The importance of yolk platelets in the embryonic and larval development of amphibians is indicated by the large proportion of oocytic material contained in these dense, subcellular structures. Of the total egg nitrogen, 69–72% is found in the yolk platelet fraction (Gregg and Ballentine, 1946; Lawrence, Miall, Needham and Shen, 1944). But the precise roles of yolk platelet lipoprotein and phosphoproteins are not yet adequately defined, nor are the mechanisms of yolk synthesis and yolk utilization well understood. A review of the present state of knowledge concerning the development, composition, organization and utilization of the yolk platelet has been presented by Ringle (1960).

A broad appreciation of the factors involved in yolk synthesis and utilization requires an adequate knowledge of the organization and composition of the yolk platelet. A number of investigations have been performed on amphibian yolk platelets in ovo as well as ex ovo, including the following: chemical analyses by McClendon (1909), Fauré-Fremiet and de Streefl (1921), Pannier (1950), Schjeide, Levi and Flickinger (1955), and Gross and Gilbert (1956)—a review of many of the early investigations is given by Needham (1931); cytochemistry by Voss (1927), Hibbard (1928), Holtfreter (1946a) and Laufer (1949)—a review of some of the cytochemical investigations on amphibian eggs is given by Brachet (1950); electron microscopy by Kemp (1956), Eakin and Lehmann (1957), Wischnewski (1957), Karasaki and Komoda (1958) and Ward (1959); immunological analyses by Cooper (1946, 1948, 1950), Flickinger and Rounds (1956), and Glass (1959); electrophoresis by Flickinger and Nace (1952), Barth and Barth (1954), and Schjeide et al. (1955); ultracentrifugation by Schjeide et al. (1955), Gross and Gilbert (1956) and Flickinger and Schjeide (1957); viscosity and flow-birefringence studies by Lawrence et al. (1944); and enzymology by Harris (1946), Brachet (1950), Pannier (1950), Recknagel (1950), Gross (1954) and Nass (1956). These studies have contributed much towards elucidating the organization and composition of the yolk platelet, but there are still many important aspects of the problem left undecided or uninvestigated.

Since yolk platelets are readily isolated from ovarian eggs, as by the homogenization-centrifugation method of Essner (1954), investigations on platelets rela-
tively free from other material are feasible. Some of the previously mentioned studies were performed on such washed, isolated platelets. Analyses on whole, washed platelet suspensions offer a means of determining the composition of amphibian yolk. By lysis with salts the yolk can be fractionated into soluble (YP) and insoluble ("ghost" material) fractions. In addition, washed platelet suspensions provide a readily available source of platelets for other types of investigation (e.g., micromanipulation). One question of importance, however, concerns the composition of the washed platelet suspension, particularly the "ghost" fraction which remains after lysis. If this "ghost" fraction is to be analyzed as a yolk platelet fraction, it must be shown to be largely yolk platelet in origin.

The studies to be reported here are primarily concerned with the following:

a. The nature of the "ghost" material.
b. The nature of the surface of the platelet.
c. The behavior of the platelet components during lysis by sodium and calcium ions.
d. The organization of the yolk components within the platelet.

**Materials and Methods**

1. **Preparation of washed yolk platelets**

Washed yolk platelets were prepared from homogenized ovarian eggs of *Rana pipiens* by the differential centrifugation method of Essner (1954). All homogenizations and centrifugations were carried out using cold solutions (refrigerator or ice bath temperatures) and chilled tubes. Buffers used were either pH 7.0 phosphate buffer (0.05 M KH$_2$PO$_4$-NaOH) or pH 7.2 Tris (hydroxymethyl) amino-methane-HCl 0.05 M buffer. Ovaries were dissected from freshly-pithed frogs, washed in several changes of buffer, and homogenized by hand with a Potter-Elvehjem homogenizer containing a small amount of buffer. The homogenate was filtered through a double thickness of cheesecloth and the filtrate diluted with 100 ml. of additional buffer for the material from each group of 2–3 frogs. The platelets were suspended and washed in 4–5 changes of buffer and in 4–5 changes of 0.1 M NaCl. Centrifugations were conducted for 4–6 minutes each at 140–150 × g. Washed platelet preparations were either used immediately or were refrigerated at 3–5° C. until used.

2. **Micromanipulation**

Micromanipulation experiments were performed using a Carl Zeiss (Jena) sliding micromanipulator. Microneedles and micropipettes were drawn by hand from 0.7–0.8-mm. external diameter glass tubing with the aid of a gas micro-burner. The techniques used were essentially those described by Chambers and Kopac (1950).

3. **Lysis in coverslip-slide diffusion chambers**

Simple chambers were constructed by affixing No. 1 thickness coverslips to glass slides by means of a thin layer of petroleum jelly applied to three edges of the coverslip. A drop of platelet suspension was placed on the coverslip and the
slide then gently pressed down on the coverslip, resulting in a platelet-containing
chamber open at one end. After 10–15 minutes the slide was inverted (i.e., the
coverslip-bearing surface placed uppermost) and the platelets adhering to the
coverslip observed with a microscope. Application of a drop of saline (1.0–2.0 M
NaCl or CaCl₂) to the open end of the chamber allowed saline to diffuse into the
chamber at a rate sufficiently slow to permit the observation of lysis of individual
platelets as the saline front passed.

4. Histological and cytochemical investigations

a. Fresh preparations

Washed yolk platelets and “ghost” material (the insoluble residue of lysed
platelet preparations) were stained in 0.05% toluidine blue O (C.I. 925) made up
in 0.05 M, pH 7.0 phosphate buffer plus 0.1 M NaCl (1:1 by volume).

b. Fixed preparations

Pieces of *Rana pipiens* ovary were fixed for 24 hours in Smith’s modification of
tellyesnicky’s bichromate mixture, washed, and imbedded in paraffin (Tissuemat
56–58.5° C. m.p.) following the method described by Laufer (1949). “Ghost”
material was fixed for 5 hours in Bouin’s picrol-formol solution (Guyer, 1936) and
washed in changes of 50 and 70% ethanol. It was then carried through the usual
higher concentrations of ethanol, ethanol + xylol, and xylol. Imbedding was done
in Tissuemat as for ovarian tissue. Paraffin sections were cut 7 μ thick and affixed
to slides with Mayer’s albumin.

*Feulgen* nucleic reaction. Slides of sectioned ovary and “ghost” material were
stained by the Feulgen method as described by Lillie (1954). Schiff’s leuco-
fuchsin was prepared with basic fuchsin (C.I. 677) by the traditional method.

*Periodic acid-Schiff* (PAS). The method employed was the short PAS
variant described by Lillie (1954).

*Heidenhain’s iron hematoxylin.* A conventional method (Gatenby and Beams,
1950) was used with minor modifications. After staining in 0.5% aged iron
hematoxylin, sections were destained in 2% ferric alum, washed for one hour in
running tap water and counterstained in 0.5% eosin-Y. Some slides previously
PAS-treated were also stained in hematoxylin, without counterstaining in eosin.

*Basic fuchsin and acid fuchsin.* Slides were stained for one hour in 0.5%
aqueous basic fuchsin (C.I. 677) or acid fuchsin and then briefly washed in distilled
water.

5. Electron microscopy of yolk platelets and YC-fragments

Washed yolk platelets were prepared from the homogenized ovarian eggs of
three frogs (*Rana pipiens*) by the homogenization-centrifugation procedure. A
portion of this washed platelet suspension was lyed in 0.5 M NaCl and centrifuged
to yield a clear, “ghost”-free yolk solution (YP₉₈). Platelet-like YC-fragments
were prepared from this YP₉₈ by a reduction in salinity, as described elsewhere
(Ringle, 1960; Ringle and Gross, 1962). In brief, the viscous, dense YC precipi-
tate, which forms following the reduction of YP₉₈ salinity to 0.30 M NaCl, was
made brittle by transferring it to 0.1 M NaCl. After the hardened YC material
was broken up by homogenization in a Potter-Elvehjem homogenizer, the resulting YC-fragments were washed in four changes of 0.1 M NaCl by centrifugation.

Portions of washed yolk platelet suspension and YC-fragment suspension were separately fixed and imbedded. All fixing, washing and infiltrating procedures were carried out in 12-ml. conical centrifuge tubes with low-speed centrifugation to facilitate separation of the fixed yolk materials from their solutions. The fixative used was buffered (pH 7.2–7.4) 1% osmium tetroxide prepared to an osmolarity of 0.14 by the method of Gross, Philpott and Nass (1958). One-half-milliliter quantities of yolk material were added to 8 ml. of fixative and fixed for 5 minutes. The fixed yolk materials were run up through changes of alcohol (30, 50, 70, 83, 95% and absolute alcohol) and then transferred to a 1:1 mixture (by volume) of absolute ethanol and n-butyl methacrylate-methyl methacrylate (4:1). After two one-hour changes of the 4:1 methacrylate mixture the fixed materials were suspended in 4:1 methacrylate containing 2% Luperco catalyst and transferred to gelatin capsules which had been previously half-filled with 4:1 methacrylate and pre-polymerized for four hours. The capsules were placed in a drying oven at 45° C. for three days and then exposed to ultraviolet light for 5 hours.

Sections were cut using a microtome described by Philpott (1955) and spread out on an acetone-water mixture. The sections were supported on a Formvar-100 mesh copper grid. Examination was done with a RCA model EMU-2C electron microscope (250 μ condenser aperture and 65 μ objective). The microscope was tested for and found free of astigmatism previous to examination of the yolk materials.

Results

1. Experiments on isolated platelets and YC-fragments

   a. Micromanipulation

   The responses of yolk platelets and YC-fragments to microdissection were similar in similar media. In distilled water or 0.1 M NaCl these yolk materials were brittle and readily dissected or fragmented by glass microneedles (Figs. 1, 2, 3). This brittleness was found not only for washed platelets and YC-fragments but also for unwashed platelets freshly extruded from crushed eggs. Like the platelets of *Rana pipiens*, platelets of *Rana catesbiana* were brittle at low salt concentrations. Occasionally, fragments of split platelets would remain feebly attached at one margin, thus indicating the presence of a pliable surface coat. This “coat” was more apparent in stored platelets and in platelets briefly exposed to 0.28–0.30 M NaCl prior to dissection.

   Platelet brittleness was also evident in simple experiments involving the crushing of yolk platelets beneath a coverslip by gentle pressure applied to the coverslip with a steel dissecting needle or pencil tip. Directly beneath the applied pressure platelets were flattened out into a thin sheet, the margins of which resembled pseudopodia. The gentler pressure at the periphery of the coverslip, however, merely fragmented the platelets.

   In 0.28–0.30 M NaCl YC-fragments and yolk platelets were soft and pliable. They became more or less spherical and were readily deformed by the microneedles, although the tendency to become globular was much reduced for platelets not freshly prepared. If these softened platelets were returned to a lower salt...
Table: NaCl Molarity and Platelet Response

<table>
<thead>
<tr>
<th>NaCl Molarity</th>
<th>Platelets</th>
<th>YC-Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>Brittle</td>
<td>Brittle</td>
</tr>
<tr>
<td>0.30</td>
<td>Soft</td>
<td>Soft</td>
</tr>
<tr>
<td>0.40</td>
<td></td>
<td>Surface Residue</td>
</tr>
</tbody>
</table>

Figure 1. Responses of isolated yolk platelets and YC-fragments to micromanipulation and lytic salt solutions. A: micromanipulation with glass microneedles. B: lysis by NaCl solutions directed by micropipettes. C: lysis in coverslip-slide diffusion chambers.

Concentration (below 0.28 M NaCl), they again became brittle (Figs. 4, 5). At NaCl concentrations above 0.30 M most of the yolk solubilized, leaving behind an insoluble surface coat which could be stretched or manipulated by microneedles.

By directing a flow of NaCl or CaCl₂ solutions with micropipettes at platelets in distilled water or 0.1 M NaCl, it was possible to observe the lysis of individual platelets and to influence the extent of their lysis. A continuous flow of 1.0–2.0 M NaCl resulted in a rapid lysis of all the platelets in the saline path. Freshly prepared, washed platelets became spherical and rapidly burst. Platelets from preparations which had been stored for several days, however, usually did not round up and, after lysis, left an insoluble residue approximately the size and shape of the platelet before lysis. If the NaCl jet was applied tangentially, a partial lysis of the platelet sometimes occurred, leaving a portion of the platelet intact.

Platelet lysis also resulted from jets of 0.1–1.0 M CaCl₂, although with this salt lysis was only occasionally preceded by a rounding of the platelet prior to the escape of solubilized yolk. If the flow of CaCl₂ was terminated prior to the escape of yolk, the platelet could be fragmented by microneedles. Fragments from these dissected platelets tended to remain attached to a pliable surface coat; and, when the flow of CaCl₂ was resumed, the fragments lysed, leaving behind a faintly visible surface coat.

The results of these micromanipulation studies, as well as the following lysis investigations, are summarized in Figure 1.
b. Lysis in coverslip-slide diffusion chambers

*Lysis of freshly washed platelets.* Lysis by NaCl was usually accompanied by an initial rounding and slight swelling of the platelet as the saline front passed, although some platelets did not round up prior to lysis. The apparent swelling may have been caused by a slight flattening of the yolk mass, since these preparations were viewed from above. Within a second or two after rounding one or more blebs appeared on the platelet surface, followed by a shrinking of the platelet surface as the bleb or blebs enlarged. Finally solubilized yolk escaped from the blebs, leaving behind a shrunken ghost of the lysed platelet (Figs. 1, 6, 7).

CaCl₂ did not ordinarily cause a marked swelling or rounding of platelets, although a few did round up prior to lysis. Lysis by CaCl₂ typically involved a marked wrinkling of the platelet interior, with the direction of wrinkling parallel to the long axis of the platelet. Then solubilized yolk appeared to escape from the entire surface of the platelet, not from one or several localized ruptures as in the case of lysis by NaCl. The residual surface coat or ghost retained approximately the size and shape of the platelet before lysis and did not show the marked shrinking which occurred after lysis by NaCl, although some shrinkage was common (Figs. 8, 9).

*Lysis of unwashed platelets.* Platelets were prepared by crushing ovarian eggs in a small quantity of buffer, about 0.05-0.1 ml. per egg (either 0.05 M, pH 7.2 Tris or pH 7.0 phosphate buffer). Most of these unwashed platelets did not lyse readily with either NaCl or CaCl₂ in the diffusion chamber. When lysis did occur it was both delayed and sporadic. Within a minute or two after the passage of NaCl some of the platelets began to show striae or wrinkles parallel to their long axes, while others rounded up slightly. These changes were followed by vacuole formation within the platelet and by an irregular swelling of the platelet. Those platelets which did lyse left behind a shrunken ghost, although many platelets were still intact or swollen 5 minutes or longer after passage of the saline front (Figs. 10, 11, 12). Prolonged exposure (5-10 minutes) to CaCl₂ caused a marked swelling of many platelets, but lysis was relatively incomplete.

After dilution of these unwashed platelet suspensions with 2-3 volumes of buffer, lysis by saline was still abnormal when compared with the lysis of washed platelets. Lysis was delayed for 10-30 seconds or more after passage of the saline front, and it occurred at random among the platelet population without detectable rounding or bleb formation. Lysis in the presence of egg cytoplasm...
resulted in the appearance of numerous lipid droplets, which occasionally clustered around the residual ghosts of lysed platelets. Platelets from these crushed egg suspensions, if washed in several changes of buffer, reacted to lytic salt solutions like platelets from the homogenization-centrifugation procedure. Thus, relatively long exposure (one hour or more) to the cytoplasmic materials of crushed eggs did not permanently alter the lytic properties of platelets.

_Lysis of stored platelets._ NaCl-lysis of washed platelets which had been stored under refrigeration (3–5° C.) for 6–10 days did not usually produce a rounding or swelling of the platelets. Instead, bleb formation occurred at one of several places on the platelet surface, and particles in Brownian motion streamed from the platelet interior into the swelling blebs. After a few seconds fluid yolk escaped from the blebs, leaving behind an insoluble ghost which retained the approximate size and shape of the original platelet. Shriveled residues of the surface blebs were also visible (Figs. 1, 13, 14, 15). During lysis by CaCl₂ the interior of these stored platelets became wrinkled in appearance, as was also true for freshly prepared platelets. After lysis by CaCl₂ particles exhibiting Brownian motion were trapped within the ghost and could be seen as long as 15 minutes after lysis (when observations were terminated).

_Lysis of YC-fragments._ Lysis of YC-fragments by NaCl proceeded initially as for freshly washed platelets by rounding up, but bleb formation was not observed. Instead, the spherical yolk mass swelled and finally burst, leaving behind a shrunken, sac-like residue. YC ghosts did not readily remain attached to the coverslip but were usually swept away by the diffusing saline. Lysis of YC-fragments by CaCl₂ proceeded as for freshly washed platelets.

2. Histological and cytochemical investigations

_a. Fresh preparations_

Washed yolk platelets of _Rana pipiens_ stained metachromatically in buffered toluidine blue O, the platelet surface staining darker than the platelet interior. “Ghost” material stained only lightly, except for scattered patches (apparently cell nuclei, insoluble surface residues of lysed platelets, and unidentified granules). Some surface residues of lysed platelets enmeshed in “ghost” substance retained the size and shape of unlysed platelets. Preparations of “ghost” material from platelet suspensions of _Rana catesbiana_ also contained larger (40 μ or more in diameter) amorphous metachromatic patches.

_Figure 10._ Yolk platelet from crushed egg in cytoplasm, slightly diluted with pH 7.0 phosphate buffer in coverslip-slide diffusion chamber. Scale marker indicates 10 μ for Figures 10–15.

_Figure 11._ Same platelet as in Figure 10 after passage of diffusing NaCl front. Note vacuoles within the platelet.

_Figure 12._ Ghost (arrow) of platelet shown in Figures 10–11. Note lipid droplets.

_Figure 13._ Washed, stored (refrigerated 10 days) yolk platelet in 0.1 M NaCl in coverslip-slide diffusion chamber.

_Figure 14._ Same platelet as in Figure 13 during lysis by diffusing NaCl. Note several blebs formed at surface of platelet.

_Figure 15._ Ghost of platelet shown in Figures 13–14 with surface bleb residue.
b. Fixed preparations

The results of the following staining procedures on fixed ovary and “ghost” material are summarized in Table I.

*Feulgen nuclear reaction.* Yolk platelets of sectioned oocytes showed a faint fuchsin color in acid-hydrolyzed slides, whereas control slides gave either a negative or much reduced reaction. Extruded platelets (i.e., platelets released from eggs cut during dissection of the ovary and fixed along with intact ovarian eggs) were stained slightly or not at all when compared with platelets fixed in ovo.

“Ghost” material contained a large number of distorted, Feulgen-positive patches, resembling the nuclei of follicle cell membranes in size and distribution.

*Periodic acid-Schiff (PAS).* Sectioned oocytes exhibited a large amount of PAS-positive granular material distributed among the platelets and adhering to platelet surfaces. Platelets in *ovo* showed distinctly PAS-positive margins, but only faintly positive or PAS-negative interiors. Extruded platelets, however, were either PAS-negative or showed only slightly stained margins (Figs. 16, 17). Follicle membranes were markedly PAS-positive and in control slides stained faintly, which was similar to the staining behavior of the bulk of the “ghost” material.

*Heidenhain’s iron hematoxylin.* Sectioned ovary counterstained with eosin showed intensely eosinophilic follicular membranes containing hematoxylin-stained nuclei. Within eggs the margins of yolk platelets were darkly stained by hematoxylin, although platelet interiors were either unstained or only lightly tinted. Extruded platelets showed less intensely stained surfaces than did platelets in *ovo*. The matrix of “ghost” material, like the follicular membranes, was eosinophilic and contained a number of hematoxylin-stained nuclei.

Oocyte sections first stained by the PAS-method and then stained with hematoxylin were much more markedly stained than sections not previously PAS-

**Table I**

*Results of histological and cytochemical staining procedures*

<table>
<thead>
<tr>
<th>Staining procedure</th>
<th>“Ghost” material</th>
<th>Ovary</th>
<th>Follicle cells</th>
<th>Platelets in ovo</th>
<th>Extruded platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclei</td>
<td>Matrix</td>
<td></td>
<td>Surface</td>
<td>Interior</td>
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<tr>
<td>Feulgen nuclear</td>
<td>++++</td>
<td>±*</td>
<td>+</td>
<td>+</td>
<td>±</td>
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<tr>
<td>Periodic acid-Schiff</td>
<td>+ + + +</td>
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<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Hematoxylin + eosin</td>
<td>H +++ +</td>
<td>E + + + H</td>
<td>E + + + H + + +</td>
<td>H ++</td>
<td>H −</td>
</tr>
<tr>
<td>PAS + hematoxylin</td>
<td>H + + + +†</td>
<td>H + + + H +</td>
<td>H ++</td>
<td>H ±</td>
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<tr>
<td>Acid fuchsin</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>±</td>
</tr>
</tbody>
</table>

* For both experimental and control sections.
** Nuclei.
†† Non-nuclear matrix.
††† Small scattered patches.
treated. Platelets in ovo showed more darkly stained surfaces as well as some stain in the platelet interiors. Extruded platelets were also stained, but less than platelets within oocytes. The ground substance of the follicular membranes did not stain with hematoxylin, nor did the bulk of the sectioned “ghost” material (which did, however, contain some hematoxylin-stained patches resembling surface residues of lysed platelets).

Basic fuchsin and acid fuchsin. The interiors of platelets in sectioned oocytes were moderately to weakly stained by basic fuchsin, whereas their surfaces were darkly stained. Platelets did not stain as intensely in acid fuchsin, although they did possess a distinctly fuchsin-colored surface. The fine granular material distributed among the platelets stained much more deeply with acid fuchsin than with basic fuchsin. As for the other staining reactions previously described, extruded platelets did not stain as intensely as platelets in ovo, except for platelets surrounded by extruded cytoplasmic material. Follicle membranes stained lightly in basic fuchsin (except for their darkly stained nuclei) but were intensely colored by acid fuchsin. “Ghost” material stained like follicle membranes with these dyes.

3. Electron microscopy of platelets and YC-fragments

Electron microscopy of osmium-fixed, washed yolk platelets of Rana pipiens revealed an orderly arrangement of yolk components within the platelet (Figs. 18, 19). Approximately 36-A wide lamellae (or bands of closely packed units) were oriented more or less parallel to the long axis of the platelet. Periodicity, the distance between centers of adjacent lamellae, was 72 A. Peripheral electron-dense material sometimes gave the appearance of an irregular outer coat, but examination showed this surface material to be only a more opaque continuation of the internal yolk (since periodic lamellae continued into this “coat”).

The organization of yolk components in YC-fragments was not as distinctly lamellar as that of platelets, although micrographs of the YC-fragments showed a compact structure as well as some indication of periodicity (Fig. 20). There was an irregular banding by linearly arranged units similar in size and appearance to the electron-dense substance comprising the lamellae of yolk platelets.

Discussion

1. Experiments on isolated platelets and YC-fragments

Washed yolk platelets and YC-fragments were rigid, brittle bodies in calcium-free, low-saline solutions (less than 0.28 M NaCl). This rigidity would be expected from the normally angular, crystal-like shapes of yolk platelets in ovo. Observations by Holtfreter (1946b) on the breakup of platelets during intracellular digestion also indicated a rigid nature for these bodies. However, Holtfreter (1946a) claimed a plastic consistency for amphibian yolk platelets, since he found that platelets squeezed between glass plates spread out into lobular sheets. This plasticity under heavy pressure applied to the entire surface of the platelet was confirmed in the studies reported here. Nevertheless, locally applied pressure with a microneedle tip revealed that platelets are sufficiently brittle to be readily split into fragments. Since YC-fragments showed the same behavior during micro-
manipulation, this suggests a similar packing of the yolk constituents in platelets and YC-fragments.

The solubilizing effects of salts on amphibian yolk are well known. As early as 1921 Fauré-Fremiet and de Streel showed that 10% NaCl (as well as strongly acid or alkaline solutions) dissolved the yolk platelets of *Rana temporaria*. Needham (1931) discussed the solubility of many kinds of yolk in salt solutions. Other investigations on the effects of ions on yolk solubility have been reported by Holtfreter (1946a), Terry (1950), Essner (1954), Gross (1954), Flickinger (1956), and Flickinger and Schjeide (1957). Yolk platelets are readily solubilized by 0.4 M concentrations of monovalent cation salts, and the soluble yolk is readily precipitated by dilution (to less than 0.28 M in the case of NaCl). Solubilization by salts of divalent cations occurs at much lower concentrations (e.g., 0.003 M CaCl₂) and prolonged exposure to lytic concentrations of calcium renders yolk permanently soluble (Gross, 1954; Flickinger, 1956). Such differences in the response of amphibian yolk to NaCl and CaCl₂ point to different mechanisms for the solubilization of yolk by monovalent and divalent cations. Observations reported here on individual platelets during lysis likewise indicate differences in the effects of NaCl and CaCl₂ (degree of platelet swelling, bleb formation, escape of fluid yolk, wrinkling, etc.).

The effect of near-lytic concentrations of NaCl (0.28–0.30 M) was a marked increase in platelet plasticity. Although there were still sufficient binding forces to prevent yolk components from going into solution, platelets readily lost their angularity and could be easily deformed by microneedles. The plastic consistency of platelets in 0.28–0.30 M NaCl was similar to that of yolk precipitated from solution by a reduction of NaCl concentration to the same molarity (YC). As reported elsewhere (Ringle and Gross, 1962) the volume occupied by this plastic YC material is equal to the volume of the packed platelets before lysis, again indicating the platelet-like organization of yolk components in YC.

Micromanipulation and observations of washed platelets during lysis showed that isolated platelets possess an insoluble surface different from the soluble material within the platelet. However, whether or not this surface material exists as such in ovo is questionable. Since the surface coat increased in prominence with time after isolation of platelets from the egg, one suspects that even the coats of freshly washed or extruded platelets may be preparation artifacts resulting from a surface denaturation of yolk components. Also, surface coats were found on the platelet-like YC-fragments prepared from solubilized yolk. Hence, one must reserve judgment concerning the presence or absence of surface coats on platelets.

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**Figure 16.** PAS-stained yolk platelets in ovo. Note staining of platelet surface. Scale marker indicates 10 μ for Figures 16–17.

**Figure 17.** PAS-stained extruded yolk platelets. Note platelet surfaces are less intensely stained than in Figure 16.

**Figure 18.** Electron micrograph of a portion of an osmium-fixed washed yolk platelet. Note orientation of yolk components in bands approximately parallel to the platelet surface. Electron-dense surface (arrow) also shows periodic banding. Scale marker indicates 0.1 μ.

**Figure 19.** Electron micrograph of platelet showing distinct periodicity (72 Å). Scale marker indicates 0.1 μ.

**Figure 20.** Electron micrograph of sectioned YC-fragment showing an irregular banding of yolk components. Scale marker indicates 0.1 μ.
in ovo from evidence involving isolated platelets. The presence of a coat on isolated platelets only indicates the possibility that such a coat normally exists in ovo.

2. Histological and cytochemical investigations

a. Nature of the “ghost” material

Washed yolk platelet suspensions in the presence of lytic concentrations of salts yielded soluble yolk and a small insoluble residue referred to here as “ghost” material. This “ghost” material was investigated by several histological and cytochemical methods to establish whether or not it was largely made up of the insoluble surface residues of lysed platelets. The results of staining reactions for both fresh and fixed material strongly suggest that the bulk of the “ghost” material consists of fragmented ovarian membranes plus only a small quantity of lysed platelet ghosts. The large metachromatic patches found in “ghost” material of *Rana catesbiana* may have been fragments of the metachromatic egg jelly layer, since this bullfrog material was prepared relatively late in the season (May 15th). Kelly (1954) has proposed that the inner metachromatic jelly layer of uterine eggs arises from the egg itself and not from the oviduct. It is of interest in this regard that homogenates of *Rana pipiens* ovarian eggs prepared late in the spring were often rendered useless as sources of platelets by a massive gummy precipitate which appeared during the first centrifugation.

b. Nature of the platelet surface

Staining reactions of fresh preparations indicated the existence of some sort of surface material different from the platelet interior. Not only did the surfaces of washed platelets stain more intensely with toluidine blue than did platelet interiors, but also toluidine blue-stained residues of lysed platelets (platelet ghosts) were found enmeshed in “ghost” material. These staining reactions support the evidence for the existence of a surface coat on platelets as found in micromanipulation and lysis experiments.

Staining reactions of platelets fixed in ovo also showed a surface coat, which was markedly stained by PAS, iron hematoxylin, basic fuchsin and acid fuchsin. However, the stained sections also revealed a tendency for cytoplasmic material to aggregate around yolk platelets, leaving large, optically clear spaces between platelets. Fixed extruded platelets (platelets released from oocytes cut during dissection and carried along with ovarian material through fixing and subsequent procedures) did not usually have as intensely a stained surface as did platelets fixed within oocytes. When a distinctly stained surface did occur on extruded platelets, these platelets were surrounded by cytoplasmic material which had been extruded along with the platelets from cut oocytes. Thus, the evidence suggests that the intensely staining coats of platelets fixed in ovo were produced by precipitated cytoplasmic components as a result of fixing techniques. Since both extruded platelets and washed platelets might also acquire similar surface precipitation coats during crushing, homogenization and washing procedures, even the occurrence of surface staining for such platelets would not prove that a coat exists for platelets in viable eggs. Of course, staining reactions leave unanswered any questions concerning a submicroscopic surface membrane.
c. Feulgen-positive yolk platelets

In addition to the Feulgen-positive nuclei of "ghost" material and ovarian membranes, a faintly positive response was noted for platelets in sectioned oocytes. Hibbard (1928) and Brachet (1950) previously reported a positive Feulgen reaction for amphibian yolk, but even in the absence of acid hydrolysis. However, results reported here for the yolk of Rana pipiens showed a greatly reduced response when acid hydrolysis was omitted. It is, nevertheless, open to question whether this Feulgen reaction of yolk is caused by DNA or is an artifact. The presence of non-nuclear DNA or DNA-precursors has been demonstrated for the amphibian egg by a number of investigators (Kutsky, 1950; Hoff-Jørgensen and Zeuthen, 1952; Sze, 1953; Gregg and Løvtrup, 1955; Finamore and Volkin, 1958; Grant, 1958; Bieber, Spence and Hitchings, 1959). But, according to Gregg and Løvtrup, the quantity of DNA that might exist in the cytoplasm of amphibian eggs could not be demonstrated by the Feulgen method. Even if caused by the presence of DNA, however, the weakly positive Feulgen response of yolk platelets in fixed oocytes does not unequivocally indicate the presence of DNA in platelets in vivo. It is possible that DNA or DNA-precursors diffuse into the platelets during fixation, as is suggested by the absence or diminution of the Feulgen stain in washed platelets and in extruded platelets.

3. Electron microscopy of platelets and YC-fragments

a. Nature of the platelet surface

Although some authors have presented electron micrographic evidence for the existence of an outer membrane on amphibian yolk platelets (Eakin and Lehmann, 1957; Wischnitzer, 1957; Karasaki and Komoda, 1958), the existence of such a membrane for mature platelets in ovo is questionable. Except for the possibility (certainly not to be disregarded) of membrane removal during isolation and preparation procedures, the failure to find a membrane for isolated platelets as reported here indicates that a differentiated surface coat may not be a normal feature for platelets in ovo. It is of interest that the membranes reported by other investigators were not a constant feature in their preparations. Since all the previous investigations were conducted on platelets fixed intracellularly, the membranes observed in these preparations might be surface-precipitated cytoplasmic material. In regard to yolk in embryos, platelets in embryonic ectodermal cells may be subject to surface alteration and enzyme deposition which would introduce another source of confusion. An electron-dense surface was sometimes visible on platelets in the study reported here, but this surface appeared to be similar or identical to the rest of the yolk platelet in periodic structure. It is possible that this electron-dense layer represents the coat found in micromanipulation and lysis studies.

b. Periodic structure of yolk

The existence of a periodic structure for yolk platelets has been reported for a number of amphibian species including Triturus viridescens (Wischnitzer, 1957), Triturus pyrrhogaster (Karasaki and Komoda, 1958) and Rana pipiens (Ward, 1959). The periodicity reported for the urodele forms, 70 Å, is similar to the periodicity for platelets of Rana pipiens as noted by Ward (personal communication).
and as reported here, 72 Å. A periodic organization of yolk components has also been reported for some invertebrates, including *Limnea stagnalis* (Wischnitzer, 1957) and *Planorbis corneus* (Favard and Carasso, 1958). It is likely that an ordered, periodic structure will be found for the yolk of many other organisms, especially in those forms with dense, crystal-like platelets.

The 72 Å-spaced lamellae found in the isolated yolk platelets of *Rana pipiens* were oriented approximately parallel to the long axis of the platelet. As also noted by other investigators for their preparations, these lamellae seem to consist of units closely packed along the length of the bend. Although electron micrograph resolution did not usually permit a clear definition of these units, near the edge of the platelet the bands were shown to be composed of individual units (Fig. 19).

The appearance of a somewhat irregular periodic structure and close packing for yolk in YC-fragments showed the similarity of YC to yolk platelets. This orientation of yolk components in YC-fragments may be important in the consideration of yolk platelet synthesis from yolk-like substances carried in the plasma of female frogs. Since yolk components can be easily precipitated from solution in vitro to form a densely packed YC material similar in organization to the yolk platelet, it is possible that yolk platelet synthesis is primarily a localized precipitation of yolk components following their transfer from the plasma to the growing oocyte.

**Summary**

1. Yolk platelets of *Rana pipiens* ovarian eggs were studied by micromanipulation, lysis, histological and electron microscopic techniques. Investigations were carried out on washed, isolated platelets; unwashed, extruded platelets; and platelets *in ovo*.

2. The presence of a surface coat was demonstrated for isolated, washed platelets by micromanipulation and lysis. However, the evidence suggested that this coat might be a preparation artifact. Histological studies also showed a surface coat for platelets *in ovo*, but evidence indicated that this coat too was an artifact.

3. “Ghost” material (the insoluble residue of platelet suspensions after lysis) was found to be largely made up of follicular and other ovarian membrane fragments.

4. Yolk platelets fixed *in ovo* were faintly Feulgen-positive.

5. Electron microscopy of osmium-fixed, washed yolk platelets showed a periodic arrangement of yolk components (72 Å periodicity of 36 Å-wide lamellae or bands of closely spaced units). An electron-dense surface was noted on some platelets, but this “coat” showed the same periodic structure as the rest of the platelet.

6. Electron microscopy of YC-fragments (platelet-like material prepared from soluble yolk by a simple precipitation process) showed a close packing of yolk components similar to the packing found for yolk platelets.

**Literature Cited**


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