# THE ORIGIN OF PROTEIN AND FATTY YOLK IN *RANA PIPIENS*

## I. Phase Microscopy

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ABSTRACT

Study of living frog oocytes with the phase microscope has shown that the early yolk appears in two forms. One of these, the protein yolk, consists of thin, dense, plate-like bodies which in face view are almost always regular hexagons. The other form, the fatty yolk, occurs as clusters of globules of varying sizes. The plate-like bodies occur both singly and in clusters. As the oocytes mature these plate-like bodies grow in size while retaining their hexagonal outline. Mitochondria have been observed to increase in length and numbers as the oocytes mature; they are rods or filaments at all stages of growth up to an oocyte diameter of 300 microns. The oocyte cytoplasm gradually becomes packed with long mitochondria, plate-like bodies, and clusters of globules.

### INTRODUCTION

The present work was undertaken as the beginning of a combined cytochemical, phase, and electron microscopical study of oogenesis in *Rana pipiens*.

There appear to be no published descriptions of the cytoplasm of the developing oocyte of this form as it appears under phase contrast optics, though practically every investigator interested in the species has examined the fresh ovary using brightfield optics. Such examinations have generally been cursory and no thorough correlations have been made between living and fixed material, except for the studies of Holtfreter (1946 *a*, 1946 *b*, 1946 *c*) which were made, however, on the mature oocyte.

The micrographs of the present paper are restricted to oocytes in the size range 50 to 200 $\mu$. It is in oocytes of this size that the earliest signs of yolk formation can be observed and the first of our combined studies will be confined to a study of this process.

### MATERIALS AND METHODS

Phase microscope studies of the ovary of the living frog were performed on pieces of ovary from *Rana pipiens* females of the 1st and 2nd year after metamorphosis. The pieces used were first freed of any large yolky oocytes, and then squashed in a drop of frog blood serum or peritoneal fluid. A thin coat of petroleum jelly was spread around the drop to prevent excessive coverglass pressure. Preparations appeared to remain healthy for 15 to 30 minutes. The larger oocytes (300 microns or larger in diameter) are often broken by any but the lightest pressure. The oocytes of tadpole ovaries were much more easily broken by coverslip pressure than oocytes of the same size range taken from 1st- or 2nd-year frogs. The appearance of oocytes of comparable size is the same, however, in both types of ovaries. It should be noted that the conditions for optimum performance of the phase microscope are hardly approached with these very thick specimens, even with squashing technique.

The observations were made with an American

Optical phase contrast microscope equipped with a 10× ocular and a 97× oil-immersion achromatic dark-medium objective of NA 1.25. The low-power darkfield micrographs were taken on the same microscope by using the 10× phase objective together with the condenser phase ring of the oil immersion objective. The images were recorded on Kodak Microfile copying film, using a Bausch and Lomb six volt ribbon filament lamp as the illuminating source together with a light green filter. The camera, a Robot IIA automatic advance camera, was used in conjunction with a Leitz Micro-Ibso photomicrographic attachment.

Study of many hundreds of preparations of the living cells proved invaluable in providing criteria of good preservation for later electron microscope investigation. The phase microscope was used also to follow each step of the preparative procedure employed in electron microscopy. Cells in squashes fixed with buffered $OsO_4$ or buffered formalin were studied carefully after the steps of fixation, fixation and dehydration, etc. For embedding, epoxy resins as well as many different mixtures of methacrylates were tried. A variety of polymerization methods was also investigated.

## OBSERVATIONS

When observed with a low-power dissecting microscope the fresh cytoplasm of young oocytes is seen to be almost optically clear until they reach a diameter of about 50 microns. Even in oocytes of smaller diameter, however, a few flecks of yellow are present. These increase in size and number until the oocytes become too opaque for good study with the phase microscope, when the diameters are 300 to 400 microns. (Fully mature oocytes are about 1500 microns in diameter.) Such flecks are highly refringent when viewed in dark field. Fig. 1 shows a living oocyte, about 130 microns in diameter, in dark field. The arrows point to refringent flecks. Fig. 2 shows an older oocyte about 200 microns in diameter; both the size and number of the flecks or patches have increased. A still older oocyte has a cytoplasm densely packed with this material.

When the cytoplasm of a young oocyte of about the diameter of that in Fig. 1 is observed at high power in the phase microscope, most of the refringent patches are seen to be groups of globules of varying diameters. Such a group is seen in Fig. 3 (*FY*, fatty yolk). Some of the patches, however, are seen to be groups of plate-like, hexagonally shaped bodies (Fig. 3, *PY*, protein yolk). (The basis for the designations "fatty yolk" and "protein yolk" is given in a subsequent paper.) Some of these plate-like bodies are seen more clearly in Fig. 4. Sometimes they seem to adhere to one another in chains of two or more (*e.g.*, see *PYA*, Fig. 4). On occasion, six or more of these bodies may be grouped together. Such groups move about in Brownian motion like a single body. The mitochondria (*M*, Fig. 4) increase in number and length during the growth of the oocyte; commonly they attain a length of 20 microns, and sometimes of as much as 40 microns in cells 300 microns in diameter. In larger oocytes the opacity of the living cells is too great for study with the phase microscope though smears of extruded cytoplasm may be examined.

The plate-like bodies (*PY*) rarely deviate in shape from a regular hexagon when seen in full face-view, and are observed to increase in size while still preserving this shape. They occur in a range of sizes in the cytoplasm of cells of any age, but in the older cells there are numbers of larger and larger bodies such as that seen in Fig. 5 (*PY*). In the youngest cells, these bodies are close to the limit of resolution of the light microscope. In cells of about 100 microns in diameter, the largest bodies are approximately 3 microns across opposite vertices.

The cytoplasmic inclusions of the living cells thus comprise mainly three types of bodies:

1. Mitochondria
2. Plate-like, hexagonally shaped dense bodies (protein yolk platelets)
3. Patches of globules of varying sizes (fatty yolk globules)

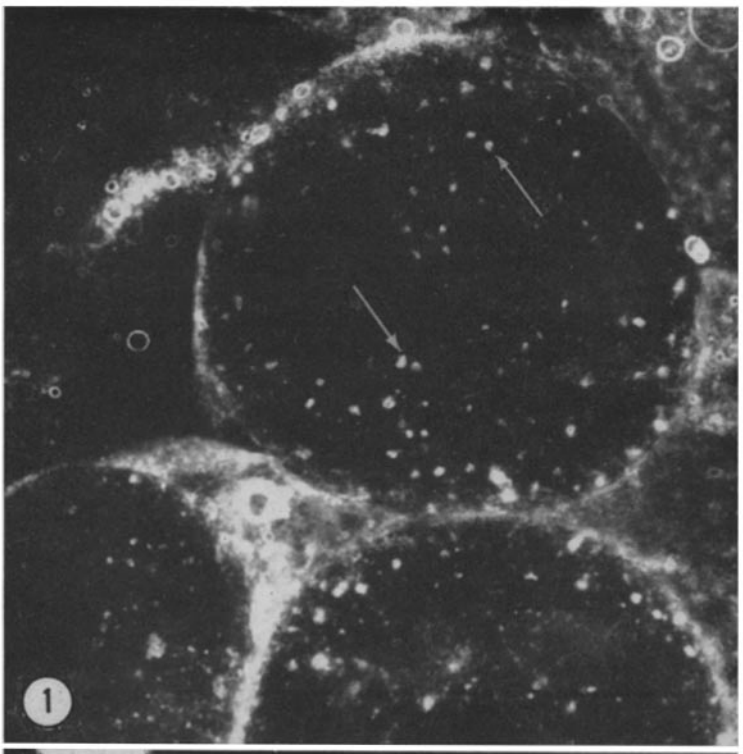
Other bodies are also present, but in much smaller quantity, and will not be referred to in this study.

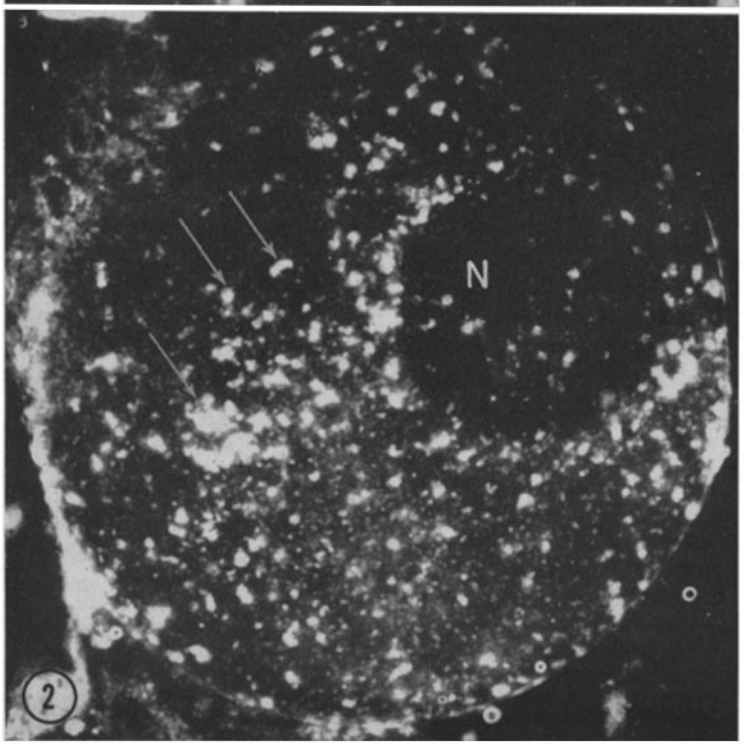
FIGURE 1

Darkfield micrograph of living oocyte, about 130 microns in diameter, showing highly refringent patches of yolk (arrows). × 480.

FIGURE 2

Darkfield micrograph of living oocyte, about 200 microns in diameter, showing increase in the number and size of refringent patches of yolk (arrows). *N*, nucleus. × 480.


1


N
2'

In the studies of fixed tissue it was quite clearly established that the embedding step (no matter what the embedding used) was responsible for poor preservation. Buffered formalin was found to preserve cells almost as well as buffered $OsO_4$, up to the polymerization step, but formalin-fixed tissue seemed always to be more damaged by the polymerization process. In general, preservation was never very satisfactory when *mature* frog ovary was embedded.

## DISCUSSION

The precise description of what appear to be, in living oocytes of the frog, the early platelets or platelet predecessors, seen as regular hexagonal plates, or more correctly, octahedral plates, is ap-

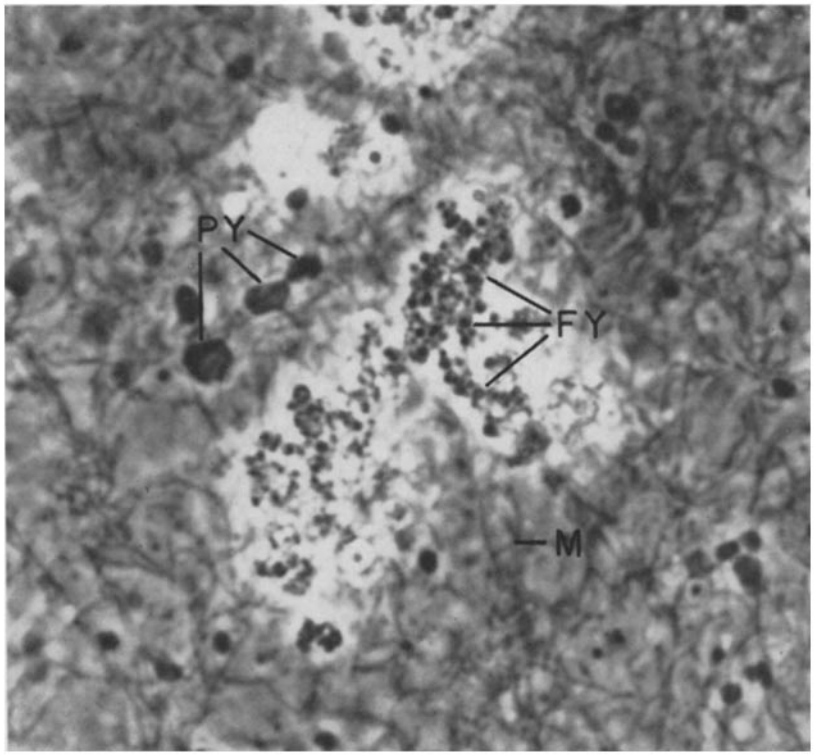



FIGURE 3

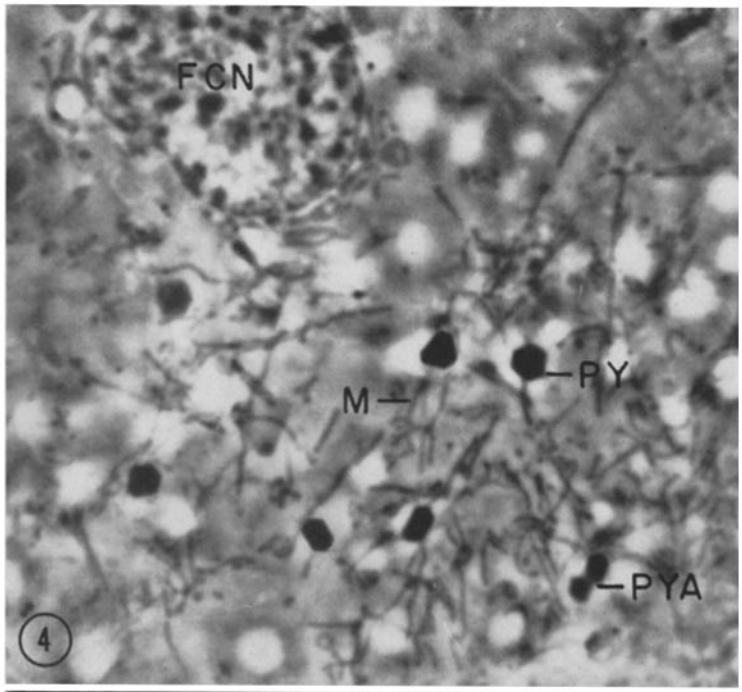
Phase contrast micrograph of living oocyte, about 100 microns in diameter, showing hexagonal bodies (protein yolk crystals) (*PY*), group of fatty yolk globules (*FY*), and mitochondria (*M*). × 2,400.

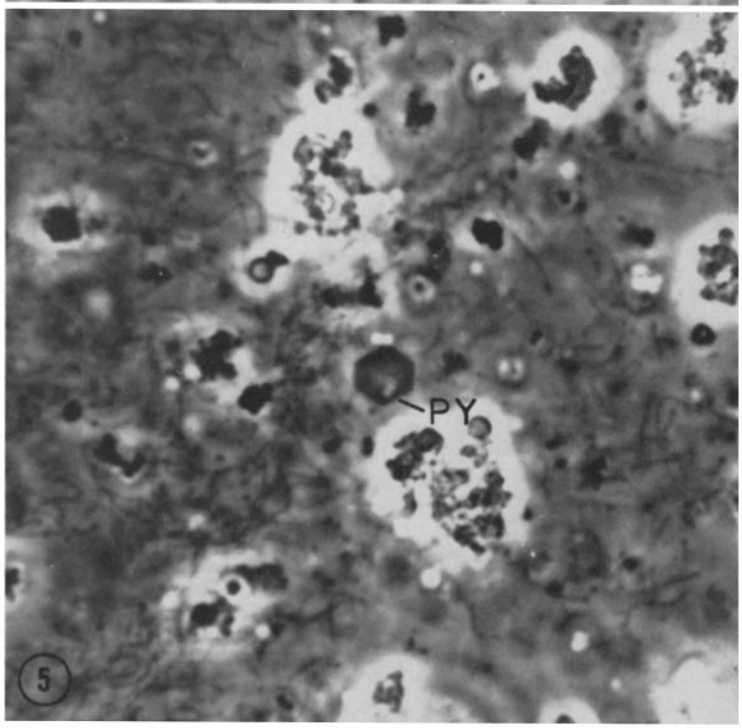
FIGURE 4

Phase contrast micrograph of living oocyte, about 100 microns in diameter, showing the hexagonal appearance of the face views of the protein yolk crystals (hexagonal bodies) (*PY*) and a pair of such bodies (*PYA*) adhering together at one edge. Single mitochondria (*M*) can be clearly seen and followed for distances up to 6 microns. In the upper left the nucleus of a follicle cell appears (*FCN*). Despite the fact that this is a very flat preparation, most of the crystals are somewhat tipped relative to the optical axis of the microscope. × 2,400.

FIGURE 5

Phase contrast photomicrograph of living oocyte, about 200 microns in diameter, showing the increased size of the hexagonal body (*PY*) in an older cell. × 2,400.


FCN
M
PY
PYA
4


PY
5

parently given for the first time in the present study. Examination of the living oocytes of four different amphibians reveals that, in all four, the early platelets are angular plates, but that in only two of these, *Rana pipiens* and *Rana catesbeiana*, do these bodies have the form of regular hexagonal plates (Ward, unpublished observations).

The mitochondria were observed to be slender filaments, and seemingly never spheres, no matter how small the diameter of the oocyte studied. They appeared to be shorter the younger the oocyte, but even in the youngest ($<25$ microns in diameter) one mitochondrial axis was longer than the other. As the oocyte matures there is clearly not only a great increase in the length of the mitochondria but also a great multiplication of these organelles. On a great many occasions mitochondria were observed to be attached to one side of the hexagonal plates, but this was thought at first to be an artifact produced in the squashing technique. A subsequent electron microscope study will show that this association is very real and more than a simple attachment.

Extruded cytoplasm of the *mature* egg reveals no sign of the long mitochondria present at earlier stages. It is possible that mitochondria at this stage are spheres rather than rods and cannot be differentiated from pigment granules. Since this transition phase cannot be studied very profitably on the living cells, clarification must await investigation of fixed material.

Holtfreter (1946 *a*, 1946 *b*, 1946 *c*) studied in some detail *in vivo* and *in vitro* the cytoplasmic constituents of the *mature* eggs and embryos of *Rana pipiens*. The former studies were performed by piercing the egg and observing the extruded masses of cytoplasm. Many physical and chemical observations were made on material isolated in distilled water and solutions of varying salt concentration and composition. (It must be pointed out that the micrographs in this paper are all of oocytes very much younger than those described by Holtfreter.) Holtfreter (1946 *c*) found the fresh platelets weakly birefringent, as had earlier workers before him. When the platelets were treated with dilute acid or alkali, they swelled in one dimension (the longest axis) and split into rodlets which were very birefringent (positive with respect to the long axis). With continued hydration the birefringence disappeared, but could be made to return if the material was treated with alcohol, xylol, or ether.

The lipoid bodies which were called "lipochondria" by Holtfreter were 2 to 4 microns in diameter and either spherical or somewhat rodlike. They were also birefringent but the direction or material source of this birefringence could not be determined. Holtfreter thought this might be due "to the presence of two kinds of oriented substances, namely phosphatides and proteins, having opposite signs of birefringence." The bodies adhered to each other in clusters and often adhered to the surface of the platelets. The bodies designated fatty yolk (*FY*) in Fig. 3 have this property of adhering in clusters and would seem to be the same as Holtfreter's lipochondria except that the stage shown here is very much earlier than the one he described.

In accordance with Holtfreter's findings, fresh smears of *mature* egg cytoplasm were seen to contain three types of bodies: pigment granules, lipoid bodies, and yolk platelets.

This paper together with the second paper in 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 form (Ward 1959 *a*, 1959 *b*).

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