THE ORIGIN OF PROTEIN AND
FATTY YOLK IN RANA PIPIENS

II. Electron Microscopical and Cytochemical
Observations of Young and Mature Oocytes

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ABSTRACT

Electron microscope studies of young oocytes have demonstrated that the plate-like, hexagonal shaped yolk bodies previously observed in living cells are wholly within the substance of oocyte mitochondria and that they remain within these mitochondria while increasing in size. These bodies possess a crystalline structure consisting of what appear to be lines, with a spacing of 70 to 85 Å, and appear very dense in the electron microscope. After formalin fixation such bodies give an intense positive test for protein, and when viewed in the electron microscope are only slightly less dense than after OsO₄ fixation. Evidence is presented for the origin of these crystals within a single crista. The clusters of yolk globules previously studied in living cells are seen to consist of several types of bodies, but an irregular dense droplet predominates. This dense material is apparently secreted by small spherical bodies which, the evidence suggests, originate from the breaking up of filamentous mitochondria and which possess an outer double membrane and sometimes internal cristallike membranes. When thin sections of young oocytes are immersed in xylol the dense globules of the clusters are dissolved, but the hexagonal bodies are unaffected, indicating that the globules are of a predominantly fatty nature, while the hexagonal bodies are of a predominantly protein nature. Examination of mature or almost mature oocytes has revealed that the main body of the yolk platelets is crystalline in nature and is surrounded by a thick matrix which, in light microscope study, masks the fact that the face view of the main body of the platelets is often hexagonal. The spacing within the main body is found to be 70 to 85 Å. The crystal laminae of this material can be resolved quite clearly into rows of particles. Dense globules of varying sizes are found in the cytoplasm between the platelets. When thin sections of these OsO₄-fixed oocytes are immersed in xylol, the material of the globules is extracted and the crystalline material of the platelets remains unaffected, indicating the fatty nature of the globules and the protein nature of the platelets. The platelets of the mature egg resemble the hexagon bodies, previously described in young oocytes, in their protein nature, their crystalline spacing, and their hexagonal outline. This is given as strong evidence for the origin of the mature platelets by the growth of the intramitochondrial hexagon bodies. The biochemical implications of this study are discussed.
INTRODUCTION

The following summary of the status of the problem of the origin of the yolk of eggs was given by Wilson (1925, p. 339), and until very recently would have been an accurate summary of the modern situation.

"In spite of numerous researches on yolk-formation, extending over a period of more than fifty years, the subject still remains in so confused a state that all statements in regard to it must be made with considerable reserve. Even in the recent literature we find the origin of the yolk-spherules ascribed to mitochondria, to Golgi-bodies, to chromidia, extruded nucleoli or nuclear fragments; while some observers consider that the yolk arises de novo in the cytoplasmic substance without discoverable relation to other formed elements. We must, therefore, conclude either that there is no general uniformity in the mode of yolk-formation, or that many of the existing accounts of the subject are erroneous."

One of the earliest cytoplasmic bodies assigned a role in the origin of yolk was the so called "yolk-nucleus" observed by Wittich (1845) in spider oocytes. This perinuclear body was described in the spider *Tegenaria parietina* by André and Roullier (1957) in an electron microscope study. Such a body does not exist in *Rana pipiens* (Ward, unpublished observations). This absence was already noted by careful light microscopists such as Gatenby (1920) and Konopacki (1927) who denied the presence of a yolk-nucleus in the frog as a result of studies using fixatives such as Flemming's without acetic acid which, in light microscopy, yields results very similar to those obtained with buffered osmium tetroxide. The term "yolk-nucleus" has often been applied to bodies in the cytoplasm of amphibian oocytes. Recently Kemp (1956) has so used it in an electron microscope study of oogenesis in *Rana pipiens*. The general ultrastructure of such bodies is not the same as that described in spiders and so avoidance of such a usage seems preferable.

It seems best to use a term such as "perinuclear zone" for the area in frog oogonia which is similar in appearance to the idiozone in spermatogonia. The fine, granular material found in this area Van Bambeke (1898) called "pallial substance," and retention of this term would appear to be useful since this component is present in very young frog oocytes after fixation with buffered OsO₄ (Ward, unpublished observations).

Though Gatenby did not publish the results of his studies on yolk formation in the amphibia, he refers to these results in a review (Gatenby and Woodger, 1920). He noted the concentration of mitochondria at the periphery of the oocyte just before the appearance of the yolk granules and concluded that, since the granules arise in intimate association with the mitochondria, the latter are probably the source of the former.

Konopacki (1927) described the relation of the mitochondria to the origin of the fatty and protein yolk in the frog in a way which appears to be quite similar to the account given in this paper. He found that the young oocyte contained two forms of mitochondria, a filamentous form which he called "chondriomite," and a rod-shaped or batonnet form which he called "chondriilont." Konopacki believed the chondriomites were a form of mitochondria primarily because of their staining reactions. When they were very small they stained totally red with Altmann's acid fuchsin like the other mitochondria, but as they grew in size their interiors were negative to the stain, and their exteriors positive. The growth of these bodies revealed that they became the yolk platelets. The completely mature platelets still possessed a very faint exterior rim of stain. Some of the chondriomites, on the other hand, were seen to break up into chains of small beads, and from these small beads drops of lipid appeared to form, and though there was a fuchsinophilic rim on these droplets at the start of their formation, they soon became completely fuchsin-negative.

Nath (1931) has postulated the origin of the yolk platelets in *Rana tigrina* as intimately associated with the mitochondria but states that fatty yolk is completely absent. In this paper he is concerned mainly with a demonstration of the vacuome, which he finds very highly developed in *R. tigrina* but extremely scanty in *Rana cyanaophyctis*. We have as yet observed nothing corresponding to a vacuome in *Rana pipiens* and, on the other hand, have observed the presence of large amounts of fatty yolk. Since Nath himself finds a great difference in the development of the vacuome in the two species of frogs he studied, it may be that species differences in yolk formation are sometimes much greater than would be anticipated.

It is of some interest to review the highly varied..."
results of some light microscopists who worked on the problem of yolk formation in several other species using techniques which yielded results very similar to those obtained with buffered OsO₄ in the present study. The outstanding workers in this field have been Hirschler, Gatenby, and Nath. The results of the first author on two very different species will suffice to illustrate the different results obtained. In the egg of Ascaris, Hirschler (1913) found a situation very similar to that which Konopacki found in the frog. Mitochondria swell and develop a crystal violet-negative interior which enlarges until mature yolk granules possessing a thin crystal violet-positive rim are produced. In this case the Golgi dictyosomes are present throughout oocyte growth but seem to take no part in yolk formation. On the other hand, in an ascidian, Ciona, Hirschler (1916) found the occurrence of two routes of yolk formation leading to the production of yolk granules. One was an enlargement and direct transformation of mitochondria into yolk, and the other was a fusion of Golgi bodies with swollen mitochondria and the transformation of this complex into yolk.

The last result resembles closely that found by Favard and Carasso (1958) in Planorbis by means of modern electron microscope techniques. In this snail, two methods of origin for the yolk granules were described:

1. A direct transformation of mitochondria into yolk granules.
2. A fusion of mitochondria with certain cytoplasmic multiple-membrane-surrounded vesicles. (These vesicles are believed by Favard and Carasso, however, not to be related to the Golgi dictyosomes.)

In general, the work of many observers on many species has implicated four organelles in the origin of yolk: the mitochondria, the Golgi bodies, nucleolar extrusions, and the ground substance of the cytoplasm. The electron microscope has clearly shown the involvement of the mitochondria (Favard and Carasso, 1958; and this paper). Certainly Gatenby's findings in Patella (Gatenby and Woodger, 1920), that Golgi batonettes surround the developing yolk granule as it is formed, would lead us to expect the involvement of this organelle in the formation of yolk in some species. Also, Nath, in a long series of papers, has implicated the Golgi bodies in the origin of fatty yolk, a view also long supported by Gatenby. In a brief electron microscope study on the origin of yolk in the snail, Cipangopaludina malleata, Yasuzumi and Tanaka (1957) have implicated the Golgi membranes in yolk formation. “Chromidial bodies” possibly originating from juxtanuclear double lamellae are enveloped by Golgi membranes and form a complex yolk body which accumulates lipid and an electron-transparent material. The lipid portion is then extruded and the remaining multilayered, electron-transparent, filled body is the yolk granule. The direct relation of the nucleolar extrusions to yolk formation has not been demonstrated convincingly at the light microscope level and, so far, electron microscope studies have given no support to this view.

There is no question that morphological study of the origin of yolk has often led to disagreement because of the difficulties in determining the origin of granules or droplets which, at their earliest stages, are at the limit of resolution of the light microscope and which might very well be considerably below this limit at still earlier unobservable stages.

Some of the classical cytologists thought these problems were solved by the staining techniques they used which served to designate certain bodies as mitochondrial, etc., but the interpretation of their results involved unproved assumptions. In their studies have given no support to this view.

“Loyez (1909), Hirschler (1916) and Harvey (1927) all reported that mitochondria enlarge as they transform into yolk. Their material was stained in haematoxylin, acid fuchsin or crystal violet. It has been our experience, and it was also theirs, that these stains do not differentiate young yolk granules from mitochondria. Using these stains they could see in the young oocytes only what they took to be mitochondria; and in older oocytes they could see, in addition, some bigger granules which appeared to them like enlarged mitochondria and which eventually became definitive yolk granules. We have studied oocytes treated with the same techniques as theirs, and we must admit that the picture one can see in such material does appear to favor a mitochondrial origin of yolk granules. But we must not lose sight of the possibility that there may be scattered among the mitochondria a different class of granules which, though showing the size, appearance and tinctorial quality of mitochondria, are really unrelated to them. That in the oocytes of
Boltenia siliosa there really exists such a class of granules is borne out by the evidence we have presented in this paper. So it could well be that in reporting what they believed to be mitochondria enlarging while transforming into yolk granules, the above-mentioned authors were really describing the enlargement of the yolk granules which have no morphological relation to mitochondria at any stage of their development."

In the present work, as well as that of Favard and Carasso (1958), the techniques of electron microscopy have enabled the investigators to visualize yolk granules or droplets below dimensions resolvable by the light microscope. Further, in this modern work, organelles such as mitochondria are identifiable by their characteristic ultrastructure rather than their tinctorial affinities.

Thus the solution of the problem is seen to have been beyond the techniques available to the classical cytologists. Nevertheless, one cannot fail to be impressed by the accuracy of observations of such competent observers as Konopacki (1927), for example, who so precisely described the major details of yolk formation in *Rana fusca* by utilizing to the utmost the scientific tools available.

**MATERIALS AND METHODS**

**Electron Microscopy**

It was found impossible to get adequate preservation of young frog oocytes if ovaries of either young or old females were used. Success was obtained only with the ovaries of tadpoles or newly metamorphosed frogs. The ovaries of tadpoles younger than Stage 17 contain few, or none, of the yolk-forming centers, and so are not considered in the present study. The range of oocyte sizes is similar in ovaries of tadpoles of any stage from Taylor and Kollros Stage 17 to the newly metamorphosed frog. Sections of such ovaries are, therefore, the basis for the electron micrographs of the early formation of yolk.

For observation of yolk in the mature egg, single ovarian eggs of sizes from one to 1.5 mm in diameter were embedded. The completely mature eggs (1.5 mm in diameter) were extremely difficult to fix and embed satisfactorily. Success was obtained only when formalin fixation was used without prior or post-OsO₄ fixation. Eggs 1 mm in diameter, however, were successfully fixed with OsO₄. Eggs 1 to 1.5 mm in diameter are designated as "mature" in this paper. The preservation of the "mature" eggs still leaves much to be desired but was adequate for the purposes of this study. Both the young ovaries and the "mature" eggs were fixed in the following two fixatives:

**Buffered OsO₄ Fixative (Fixation Time—15 Minutes)**

**Buffer:** A phosphate buffer of pH 7.4 was prepared by combining M/30 Na₂HPO₄ (four parts) with M/30 NaH₂PO₄·H₂O (one part). To each 100 ml of buffer the following salts were added:

- NaCl 0.044 gr
- KCl 0.012 gr
- CaCl₂ 0.0001 gr

One-half gram of OsO₄ was added to 25 ml of the buffer-salt mixture.

**Buffered Formaldehyde Fixative (Fixation Time—24 Hours)**

**Buffer:** A phosphate buffer of pH 7.4 was prepared as described in the previous section for the OsO₄ fixative. **Formaldehyde:** A 7 per cent formaldehyde solution was prepared from methanol-free formaldehyde kindly provided by the Heyden-Newport Co., Garfield, New Jersey. To each 100 ml of this formaldehyde solution 0.030 g of NaHCO₃ was added. Phosphate buffer solution and this neutralized formaldehyde solution were then combined in the proportions 1:1. To each 100 ml of the buffered formaldehyde the following salts were added:

- NaCl 0.149 gr
- KCl 0.012 gr
- CaCl₂ 0.0001 gr

The final concentration of formaldehyde is 3.5 per cent, which is close to 10 per cent formalin, and the final pH is 7.4.

After OsO₄ fixation the ovaries and eggs were rinsed briefly in buffer-salt solution and postfixed overnight in the buffered formalin fixative (Ornstein and Pollister, 1952).

**Embedding**

The young ovaries of the tadpole or newly metamorphosed frog were embedded in a mixture of 75 per cent butyl methacrylate and 25 per cent methyl methacrylate. The inhibitor was removed by shaking the mixture of monomers with a solution of NaOH, drying with Drierite or Linde molecular sieve, and finally filtering over bone black. Polymerization was initiated by adding 1 per cent of dry 2,4-dichlorobenzoyl peroxide. The latter was obtained as the residue remaining after absolute alcohol extraction of Lupercio CDB. This residue was dried by pumping it for 3 days with a mechanical pump. (Only small
amounts are so prepared since dry 2,4-dichlorobenzoyl peroxide is potentially a very dangerous combustible.) Dry uranyl nitrate was added to the final wash and to the embedding mixture in a concentration of 0.075 per cent (Ward, 1958). Polymerization was accomplished in gelatin capsules at room temperature. To prevent inhibition of polymerization by air renewal, a lightly moistened empty capsule bottom was pressed into the top of the filled capsule. Such capsules harden in 2 to 4 days at room temperature.

The mature eggs were also embedded in a monomer mixture of 75 per cent butyl methacrylate and 25 per cent methyl methacrylate with 1 per cent dry 2,4-dichlorobenzoyl peroxide as a catalyst. In this case, however, 5 per cent of a plasticizer, dibutyl phthalate, was added to the monomer. Dry uranyl nitrate was added in a concentration of five times that employed for the young ovaries, or 0.375 per cent.

The dehydration and embedding schedule was:

- 50 per cent ethyl alcohol 30 minutes
- 100 per cent ethyl alcohol 30 minutes (three changes)
- 100 per cent monomer mixture 30 minutes
- 100 per cent monomer mixture and uranyl nitrate, plus catalyst (and plasticizer for mature eggs) 30 minutes
- Embedding at room temperature.

A few attempts were made to embed material in Araldite, Epon, and Vestopal W, but these all resulted in poor preservation.

Tissue blocks were oriented by mounting with carnauba wax on nylon rods (Gettner and Ornstein, 1956) and sectioned with a diamond knife (Fernández-Morán, 1953) on a Porter-Blum microtome (Porter and Blum, 1953) or a Gettner-Ornstein microtome (Gettner and Ornstein, 1953). Sections appearing either white or very light yellow in reflected light were picked up on collodion-covered grids which had been stabilized by a very light coating of evaporated carbon.

**Electron Micrography**

A Philips 100 A electron microscope with a carefully selected but uncompensatable objective lens was used for all micrographs, with the exception of Fig. 13, which was taken after the installation of a Canalco electrostatic compensator for astigmatism. All micrographs were obtained with the instrument operating at 80 kv, and taken on Kodak 35 mm Microfile Copying Film. Film magnifications ranged from 2,000 to 23,300. The magnifications were calibrated with a carbon replica of a diffraction grating of 28,800 lines per inch obtained from Ernest Fullam, Inc. Magnification of the photographic enlarger used was also checked.

The best operating resolution attained was between 20 and 30 A, measured as fringe width on holes in a substrate film.

**Cytochemistry**

For the cytochemical tests employed, ovaries from tadpoles of Taylor and Kollros Stages 17 to the newly metamorphosed frog (Taylor and Kollros, 1946) were employed as well as single ovarian eggs 1.5 mm in diameter. All tissues were fixed in the buffered formalin fixative described under the section headed *Electron Microscopy*, dehydrated in alcohol, passed from a methyl benzoate-parlodion mixture to pure benzene and embedded in paraffin. Sections were made at 5 microns. Bonhag’s modification of the Mazia-Alfert Bromphenol blue method (Pearse, 1960 a) was used to test for protein, and the Perls Prussian blue test (Lison and Bunting’s modification, Pearse, 1960 b) was used to test for iron. In addition, sections were also treated for varying times up to 2 hours either with 30 per cent H₂O₂ or a saturated solution of NaHSO₃ before applying the Prussian blue test.

For the differentiation of fatty yolk from protein yolk, thin sections of methacrylate-embedded tissue were picked up on carbon-coated grids and immersed in xylol for 2 hours. They were then air dried and examined in the electron microscope.

**Observations**

**Relation of the Mitochondria to Protein Yolk Formation**

Fig. 1 shows two oocytes about 100 microns in diameter at low power which contain a cluster of globules (FYC, Fatty Yolk Center), mitochondria (M), and hexagonal bodies of protein yolk crystals as seen in section (PY). Since the last are thin plates, most of the profiles are rods of varying length and width. Only rarely does one see either a full face view of the plate or the view that results from sectioning normal to the face.

Fig. 2 shows a group of sectioned hexagonal bodies just under the oocyte cell membrane. One observes on oocytes of diameters between 400 microns and 1000 microns are not presented in this paper.

The justification for the use of the terms “protein yolk” and “fatty yolk” is given under the section of Observations headed Cytochemistry.

These three cytoplasmic components have been described in a previous phase microscope study of the living oocyte (Ward, 1962).
of these \((PYF)\) has been sectioned almost parallel to the face while the others have been cut more or less obliquely. The low density of the face view as contrasted with that of the oblique views is due to the flat, plate-like form of the hexagonal bodies. Sections normal or oblique to the face usually contain the body throughout the section's thickness. In the semi-face view \((PY)\) of Fig. 3, at the upper left, the density of part of the face closely approaches that of the oblique view \((PY)\) at the lower right. This would be the result of the former body \((PY, \text{ upper left})\) traversing the whole thickness of the section at this place.

The body in face view in Fig. 2 measures about 1 micron between the most widely separated opposite vertices of the face. The smallest of these bodies observed are about 0.2 micron between opposite vertices. Measurements of the widths of the rod-shaped profiles suggest a minimum thickness of about 300 A. Since the bodies are growing and since the rod-shaped profiles may be small parts of relatively large bodies or large parts of relatively small bodies, the thickness cannot be related to a body of a definite face size. A measurement of 0.2 micron thickness was obtained on a body 1.25 microns between opposite vertices of the face by serial sectioning. The ratio of the lengths in this case is approximately one to six.

The two hexagonal bodies in Fig. 3 are seen to be within a matrix, the mitochondrial nature of which is shown by the characteristic crista structure outside the region of the hexagonal body.

In Fig. 4 one of the hexagon-shaped bodies is seen at higher power in almost full face view. It is wholly within the mitochondrial cortex. Often these bodies are obviously incompletely surrounded by a mitochondrial cortex or are free in the cytoplasm surrounded by no mitochondrial substance. It has been observed, however, that the more poorly preserved the cell is in general, the more often are such mitochondrial coats incomplete. Furthermore, serial sections of the best preserved cytoplasm have demonstrated that these bodies are completely within the substance of the mitochondria. Study of many sections of the bodies suggests that they arise and grow between the two membranes of a single crista. In Fig. 4, \((C)\) is believed to be the crista membrane. In Fig. 5, \((CN)\) appears to be the neck of the crista, with the crista membrane \((C)\) turning and approaching contact with the outer mitochondrial membrane.

**Ultrastructure of Hexagonal Bodies**

The material of the hexagonal body at higher magnification (Figs. 4 and 6) is seen to be of a

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**Key to Abbreviations**

- C, Crista membrane of mitochondrion
- CN, Neck of crista of mitochondrion
- DB, Dense body
- EF, Extracted fatty yolk
- ER, Endoplasmic reticulum
- FCN, Follicle cell nucleus
- FY, Fatty yolk
- FYA, Fatty yolk inside mitochondria
- FYB, Fatty yolk extruded by mitochondria
- FYC, Fatty yolk center
- FYM, Fatty yolk mitochondria
- GD, Golgi dictyosome
- IL, Inner layer of yolk platelet
- LD, Lipid droplet
- M, Mitochondria
- ML, Matrix layer of yolk platelet
- MC, Mitochondrial cortex
- NC, Nucleolus
- NM, Nuclear membrane
- OL, Outer layer of yolk platelet
- PC, Pigment granule
- PY, Protein yolk
- PYF, Face view of hexagonal body
- YP, Yolk platelet

Figs. 1 through 18 are electron micrographs of *Rana pipiens* oocytes. Fixation was performed with 2 per cent buffered OsO4, followed by overnight fixation in buffered formalin, except where otherwise noted.

**Figure 1**

Two adjacent oocytes of tadpole ovary. Part of a fatty yolk center \((FYC)\) and sections of hexagonal bodies \((PY)\) may be seen. \(\times 4,500.\)
Group of hexagonal bodies cut in various planes but mainly oblique to that of the face view or edge view. *PYF* is a face view and is of less density than the oblique cuts, because of flat, plate-like shape (see text). \( \times 9,000 \).

Crystaline nature. It consists of dense laminae which have an average spacing of between 70 and 85 Å. Examination at the higher magnification (Fig. 6) suggests that these laminae have a particulate nature, but the preservation is not adequate to establish their precise morphology. Extensive through-focal series were used to no avail to settle this point. The angle these laminae make with one another at different levels is 65 degrees, and, therefore, close to that of ideal hexagonal packing and, of course, the outlines of the crystals themselves are hexagonal. Occasionally, these crystals occur in groups or nests within a greatly enlarged mitochondrial cortex. Part of such a group is seen in Fig. 7. In such a nest the crystals do not all lie in the same plane, but form a sort of pleated structure in which, at *A* and *B* in Fig. 7, one can follow individual crystals of the aggregate leaving the plane of section. Though they are not all precisely in the same plane, the crystals of such a group are usually oriented roughly in a co-planar fashion, their edges never being at right angles to one another.

Fig. 8 shows what appears to be the formation of two young crystals side by side. In cells like that of Fig. 8 possessing predominantly small...
crystal profiles, almost all sections show the crystals within the mitochondrial cortex. In the larger profiles the mitochondrial cortex appears thinner and is apparently more easily damaged. Fig. 8 is a micrograph of a section of tissue fixed only in buffered formalin, so we may be sure the high density of these crystals is primarily intrinsic and not due to substances such as osmicated lipid.

**Relation of the Mitochondria to Fatty Yolk Formation**

Fig. 9 shows a part of a fatty yolk center at higher power. It consists mainly of two components: vaguely delimited dense droplets and much less dense, sharply bounded spheres. The dense droplets which are of various sizes are, in general, the larger component which is so conspicuous in phase microscope studies of the living cells (Ward, 1962). The departure from spherical shape shown by the droplets is probably due to preservation artifacts. When studied at very high magnifications, these droplets appear to have no definite pattern in their ultrastructure. The other, less dense, component consists of sections of spherical bodies which vary in size from about 0.5 micron in diameter (FYM) down to quite small and extremely dense bodies (DB). The large spheres have double limiting membranes somewhat like the boundaries of mitochondria and a few dense internal granules. In smaller spheres the membranes are less evident, and the number of internal granules increases, the smallest bodies (DB) being apparently mainly a mass of these granules surrounded by either a single membrane or no membrane.

Indications that the resemblance of the larger spheres to mitochondria is more than superficial can be seen in Figs. 10 and 11. These bodies apparently arise by a break-up of the long, filamentous mitochondria into beads or spheres. In Fig. 10 a filamentous mitochondrion may be seen in the process of forming these spheres (FYM). The spheres appear to give rise to the dense droplets. The dense material of the droplets can be seen in very thin section within spheres, as in Fig. 11 at FYA. One of these spheres clearly contains some crista-like membranes. In Fig. 10, FY is believed to be a precocious accumulation of the droplet material occurring before sphere formation. The droplet material is apparently excreted through a break in the sphere membrane (see FYB in Fig. 11). The significance of the gradual accumulation of the dense granules and eventual formation of the dense bodies (DB) is not known.

**The Mature Egg Cytoplasm**

A section of the cytoplasm of an OsO4-fixed egg 1 mm in diameter is seen in Fig. 12. Yolk platelets (YP), lipid droplets (LD), swollen mitochondria (M), and pigment granules (PG) may be observed. Many of the lipid droplets do not possess a distinct limiting membrane, while others have a membrane which seems to consist of cytoplasmic material condensed or precipitated on their outer surfaces. The ultrastructure of the lipid droplets appears to have no definite pattern when they are studied at high magnifications. The yolk platelets possess three distinct layers: an inner one (IL) which seems to be a differentiated part of the main body of the platelet, a fairly thick matrix (ML), and an outer layer (OL). These layers often surround the main body of the platelet incompletely. The outer layer (OL) varies in appearance and is sometimes absent. It is not yet certain whether this is due to technical factors or to other causes.

It is at once apparent that, though the predominant profile of the platelet is more or less oval in outline, its actual main substance is more or less hexagonal in outline in many instances. The layers surrounding the main body of the platelet have concealed this shape in studies made with the light microscope, in which platelet outlines appear spherical or ellipsoidal. The actual

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**Figure 4**

Hexagonal body sectioned almost parallel to face showing its crystalline nature, the surrounding mitochondrial cortex (MC) and membrane (C) believed to be the limiting membrane of a greatly enlarged crista. X 100,000.
shape of fresh, mature yolk platelets when examined with the light microscope varies, but they have been described as predominantly oblong, with a thickness about 30 to 50 per cent of the long axis (Hohfreter, 1946 a). This relatively great thickness results in our obtaining many electron microscope profiles of the main body of the platelet with a polygonal appearance, at least at one end.

Ultrastructure of the Yolk Platelets

Fig. 13 shows the edge of one of the yolk platelets at high power. Only the middle and inner layers are shown. The main body of the platelet is seen to consist of regularly spaced laminae whose average spacing is between 70 and 85 Å, agreeing with that found for the hexagonal bodies of earlier oocytes. At various points within the main body and especially at the edges, these laminae can be well resolved into rows of particles. The packing of these particles is often that for a hexagonal crystal, but packing arrangements are also encountered which suggest that there may be some departure from the hexagonal shape. In general, while the hexagonal shape is sometimes somewhat obscured in the mature protein yolk platelet, it is quite clear in the most favorable electron micrographs. This fact plus the similarity of crystalline spacing offer strong evidence that the protein yolk platelets of the mature egg are derived by growth from the small hexagonal bodies of the young oocyte.

When formalin is the sole fixative employed, the particles of the laminae of the inner layer (IL) are found to be only slightly more dense than the rest of the main body of the platelet. The increased density of the OsO₄-fixed preparations is, therefore, an effect of the osmium fixation. Whether it represents the result of greater osmophilia of the outer particles over the inner ones, a zone indicating the extent of OsO₄ penetration, or a differential higher sublimation of the inner body of the platelet under the electron beam is not known. With either formalin or OsO₄ fixation, however, the resolution of the laminae into particles seems to occur mainly at the edges, suggesting that thinness and less heating by the beam combine to preserve the ultrastructure best. (The large platelets are thick and very dense, and consequently a great deal of absorption of electron beam energy takes place except at the edges.) Blanched of the interior regions of the platelets has been observed, and there is thus very good reason to suppose that the relative lack of density of their interiors as well as the disorder and running together of particles giving rise to line images in these regions is an artifact. Certainly, in the best preserved specimens a particulate nature is suggested throughout the whole of the main body of the platelet.

The matrix (ML) seems to consist of a tangle of particles and fibers of extremely high density after either formalin or OsO₄ fixation.

The appearance of the ultrastructure of a platelet in an egg 1.5 mm in diameter which was fixed only in formalin is seen in Fig. 14. The spacing of the laminae in such platelets has been found to be also between 70 and 85 Å. The matrix (ML) of the platelet in Fig. 14 has only been partially preserved, but again is seen to consist of particulate and fibrous components. As in the case of the hexagonal bodies of the young oocyte, the density of the crystalline array does not depend on osmophilia.

In general, the fatty yolk is not preserved in young or mature eggs after formalin fixation. The observations reported for OsO₄-fixed tissue other than those on the fatty yolk have all been confirmed on formalin-fixed material.

Cytchemistry

Both the hexagonal bodies of the young oocyte as well as the platelets of mature eggs gave a strongly positive reaction for protein with the Mazia-

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**Figure 5**

Oblique view of hexagonal body showing what is believed to be the neck of the crista (CN). C is presumed to be the crista membrane which turns and becomes continuous with the inner mitochondrial membrane. The course of the membrane on the other side of the neck is difficult to follow. The crystalline lattice is not visible, probably because critical focus was not achieved. × 85,000.
Alfert Bromphenol test. The Perls Prussian blue test for iron performed on these same bodies was negative even after long treatment (2 hours) with H₂O₂ or NaHSO₃. For both of these tests paraffin-embedded tissue was used. The same results were achieved with sections of methacrylate-embedded tissue, but the weaker coloration obtained with the protein test leads one to suspect some "masking" effect due to the presence of the embedding.⁶

From the earliest days (Van Bambeke, 1898), OsO₄-fixed yolk has been differentiated as protein or fatty yolk by its differential solubility in xylol or turpentine. Fatty yolk is soluble in these solvents, while protein yolk is not. This test was applied to thin sections of OsO₄-fixed tissue, with the results shown in Fig. 15. The dense globules (PY) of Fig. 9 were completely extracted by xylol (Fig. 15, EF), while the density of the hexagonal bodies (FY) appeared unaffected. Fig. 16 shows one of the latter after xylol treatment, at high magnification, revealing little or no effect on its structure or content. These negative results confirmed those obtained by the Mazia-Alfert Bromphenol test for protein.

As a consequence of these two tests and for other reasons involving correlations with the known chemical composition of the inclusions of the mature egg, the globules have been designated fatty yolk, and the hexagonal bodies, protein yolk.

Sections of almost mature OsO₄-fixed eggs were tested with xylol in the same manner as the sections of early oocytes, with the results seen in Fig. 17. The dark, osmiophilic droplets (LD of Fig. 12) were completely extracted, indicating their fatty nature, but the yolk platelets and pig-

⁶ The embedding can be removed presumably by solvents such as CC₄. If the molecular weight is very high, however, all common solvents are without effect. Immersion of our material in solvents did not change the test results. One could occasionally observe an individual platelet with an intense reaction, however, perhaps because it contained little or no polymer.

DISCUSSION

Mitochondria and Yolk Formation

One of the first problems we found of interest was the great elongation as well as the multiplication of mitochondria. Though this is not the topic of interest in this paper, it may be remarked that the evidence points to a growth by self-elongation, rather than fusion, followed by multiplication by fission. During this elongation and multiplication both the process of fatty yolk formation and that of crystal formation go on side by side, involving mitochondria otherwise indistinguishable from one another, except for the fact that those concerned in fatty yolk formation usually fragment into spheres before fatty yolk deposition begins.

The earliest yolk of either sort appears somewhat randomly placed in the cytoplasm at a time just after the mitochondria have left their juxtanuclear position. Soon after this, there is a concentration of mitochondria at the periphery of the oocytes and, as noted by all workers studying the early formation of yolk in the frog, there is a very active synthesis of yolk in this region. There appears to be some tendency for mitochondria to pair or lie close to one another along their lengths at this time (Figs. 2 and 3).

No useful information has been obtained as yet concerning intramitochondrial events just prior to the production of the smallest crystals (about 0.2 micron across opposite vertices in face view). One would expect that a production of the macromolecules comprising the crystals would be seen just before actual crystal formation, similar

FIGURE 6

High power view of the crystal of Figure 4 showing the lattice laminae. Spacing is 70 to 85 A. Packing angle is 65°. Irregularities suggest preservation is not quite good enough to reveal the true nature of the laminae. The significance of the dark masses, lower left and upper center, is not known. They are not a constant finding. × 233,000.
to the findings of Favard and Carasso (1958). Perhaps a combination of difficulties, such as the very short duration of this stage in the frog plus inadequate resolution in our micrographs, have combined to conceal these events.

In cells about 50 to 100 microns in diameter, most of the yolk crystals occur singly in filamentous mitochondria, often at the ends of the latter. As the oocyte grows larger, however, more and more groups of mitochondria come into contact, giving rise to groups of crystals each of which is surrounded by a large, irregular, sheet-like mitochondrial cortex (Fig. 7). It is not clear whether the mitochondria become confluent and so form the large crystal groups. So far, the evidence is against this, for whenever mitochondria are found in contact they are, nevertheless, almost always seen to be separate, except for a few very small points of contact. It appears rather that such groups arise within a single mitochondrion. This would entail a truly enormous surface increase in the cortex material. It has not been possible, as yet, to discover any evidence concerning the mechanics of this increase.

Fatty yolk formation is occurring at the same time as protein yolk formation. In regard to fatty yolk formation, the following positive statements of observation may be made without relating them to each other:

1. Filamentous mitochondria can be found at the cell’s periphery seemingly breaking up into spheres.
2. Fatty yolk centers consist of droplets of lipid and a variety of spherical bodies. Some of the latter are mitochondria containing lipid. The lipid nature of the droplets and the material within the mitochondria has been established by xylol extraction.
3. Lipid accumulation in the mitochondria is accompanied by the loss of mitochondrial membranes.
4. Images have been obtained suggesting that lipid material may be continuous through breaks in the outer mitochondrial membranes.

It has not been possible to relate these observations experimentally and so a hypothesis consistent with the observations is formulated as follows:

The sequence proposed for fatty yolk production is, first, fatty yolk mitochondria formation; second, accumulation of lipid within fatty yolk mitochondria; third, secretion of lipid by fatty yolk mitochondria. It seems clear that the three steps must follow each other very rapidly, for, even in the youngest cells, if they contain any fatty yolk centers at all, the three steps are represented in these centers. Groups of spherical mitochondria completely filled with lipid have been observed, and other groups have been seen apparently in the process of extruding this lipid, but one cannot be certain that a given group of spherical mitochondrial profiles containing no lipid represents the first step in the sequence just described.

The fact that deposition of lipid occurs occasionally even before sphere formation suggests that the required machinery is present within the mitochondrion before this process begins. Sphere formation is, however, probably the first step in the breakdown of continuity within the membrane system. The replacement of the internal membranes by lipid suggests strongly the possibility that the lipid of the membrane system itself contributes, at least in part, to the formation of the fatty yolk. The groups of granules which appear to be the end product of sphere formation are, perhaps, the mitochondrial proteins remaining after loss of lipid from the lipoprotein complex. Though the reason for the high density of these granules is unknown, it is tempting to speculate that this may be the result of the assumption of a very compact, globular form by these proteins when they are released from their complex with the lipid.

Despite the evidence given in this paper as well as additional unpublished material supporting the hypothesis presented, no success has as yet been achieved in following the sequence of steps in fatty yolk formation. Until the steps are

**Figure 7**

Nest of hexagonal bodies within extensively developed mitochondrial cortex (MC). At A and B are crystals whose orientation is fairly oblique to the plane of the section, and most of the substance of these bodies lies outside the section. × 135,000.
followed sequentially our synthesis must remain inferential and based on circumstantial evidence. It is hoped that more intensive study of the very young cells may provide a better sequential separation.

For a long time, pathologists have described and discussed the relation of mitochondria to intracellular lipid formation in pathological states. Recently, Palade (1959), in an electron microscope study, described a close association between lipid droplet formation and mitochondria in the acinar cells of the pancreas of fasting guinea pigs. He concluded, however, that such droplet-mitochondrion associations were probably an example of utilization of fat which came from the animal's fat depots via its blood circulation, rather than of production of lipid by the pancreatic mitochondria. Such lipid was presumably being used for energy production.

Pasteels et al. (1958) noted a close association of mitochondria and lipid droplets in the fertilized sea urchin egg. Lever's studies (1956, 1957) on the brown fat cells and the adrenal cortex of the rat seem to provide the only evidence other than the present study for the intramitochondrial deposition of lipid in normal cells. Napolitano and Fawcett (1958), however, did not confirm Lever's findings on this point in the brown fat cells.

Synthesis of Yolk Protein

The accumulation of protein crystals within the substance of mitochondria raises a question as to whether the protein is actually synthesized from amino acids within the mitochondrion or whether the mitochondrion acts merely as a precipitator or concentrating site for protein synthesized somewhere else. The findings of Work and his associates (Roodyn, 1961) would suggest that isolated mitochondria are at least capable of some protein synthesis, so that an intramitochondrial synthesis does not seem improbable. Further, Giudice (1960) has demonstrated an incorporation of amino acids into the proteins of isolated mitochondria obtained from unfertilized sea urchin eggs. This incorporation is found markedly reduced when mitochondria isolated from the fertilized egg or the embryo are used. On the other hand, however, tracer, chemical, and immunological studies have indicated an extra-oocyte source for at least some of the yolk proteins. A series of papers (e.g., Laskowski 1938; Cooper, 1950; and Flickinger and Rounds, 1955) have suggested the possibility that some of the yolk proteins are synthesized in the maternal frog liver. These proteins are thought to reach the eggs via the blood circulation. They must, of course, pass through the follicle cells. It must be stressed, however, that these studies do not give us information regarding the quantities of protein involved. One would expect evidence at the electron microscope level if a great deal of protein were being passed to the oocytes via the follicle cells, but even at the stage when both follicular and oocyte cytoplasmic membranes have been thrown into folds and interdigitate in intimate contact, there is no evidence for such a passage (Kemp, 1956; and Ward, unpublished observations).

The relation to the synthesis of protein yolk of the well known evidence cited by Brachet (1950), that a marked basophilia is present in the cytoplasm of frog oocytes just before the onset of yolk synthesis, remains obscure in the present study.

In any case, an extra-oocyte origin for all the yolk protein seems difficult to accept at this time.

It may be possible that the yolk-synthesizing mitochondria (if such they be) come to contain some additional amounts of RNA. On the other hand, perhaps all the protein synthesizing machin-
Crystalline Nature of Protein Yolk

Evidence has been presented previously for the crystalline nature of mature amphibian yolk platelets. Ringle (1960) has measured the period in the isolated platelets of *Rana pipiens* as 75 Å; Wischnitzer (1957) measured the period in the platelets of *Triturus viridescens* as 70 Å; and Karasaki (1959) measured the period in the platelets of *Triturus pyrrhogaster* as 72 Å. Karasaki (1959) interpreted the ultrastructure as a system of fringes, but we believe these line images to be the result of a running together of the individual particles. Many of our own micrographs show the ultrastructure as lines rather than rows of particles. It seems prudent to assign a value with a range (70 to 85 Å) rather than a precise value to the spacing. Averaging of direct measurements on the micrographs varies, depending on the tilt of the crystal. Further, variations occur in different parts of the same crystal. A more precise indication of the average value could presumably be accomplished by the use of electron diffraction. Karasaki and Komoda (1958) and Karasaki (1959) obtained by electron diffraction a value of 71 Å for salamander platelets.

Favard and Carasso measured a period of 85 Å in crystals of the yolk bodies of the fresh water snail *Planorbis*, and Elbers found a period of 50 Å in the protein inclusions in the egg of *Lymnea stagnalis*.

The relative proximity of all the reported values to each other (30 to 85 Å) is undoubtedly of more biological significance than their differences.¹

Chemical Composition of Amphibian Yolk Platelets and Fatty Yolk

There is evidence that the crystalline yolk inclusions of certain molluscan eggs are protein in nature (Elbers, 1957; and Favard and Carasso, 1958). In the case of *Planorbis*, Favard and Carasso (Favard and Carasso, 1958). One of these is by growth within sacs which are first budded off from filamentous mitochondria, and the other is by growth within multi-vesicle-containing sacs which originate from a large mass of juxtanuclear vesicles.

As presented there are several important points of disagreement between our description and that of Lanzavecchia. It would be surprising if there are significant differences between these two frogs with respect to yolk formation, but there is so little electron microscopical information on this subject that such a possibility can not be ruled out at this time. Additional cytological description concerning the origin of yolk in *Rana esculenta* will be awaited with interest.

¹ Fernández-Morán and Finean (1957) have shown that the 170 Å radial repeat found in frog myelin by x-ray diffraction is reduced to 148 Å by OsO₄ fixation. The value for the latter as measured on electron micrographs is found to be only 115 Å, however. This result raises questions as to the significance of the absolute value of the measurements on cells after the preparation techniques of electron microscopy.
Figure 9
Part of a fatty yolk center showing the fatty yolk globules (FY), spherical fatty yolk mitochondria (FYM) and dense bodies (DB) believed to be the end product of the membrane loss and subsequent collapse occurring in the fatty yolk mitochondria after their lipid secretion. X 26,000.
of the substance of isolated platelets is protein. Since the bulk of our fixed platelet is crystalline and since the density of the formalin-fixed platelet is only slightly less than that of the OsO$_4$-fixed platelet, and finally since one obtains an intense Bromphenol blue protein test in the interior of sectioned platelets, there seems no reason to doubt that the crystalline lattice consists of protein molecules. Whether each dense spot represents a single protein molecule is not known, but assuming that this is so and that the molecules are in contact and possess an electron transparent outer shell, the diameter would be close in dimensions to that of other known proteins which have been measured in the electron microscope (e.g., ferritin, edestin). Favard and Carasso (1958) raised the question as to whether the particles comprising the protein crystals which they studied in the eggs of Planorbis were ferritin-like, since the diameters were close to that of ferritin. They could, however, obtain only a weak Prussian blue test even after microincineration. In the present study a positive Prussian blue reaction was not obtained on formalin-fixed, paraffin sections even after prolonged reduction or oxidation. Favard and Carasso were inclined to a view that the iron was perhaps present in the protein macromolecules in a masked form, but it would appear that the evidence in either their case or ours is against the presence of any significant quantity of iron.

Barth and Barth (1954) as well as Flickinger and Schjeide (1957) and Ringle (1960) found by electrophoretic analysis that the protein of the isolated platelets of Rana pipiens consists of two components, one present in the greatest amount and another, which is a phosphoprotein, present in lesser quantity. The ratio of the areas under the electrophoretic peaks was approximately 10 to one, and over 90 per cent of the phosphorus present was found in the smaller component.

There is also lipid present in amounts ranging from 1.62 per cent (Gross and Gilbert, 1956) to 18.9 per cent (Schjeide, Levi, and Flickinger, 1955), depending on the method of extraction used. Using a method for the removal of lipid from serum lipoproteins, Ringle (1960) determined the amount of lipid as approximately 10 per cent.

Phosphorus to nitrogen ratios have indicated a significant amount of phosphorus in amphibian yolk. Ratios of 0.093 to 0.132 were found for the platelets of Rana fusca by Panijel (1951), for example. He found about 70 per cent of this phosphorus to be in phosphoprotein, and the remainder mainly in phospholipid.

Finally, the presence of varying but small amounts of nucleic acid, mainly pentose nucleic acid, has been found by most investigators analyzing yolk platelets. A low value for PNA-P/N-(total) of 0.00022 has been reported by Ringle (1960) for Rana pipiens, while Panijel (1951) has reported a much higher value of 0.01 for this ratio in Rana esculenta.

Of special interest are the major differences between a small platelet fraction (length of long axis one micron and below) and a large platelet fraction (length of long axis 1 micron to 17 microns) analyzed by Panijel. The small platelets have more RNA phosphorus (ratio of µg. of P per mg. N of the suspension being 112 for the small platelets and 30 for the large platelets) than the large platelets. On the other hand, the large platelets contain more phospholipid than the small platelets (the ratio of µg. of phospholipid

**Figure 10**

Formation of the spherical fatty yolk mitochondria from a branched, filamentous mitochondrion. In one arm of the latter pinching off of the spheres may be seen (FYAM), while in the other at FY (right side) fatty yolk is seen depositing precociously before sphere formation. X 35,000.

**Figure 11**

Thin section showing fatty yolk within spherical fatty yolk mitochondria at FYA. Remnants of cristae may be seen. Secretion of the lipid seems to take place through a break in the membrane such as that seen near FYB. X 55,000.
phosphorus per mg N of the suspension being five for the small platelets and 18 for the large).

It would seem likely from the cytological picture that the main body of the platelet consists of the major peak found by electrophoretic analysis, though we cannot rule out the possibility that the phosphoprotein fraction is also included. The other constituents might well be present in the cortex of the platelet.

There would appear to be substantial changes in the mitochondrial cortex as the hexagonal body matures. One change is a large increase in the electron density of this material as seen after formalin or OsO₄ fixation. This may be a reflection of the concentration of phospholipid found in the larger platelets. The decrease in RNA in the large platelets is difficult to harmonize with the changes occurring between large and small platelets. The mitochondrial coats of the young platelets do not contain unusual numbers of granules which could be assumed to be ribosomes, and if these coats are indeed richer in RNA, such RNA must be present in an agranular form. In view of the considerable differences found by different investigators for the RNA content of yolk platelets, it is perhaps somewhat too soon to attempt to fit the cytological picture with these chemical results.

Holzfretter (1946 a to c) has stressed that the lipid inclusions of the mature frog egg, his "lipochondria" which are mainly lipid, do, however, contain protein as well. This protein is mainly found in the cortex of the lipochondria, though some evidence suggests the possibility of an internal protein framework as well. He found the lipochondria to be rather easily transformed into fat droplets or "liposomes." Holzfretter noted that fixation such as that of Champy would cause this transformation. However, 1 per cent OsO₄ was found to preserve the lipochondria morphologically intact.

The present study shows large variability in the appearance of the lipid yolk. Some droplets possessed a thick coating, but most seemed to have no discernible covering or membrane. The coverings when present seemed to be a condensation of surrounding cytoplasmic material rather than a part of the lipid droplet. Such coats can be seen, in Fig. 17 of this paper, outlining the sites of the xylol-extracted droplets.

Technical difficulties in the handling of this material are still formidable and much still remains to be done. It is hoped that eventually the origin of the protein, fatty and carbohydrate stores of the egg will be presented in an uninterrupted, tight sequence in the manner of classical cytology.

This paper together with the first of the series contains the material of a thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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The substance of this investigation was previously published in abstract (Ward 1959 a and b).

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LITERATURE CITED
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FIGURE 12
Section of cytoplasm of "mature" egg 1 mm in diameter. The large yolk platelets (YP) are seen to possess a main body often hexagonal or partially hexagonal in outline. Around this main body are three layers: an inner one (IL), a fairly thick matrix (ML), and an outer layer (OL). Dense pigment granules (PG) may be seen and somewhat distorted mitochondria, (M). The very dense globules (LD) are lipid droplets. × 8,000.
FIGURE 14
An edge of a yolk platelet of a mature egg 1.5 mm in diameter, fixed with formalin alone, seen at high magnification. The outer layer is absent (though present on other platelets in this same cell), and the matrix layer (ML) has only been partially preserved. As in the case of the OsO₄-fixed platelet the main body of this platelet shows a spacing of 70 to 85 Å, and resolution of the laminae into particles is seen best at the edge. An inner layer is not sharply delimited, though the density of the outer planes or particles is somewhat higher than the rest of the main body. The matrix layer (ML) is seen to consist again of a tangle of particles and fibrils though it has lower density than that of the OsO₄-fixed platelet. X 233,000.

FIGURE 18
An edge of a yolk platelet seen at high magnification. Only the main body of the platelet, the inner layer (IL) and the matrix (ML) are shown. The inner layer (IL) appears to be a continuation of the main body. The spacing of the crystal laminae is 70 to 85 Å, and the laminae are seen to consist of particles at the edge of the main body where conditions are best for resolution and absence of electron beam damage. The particles measure 35 to 40 Å in diameter, but may be the dense center of a macromolecule possessing an electron-transparent outer shell. The dense matrix layer seems to consist of a tangle of fibrils and particles. X 233,000.
Figure 16

Ultrastructure of hexagonal protein crystal seen at high magnification to show little or no effect on either the density or spacing of the crystal laminae after xylol extraction for 2 hours. × 233,000.

Figure 15

Appearance of the cytoplasm of a young oocyte after immersion of the thin section in xylol for 2 hours. There is widespread damage of fine structure, but most of the organelles retain their customary outlines. The fatty yolk is completely extracted leaving only the outlines of its previous location (EF). The dense bodies (DB) and the hexagonal protein crystals (PY) seem little affected though the mitochondrial matrices of the latter are severely damaged and sometimes missing. × 15,000.
FIGURE 18

Ultrastructure of two adjacent yolk platelets seen after xylol extraction for 2 hours. Little or no effect on density or spacing is apparent in either the matrix layer (ML) or the main body of the platelet. The edge of the platelet on the right was apparently bent and so is out of focus, giving rise to a negative image of the particles comprising the laminae. × 233,000.

FIGURE 17

A low power view of the cytoplasm of a “mature” egg like that of Fig. 1 seen after xylol extraction for 2 hours. The platelets (YP) and their matrix layers (ML) as well as the pigment granules (PG) seem relatively unaffected. The outer layers of the platelets and the lipid droplets are extracted, the latter leaving only their outlines (EF) behind. × 8,000.
LITERATURE CITED


17. Hirschler, J., Uber die Plasmakomponenten (Golgiischer Apparat Mitochondrien u.a.) der weiblichen Geschlechtszellen (zytologische Untersuchungen am Ascidien-Ovarium), Arch. mikr. Anat., 1916, 89.


44. VAN BAMBEKE, C., Contributions à l'histoire de la constitution de l'œuf, Recherches sur l'ovocyte *Phoca plus phalangiaoides* (Feussel), *Arch. Biol.*, 1898, 15.

45. WARD, R. T., Prevention of polymerization damage in methacrylate embedding media, *J. Histochem. and Cytochem.*, 1958, 6, 396.


47. WARD, R. T., Origin of yolk in *Rana pipiens*, *J. Appl. Physiol.*, 1959 b, 30, 2041.


51. WITTICH, W., De observationes quoedam de araneae ex ovo evolutione, Halis, 1845.


R. T. WARD Origin of Protein in *R. pipiens* II 341