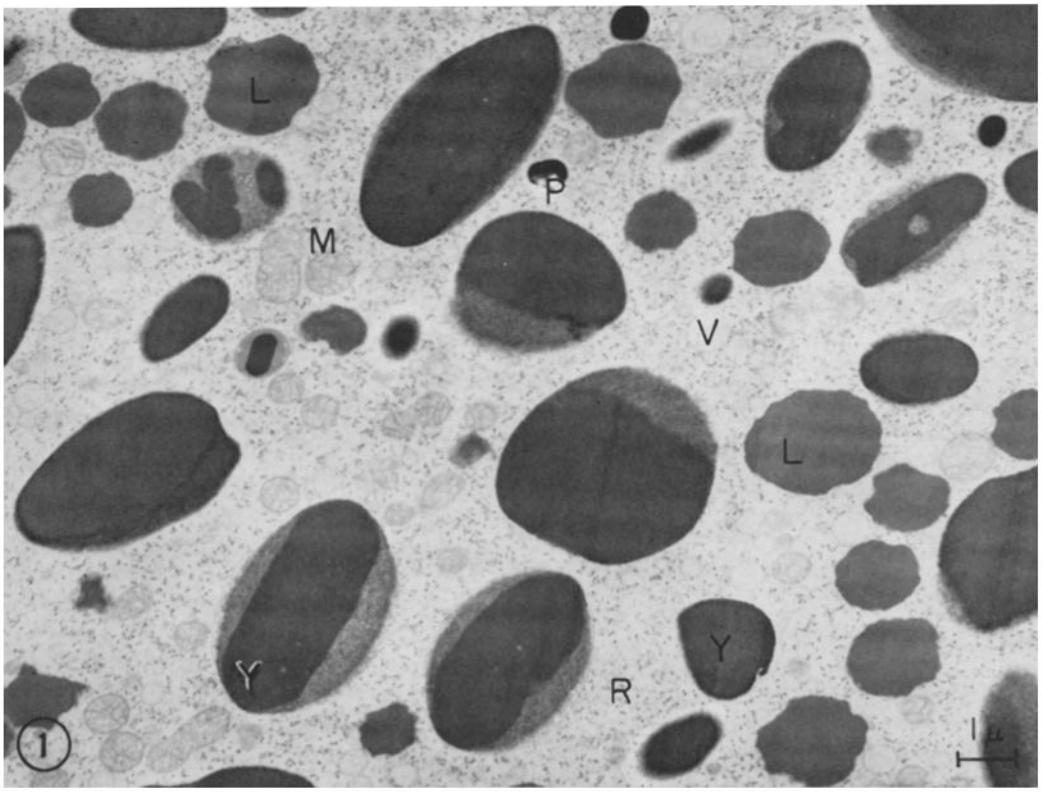
As previously mentioned, when ovarian yolk platelets were isolated in salt solutions by the modified Essner method described by Ringle and Gross (17), platelets were obtained which contained less pigment and nuclear contamination than those obtained by the methods of Panijel (15) and Flickinger (4). Nevertheless, what appeared to be extracted nuclei and membranes were occasionally found either free or attached to the platelets when viewed with the electron microscope (Fig. 3). Much of this probably was derived from follicle cells, and the use of body cavity eggs would undoubtedly have reduced even further the amount of contamination observed. Very rarely, pieces and fragments of what seemed to be superficial layer material were also found among the platelets, but mostly this structure and the outer membrane were absent. Furthermore, platelets isolated by this method were relatively fragile, and under high magnification the crystalline main body at times showed signs of cracking, lamination, and fragmentation (Fig. 4). Although the lamination or "splitting" along lamellar planes observed in these platelets was sometimes quite striking, many fragments still displayed a clear indication of a hexagonal packing of units or the usual band patterns (see

FIGURE 1

Part of a blastomere in the presumptive ectoderm area of an early *R. pipiens* embryo. Yolk platelets (*Y*), lipoid bodies (*L*), mitochondria (*M*), pigment granules (*P*), vesicles (*V*), and many ribonucleoprotein particles (*R*) can be identified. × 8000.

FIGURE 2

Higher magnification of two yolk platelets within a blastula cell of *R. pipiens*. Each platelet is limited by a single membrane (arrows) and is composed of a dense main body (*B1, B2*) surrounded by a "superficial layer" (*S*) of particles or fibrils approximately 50 A in diameter. Each main body shows a very regular system of parallel lines. The spacing between lines is 68 A in *B1* and 75 A in *B2*. Part of a lipoid body can be observed at *L*. × 80,000.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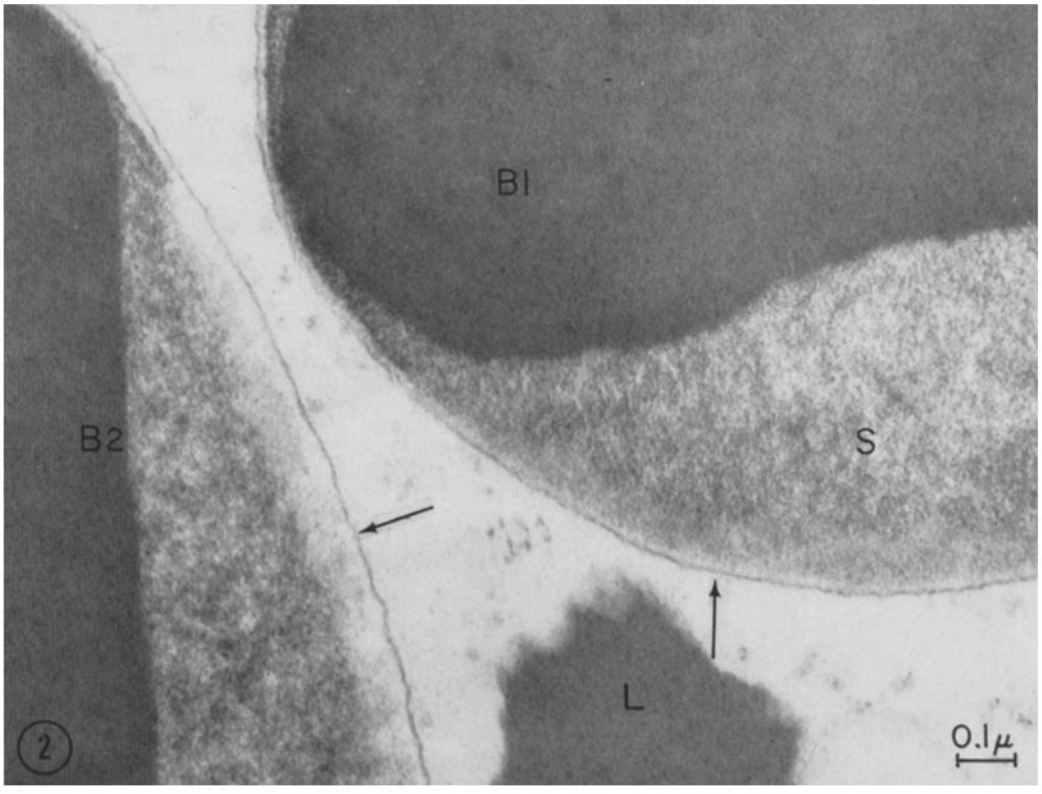
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reference 10), indicating that the basic structure of the main body was not altered. At times, amorphous or distinctly fibrous material, frequently around the surface of the main body, was also seen and was noted as "denatured material."

Attempts were also made to isolate platelets using sucrose solutions without PVP. Again, the superficial layer was mostly lacking from platelets isolated by such methods. Furthermore, when pure sucrose solutions were used, mild aggregation frequently occurred. When the sucrose solution contained 2 mM $CaCl_2$, extensive aggregation occurred and the platelet preparations were always dark gray owing to pigment contamination. Similarly, when "clean" platelets isolated by other methods were placed in 0.25 M sucrose–2 mM $CaCl_2$, clumping and aggregation could be observed both visually and with the electron microscope.

When sucrose solutions which contained 2 mM EDTA were used, aggregation was completely eliminated and true suspensions were routinely obtained with rehomogenization. Examined with the electron microscope, sucrose-EDTA preparations of yolk platelets prepared from body cavity eggs appeared to contain even less cytoplasmic contamination than the sucrose-PVP preparations described below. However, the superficial layer was again absent, and damage to the main body was more marked and consistent than in any other type of preparation. Lamination and fragmentation of this structure were extensive, and evidence of crystallinity in free pieces, as shown in Fig. 4 for the preparation of Ringle and Gross, was rare.

Isolation of Yolk Platelets Employing Sucrose-PVP

When yolk platelets were isolated in sucrose solutions containing PVP as described, nuclear contamination seemed to be absent. In addition, the platelets still maintained most of their superficial layer and showed little or no evidence of cracking or lamination (Figs. 5 and 6). Fig. 5 shows that the superficial layer was not maintained strictly intact in several cases, while Fig. 6 indicates that the outer membrane has been lost (in only a few cases, isolated platelets together with their outer membranes have been seen). We do not know how much of this loss may be due to the suspension medium, the manipulative procedures of isolation, or the processes of fixation and embedding. Occasionally platelets have been observed with the same damage to the superficial layer *in situ*, and we have also observed what seemed to be a fragmentation of the outer membrane to form a series of small vesicles (10). Sometimes such vesicles can be seen among the isolated platelets, and their derivation is thus problematical. We believe, however, that to date the sucrose-PVP method best preserves the superficial layer and that the platelets obtained are generally more free from contamination than those obtained by previously published methods; an optimal concentration of about 5 per cent PVP appears to be needed, although this opinion needs confirmation by more experimentation.

Further Treatment of the Isolated Platelets

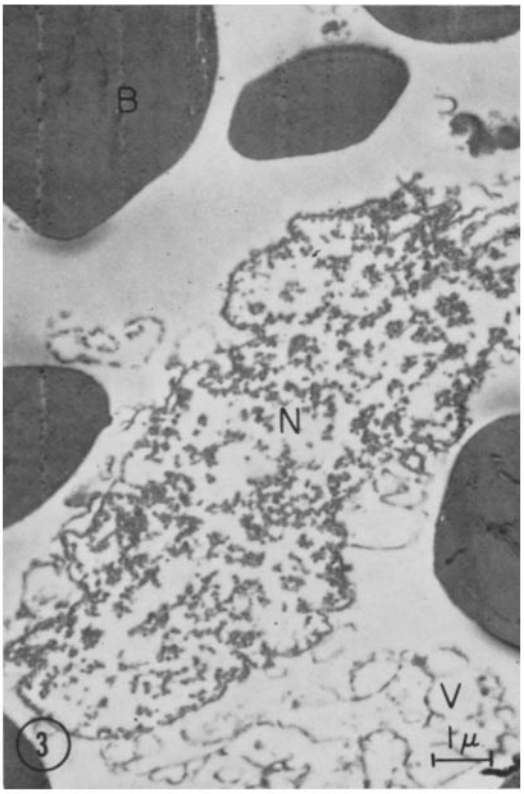
When a 20-ml sucrose-PVP homogenate representing 250 ± 20 eggs is used in each tube, a final pellet is obtained which has a wet weight

FIGURE 3

Electron micrograph of platelets isolated by the method of Ringle and Gross, selected to show what appears to be an extracted nucleus (*N*) and membranous components (*V*). Note that the superficial layer of the yolk platelets has been lost and only the main body (*B*) is preserved. × 8000.

FIGURE 4

Fragments of the main body of yolk platelets prepared by the method of Ringle and Gross. The isolated pieces frequently still show evidence of crystallinity, from an almost hexagonal arrangement of 50 A particles (*a*, spacing between particles is 85 A) and a crossed band (rhombic) system (*b*, spacing is 72 A in each system) to the more frequently observed parallel lines at *c* (81 A), *d* (71 A), *e* (79 A), *f* (64 A), *g* (73 A), and *h* (74 A). Note, however, an incipient breakdown of the crystalline structure along lamellar planes at *c* and what appears to be denatured material (arrows). × 80,000.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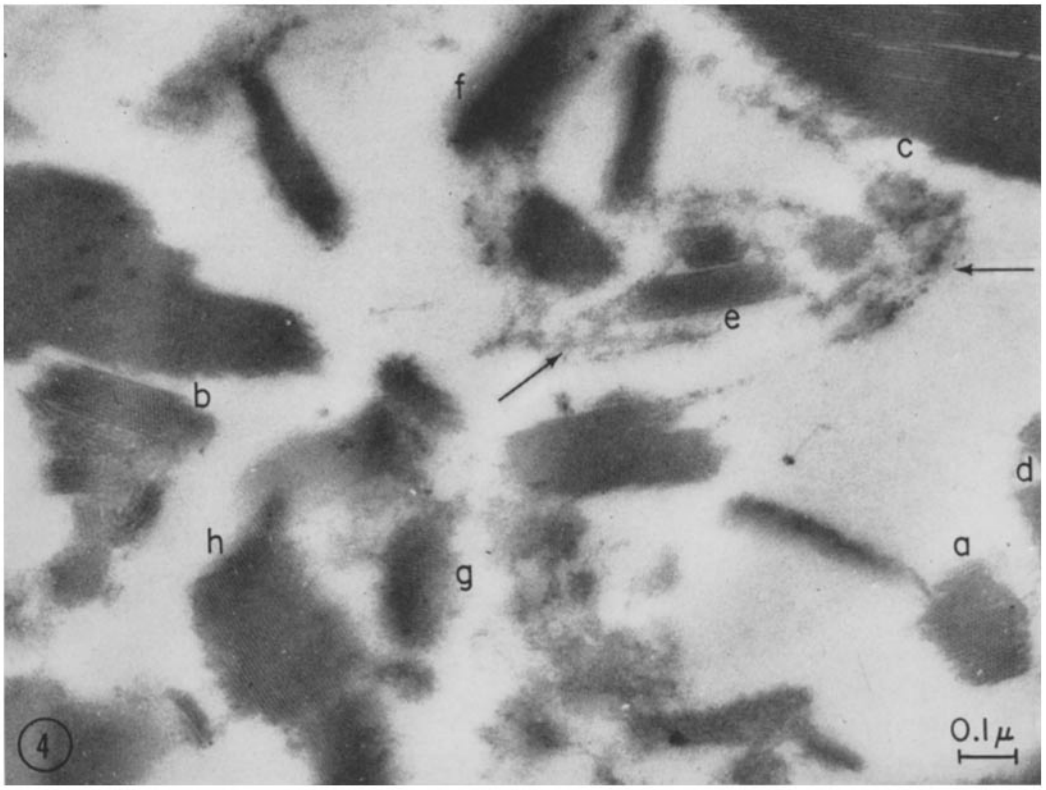
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of 440 ± 30 mg (21 determinations). Sucrose can be eliminated from the final pellet by thorough dialysis against a salt or distilled water solution, but PVP still remains within the dialysis bag as a contaminant. Dilution of the pellet with 0.25 M sucrose (or distilled water) and subsequent centrifugation remove PVP (and sucrose) from platelets, but also most (if not all) of the superficial layer.

Figs. 7 and 8 represent platelets which have been dialyzed for 24 hours against distilled water and finally washed free of PVP by a resuspension in distilled water. It was important to observe such platelets because this treatment removes about 18 per cent of the dialyzed platelet weight but at the same time extracts very little of the proteins of the crystalline matrix (23). Platelets thus treated with distilled water tend more to develop cracks within the main body, but this structure does not fragment into small isolated pieces or show signs of severe lamellar breakdown. Fig. 7 shows part of a treated platelet which is still intact and which now lacks both the outer membrane and the structure previously identified as the superficial layer. There is, however, a new matrix composed of a dense granular material without ordered structure which surrounds the exterior of the crystalline main body. The granular units of this matrix appear very similar to the components of the crystalline lattice. This structure is generally found in sucrose-PVP–isolated yolk platelets which have been treated with distilled water, and occasionally can be seen in platelets treated with simple sucrose solutions. Still less frequently, it can be found as a component of platelets isolated in 0.1 M NaCl.

If the platelets which were treated with distilled water (or platelets which were isolated according to Ringle and Gross) are dissolved in 0.5 M NaCl and the resulting solution is centrifuged to remove any residue, the small amount of insoluble material obtained is composed of both vacuolated sheets and fibrous components (Fig. 9). When the dissolved material is slowly reprecipitated by dialysis against 0.3 M NaCl and then 0.1 M NaCl (17), the "reconstituted" yolk obtained displays a nonordered granular appearance not unlike the new matrix surrounding platelets treated with distilled water (Fig. 10). We have not yet found any indication of periodicity in such reconstituted fragments, as was described by Ringle and Gross (17).

SUMMATION AND CONCLUSIONS

Evidence has been presented that yolk platelets isolated in the presence of PVP more closely approximate those observed *in situ* than do platelets obtained by previously published methods. Specifically, the superficial layer is maintained, and it is suggested that at least part of this component may subsequently be extracted by treatment with distilled water.

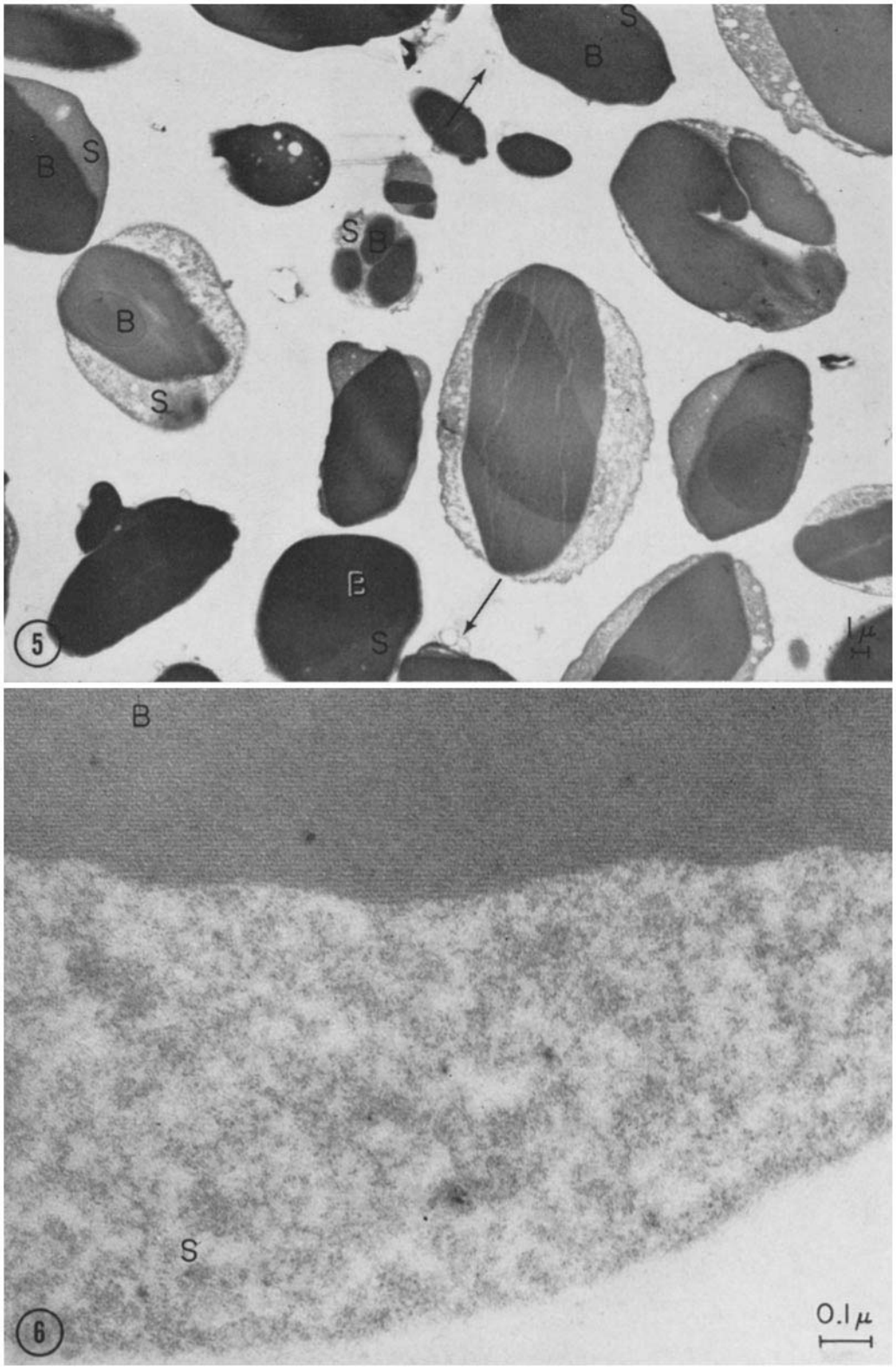
Ohno, Karasaki, and Takata (14) observed that yolk platelets within the intact cell stained metachromatically at their periphery with toluidine blue and gave a positive test in this region with the periodic acid–Schiff (PAS) reaction. This would indicate that the periphery, and hence most likely the superficial layer, contains a polysaccharide material. Takata and Ohno (22) have isolated such a substance (an amylose-like polyglucose) from the yolk platelets of *Triturus pyrrhogaster* and suggest that it is derived from the superficial layer. Ringle and Gross (17) also observed hematoxylin and PAS staining at the periphery of yolk platelets within the oocyte, but they noticed that the platelets lost much of their staining ability when extruded from their

FIGURE 5

A very low power micrograph of yolk platelets isolated in sucrose-PVP medium. Each yolk platelet is comprised of a main body (*B*) and superficial layer (*S*), the latter being rather variable in appearance. A few vesicular membranes (arrows) are present in this fraction. × 3000.

FIGURE 6

High power micrograph of part of a platelet isolated in sucrose-PVP medium. No limiting membrane is present, and the superficial layer (*S*) is composed of many particles or fibrils approximately 50 A in diameter. The similarity of these materials to the units of the crystalline main body (*B*, spacing between parallel lines 92 A) is suggested by a close examination. × 80,000.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environment. The staining reactions were therefore ascribed by these authors to a precipitation of cytoplasmic components around the yolk platelet during fixation *in situ*. Our results would indicate rather than the extruded platelets probably lost their outer membrane and most of their superficial layer. If platelets are isolated in a sucrose-PVP medium, they maintain their surface staining properties (14, 22).

A further point concerns the "membrane" or "ghost" material of isolated platelets originally described by Gross and Gilbert (8) and later identified by Ringle and Gross (17) as being an artifact of preparation. We have frequently observed what we have designated as "denatured material" on the surface of the crystalline main body of isolated platelets when they were exposed to a medium lacking PVP, as indicated in Table I. Such material is generally fibrous or amorphous and vacuolated in appearance, and a similar material is observed in the insoluble residue derived from the main body of platelets dissolved in 0.5 M NaCl (Fig. 9). A precipitation of the material soluble in 0.5 M NaCl and subsequent resolubilization produces another crop of insoluble material, particularly if the precipitated yolk is allowed to stand for a while. This material thus seems to correspond rather well to the "ghost" substance described by Ringle and Gross (17), and we agree that it is probably the result of a "surface denaturation of yolk components." Ringle and Gross then conclude (p. 277), however, that "Although some authors have presented electron micrographic evidence for the existence of an outer membrane on amphibian yolk platelets [2, 12, 25], the existence of such a membrane for mature platelets *in ovo* is questionable," presumably because the membrane found around platelets *in ovo* may be "surface-precipitated cytoplasmic material" formed during fixation in a manner analogous to the formation of "ghost" substance on the surface of the isolated main body component of yolk platelets. The membrane to which we have referred in this paper (Fig. 2; Table I), which surrounds the superficial layer and hence is analogous to the membrane previously described by electron microscopists (2, 10, 12, 25), and which is different from the "ghost" fraction, surface "coat," or "electron-dense layer" of Ringle and Gross (17), may indeed prove to be an artifact since it is not invariably present in all preparations. We prefer to think that it is not an artifact, however, because it can generally be seen in cells which appear to be well preserved (most clearly seen in reference 10, Figs. 1 and 11). Since we have only rarely observed this membrane around isolated platelets, we tentatively conclude that it is broken up into vesicles and lost during the double suspension and centrifugation involved in the isolation procedure.

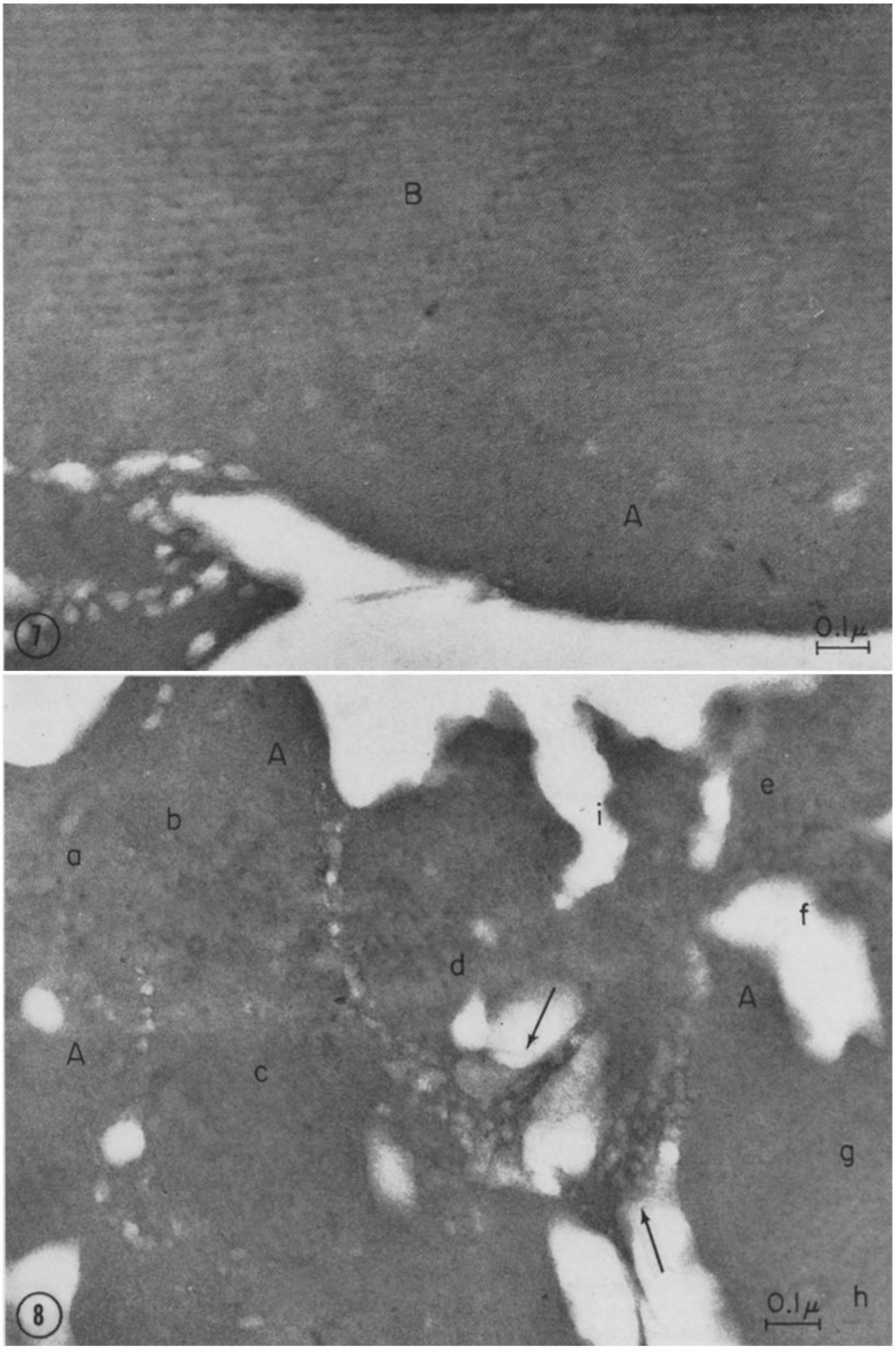
The new dense granular matrix without ordered structure, which is found surrounding the main body component of platelets isolated in a sucrose-PVP medium and treated with distilled water, is also an entity distinct from the substance we have designated as "denatured material." Its distinctive features reside in its relative thickness and its

FIGURE 7

Part of an intact platelet which has been extracted with distilled water following isolation in a sucrose-PVP medium. The main body (*B*) displays a parallel band system of 75 A spacing. Peripherally, the regular band pattern changes into a region (*A*) without ordered structure, which seems composed of dense granular materials approximately 50 A in diameter. The component originally described as the superficial layer is absent. Note the moiré fringe pattern imposed over the normal periodic pattern, probably indicating some degree of shifting within the crystalline lattice. × 80,000.

FIGURE 8

Part of a severely distorted platelet which has been extracted with distilled water following isolation in a sucrose-PVP medium. Evidence of crystalline structure within the irregular mass can be seen by the periodic spacings at *a* (distance between 50 A particles is 85 A), *b* (73 A), *c* (72 A), *d* (71 A), *e* (65 A), *f* (72 A), *g* (66 A), *h* (72 A), and *i* (69 A). Note that the crystalline areas are oriented independently of one another and among these areas can be seen dense granular material (*A*) without ordered structure, similar to the peripheral material in Fig. 7. "Denatured material" is indicated by arrows. × 80,000.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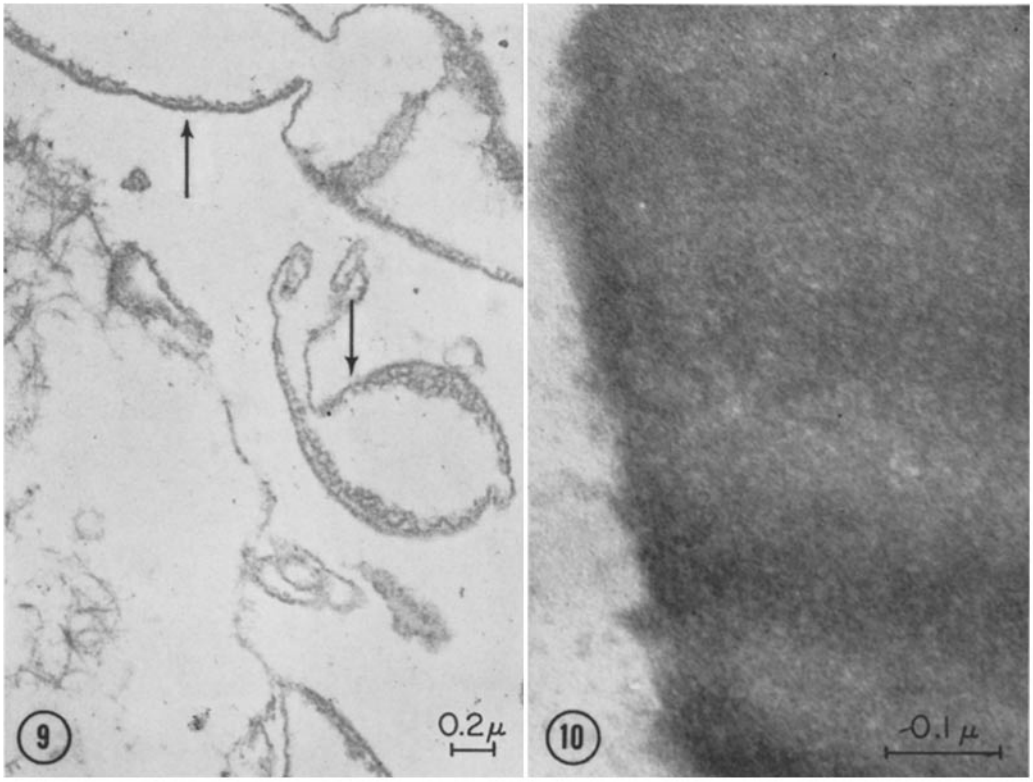

FIGURE 9

Micrograph of 0.5 M NaCl-insoluble material derived from yolk platelets. Vacuolated "sheets" (arrows) and fibrous material (at left of figure) comprise the elements generally seen in these preparations. × 30,000.

FIGURE 10

Micrograph of "reconstituted" yolk. Close examination reveals that such yolk is composed of granular elements approximately 50 A in diameter. × 150,000.

dense and granular appearance. The identification and origin of this substance are uncertain, but two likely alternatives may be postulated:

1. The new layer around the main body may be derived from a component of the superficial layer which, after gradual extraction with distilled water during dialysis, condenses onto the crystalline matrix. The granular components of the superficial layer resemble the units of the new matrix (compare Figs. 6 and 7). Perhaps the removal of an interfering or enveloping substance(s) allows the units of the superficial layer to coalesce onto the main body, although in a non-crystalline manner. That this structure is not found to any great extent in 0.1 M NaCl-isolated platelets may thus indicate a more drastic extraction of the superficial layer during the isolation process.

2. The new dense material may be derived from the main body itself by a process through which the crystalline structure in distilled water becomes disorganized but not fragmented. Evidence for this might be inferred from Fig. 8, which represents an extreme example of damage to a yolk platelet incurred by distilled water treatment. This treatment does not fragment the platelet to the same extent as that seen in Fig. 4. Rather, there is an increased tendency for the crystalline main body to become disorganized so that patches of the crystalline material shift with respect to one another, without necessarily

separating into isolated fragments. Between and surrounding such patches can frequently be seen non-ordered granular material similar to that shown in Fig. 7 and similar to the non-ordered granular appearance of "reconstituted" yolk (Fig. 10).

Unfortunately, however, one cannot decisively choose from the microscopic evidence which of these two interpretations is correct, because the granular units of the superficial layer, the main body, and the new matrix are all of the same size, *i.e.*, about 50 A in diameter.

Finally, when the superficial layer is lost from yolk platelets, the residual main bodies tend to aggregate and, as mentioned above, to form a surface coat of denatured material which may trap or stick to other substances in the homogenate (13). This effect is counteracted by the presence of EDTA and enhanced by the presence of calcium. It is interesting that EDTA also promotes the fragmentation of the platelet, whereas a concentration of calcium only slightly higher than that used in the described isolation procedure causes a solubilization of the yolk (3, 7). To what extent calcium may thus function in the maintenance of the crystalline matrix is unknown. This function has been suggested for the integrity of chicken yolk granules by Schjeide and Urist (21), and Flickinger and Schjeide (5) have also indicated that the phosphoprotein from amphibian yolk binds calcium. Further studies on the effects of calcium, EDTA, and other substances on the crystalline structure of amphibian yolk are needed for a clear understanding of this phenomenon.

We wish to express our gratitude to Dr. Norman Anderson for his encouragement and for making available to us the facilities of his laboratory. We would also like to thank Dr. Kenzo Takata for helpful discussions concerning his work and ideas prior to their publication. During the course of this work, Dr. Wallace was a Postdoctoral Research Fellow of the National Cancer Institute, United States Public Health Service.

The Oak Ridge National Laboratory is operated by Union Carbide Corporation for the United States Atomic Energy Commission.

Received for publication, November 2, 1962.

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