Histochemical and Morphological Studies of Lipids in the Oogenesis of *Pheretima posthuma*

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With one plate (fig. 2)

Summary

A study of the oocytes of the earthworm, *Pheretima posthuma*, examined fresh under the phase-contrast and interference microscopes as well as by histochemical techniques, has revealed that there are two types of lipid bodies in the cytoplasm. The lipid bodies of the first type (L_1) are smaller, appear as homogeneous, dark granules under the phase-contrast microscope, and have a protein-phospholipid core surrounded by a thick sheath of phospholipids only. The lipid bodies of the second category (L_2), which arise as a result of growth and chemical change in L_1 bodies, have a pure phospholipid core surrounded by a thick triglyceride sheath. They give a ringed appearance under the phase-contrast microscope. The study under the interference microscope shows that this ringed appearance is an optical artifact. The lipid spheres present in the follicular epithelium contain phospholipids only.

The mitochondria are in the form of minute granules. They remain unchanged throughout oogenesis.

Some vacuoles devoid of any lipids, proteins, or carbohydrates have been observed. They also remain unchanged.

Pure triglyceride spheres, yolk globules, nucleolar extrusions, as well as cholesterols and cholesteryl esters are absent.

Introduction

THE inadequacy of the osmium and silver techniques for the so-called Golgi apparatus has been brought out by the confusion that still exists with regard to our knowledge of the chemistry of the cytoplasmic inclusions of the earthworm egg, in spite of at least a dozen papers previously published on the subject (see Nath, 1957).

In our present study of the cytoplasmic inclusions of the egg of the earthworm, *Pheretima posthuma*, we have employed phase-contrast and interference microscopy on living material, and histochemical techniques. Our studies have once more emphasized that the so-called Golgi techniques of osmium and silver are inadequate, indeed misleading, in studies of the chemistry and morphology of cytoplasmic inclusions.

Material and Technique

The specimens of *P. posthuma* used in the present study were collected during the months of July and August, 1956 and 1957. Material was also collected during the remaining months of the year for study of seasonal variations in the ovaries. The animals were dissected alive in the physiological solution of Baker (1944), i.e., 0·2 ml of 10% anhydrous $CaCl_2$ in 100 ml of

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0·7% NaCl solution, and the ovaries were transferred directly to the various fixing fluids.

The fixative employed for almost all the histochemical tests was formaldehyde-calcium followed by postchroming, as recommended by Baker (1946) for his acid-haematein technique. The ovary was embedded in gelatine for the cutting of frozen sections. A complete list of the histochemical reactions used for the detection of lipids, proteins, and carbohydrates is given in table 1 on page 477.

The action of lipid-solvents was also investigated. For details of the methods used, see Nath and others (1958). Fresh ovarioles were in some cases exposed to the solvents. In other cases ovaries were fixed in formaldehyde-calcium, and sometimes postchromed; gelatine sections were prepared, and the solvents acted upon these. Hot solvents could not be used as it is impossible to manipulate the ovaries on account of their very small size, even in a micro-Soxhlet extractor.

Most of the histochemical reactions listed in table 1 were also employed on centrifuged material. The ovaries for this purpose were centrifuged for one hour in Baker's physiological solution. A Christ (W. Germany) electric centrifuge was used at 5,000 rev/min. The ovaries were subsequently fixed by the usual formaldehyde-calcium/postchroming technique. The stratification achieved was excellent.

Living ovaries were studied by phase-contrast and interference microscopy. The ovaries were mounted in a drop of Baker's physiological solution, without teasing. The pressure of a no. 1 coverslip was sufficient to flatten the oocytes satisfactorily. The margins of the coverslip were sealed with vaseline to avoid evaporation. A Zeiss 'W' microscope with Ph 100/1·25 objective and K 8x eyepiece was used for phase-contrast studies. A Zeiss micro-reflex attachment with Contax camera was used for photomicrography. Kodak panchromatic 35-mm film was used. A Charles Baker instrument with both shearing and double-focus objectives was used for interference microscopy.

The term 'lipid' is used in this paper to mean triglycerides and all other cellular constituents having similar solubilities (phospholipids, etc.).

Results

Morphological observations

The germ-cells in the ovary of *P. posthuma* are arranged in the form of filaments, radiating from the point of septal insertion of the ovary. Undifferentiated germ-cells and oogonia are present at the proximal end of each filament (ovariole), whereas the oldest oocyte occupies the distal end. All the developmental stages of an oocyte are arranged serially in a single ovariole; this greatly facilitates the study of oogenesis.

The use of the various physical lipid colorants has revealed that the lipids are present in the form of homogeneous, spherical bodies in the cytoplasm during all stages of oogenesis. The number and size of these lipid spheres increase considerably with the growth of the oocyte. In fact, the whole

TABLE 1. *Table showing the histochemical reactions of the oocytes of* Pheretima posthuma

Technique	*Fixative*	*Embedding medium*	*Thickness of sections* (μ)	*Reference*	*Mitochondria*	*Bodies in the follicular epithelium*	L_1	L_2	*Vacuoles*
Sudan black B:									
in 70% ethanol	FS+PC	G	10	Baker, 1949, 1956	++	N	+++	A	−(P)
	FCa+PC				++	++	+++	+++ (Some ringed)	,,
in 70% ethanol at 60° C	,,	G	10	Chiffelle and Putt, 1951	++	++	+++	+++	,,
in propylene glycol at 60° C	,,	G	10	Krishna, 1950	++	++	+++	+++	,,
— after treatment with cold acetone	Fr. and FCa	G	10	Pearse, 1954	++	++	+++	++(R)	,,
— — cold ethanol	,,	G	10	,,	−(P)	O	−(R)	O	,,
— — cold ether	,,	G	10	,,	−(P)	O	−(R)	O	,,
— — methanol/chloroform	,,	G	10	,,	−(P)	O	−(R)	O	,,
Sudan III and IV:									
in 70% ethanol/acetone	FCa+PC	G	10	Kay and Whitehead, 1941	+	++	++	+++	
in 1% gelatine suspension	,,	G	10	Govan, 1944	++	++	++	+++	,,
Fettrot in 70% ethanol	,,	G	10	Pearse, 1954	+	++	++	+++	,,
Nile blue	,,	G	10	Cain, 1947, 1948	blue	blue	blue	violet	,,
Acid haematein (AH)	,,	G	10	Baker, 1946	++	+++	+++	++(R)	,,
— after pyridine (PE)	WB+PE	G	10	,,	−	O	++(R)	O	,,
Fischler's reaction	FS	G	10	Pearse, 1954	−	−	−	−	,,
Periodic acid / Schiff (PAS)	FCa+PC	G	10	Hotchkiss, 1948 Pearse, 1954	−	−	−	−	,,
,, ,,	Carnoy	P	7½	,,	−	−	−	−	,,
Mercuric bromphenol blue (Hg–BPB)	FCa+PC	G	10	Mazia and others, 1953	++	−	+(R)	−	,,
,, ,,	Carnoy	P	7½	,,	++	−	+(R)	−	,,
— after treatment with ethanol	Fr. and FCa	G	10	,,	+++	−	++(R)	−	,,
— — pyridine	WB+PE	G	10	,,	+++	−	++(R)	−	,,
Performic acid / Schiff (PFAS)	FCa+PC	G	10	Pearse, 1951	−	+++	−	+++	,,
— after bromination	,,	G	10	Lillie, 1952	−	−	−	−	,,
Plasmal reaction	,,	G	10	Hayes, 1949 Cain, 1949	−	−	−	−	,,
Cholesterol reactions	,,	G	10	Schultz, 1924 Gomori, 1952 Romieu, 1927 Pearse, 1954	−	−	−	−	,,

Key: A = Absent; FCa = formaldehyde-calcium; FS = formaldehyde-saline; Fr. = fresh material; G = gelatine; N = not observed; P = paraffin; −(P) = present but negative; PC = with postchroming; (R) = reduced in size; WB+PE = weak Bouin's fluid followed by pyridine extraction; + = weak reaction; ++ = moderate reaction; +++ = strong reaction; − = negative; O = dissolved.

process of oogenesis in earthworms, so far as the cytoplasm is concerned, is restricted to the synthesis and growth of the lipid spheres. There is no indication of the presence of any protein or carbohydrate yolk at any stage of oogenesis.

The cytoplasm of the earliest cell studied in an ovariole reveals the presence of four or five minute lipid granules (fig. 1, A, B). With the growth of the oocyte, the number of the lipid granules increases and the cytoplasm now also shows a mass of mitochondria near the nucleus (fig. 1, C). Gradually the mitochondria and the lipid spheres become uniformly scattered throughout the cytoplasm of the developing oocyte (fig. 1, D–G). The mitochondria do not change at all even in the oldest oocyte studied (fig. 1, H, I). The size of the largest lipid sphere in the oldest oocyte studied is approximately $3{\cdot}5\,\mu$ (fig. 1, I).

Even in the youngest oocyte studied, the cytoplasm reveals the presence of some vacuoles in all the preparations. The number of these vacuoles increases considerably, and some of them invade the central regions of the cytoplasm also. These vacuoles persist even in the oldest oocyte without any visible change (fig. 1, A–I).

The nucleus remains small throughout oogenesis, and is almost invariably excentric. It always contains a single nucleolus.

In very young oocytes the small lipid globules appear by phase-contrast or interference microscopy as homogeneous bodies giving a high phase-change. One or two larger spheres that are present at this stage show a ringed appearance: a grey medulla is seen to be surrounded by a very thick, dark, cortical sheath when positive phase-contrast microscopy is used (fig. 2, A, B). The histochemical tests described in the later part of this paper have shown that these larger bodies are chemically different from the smaller ones. They have been called L_2 bodies, while the smaller ones, which appear homogeneous under phase-contrast, are called L_1. There is a gradual increase in the number and size of the L_2 bodies, which continue to give a ringed appearance under the phase-contrast microscope (fig. 2, C, D). The mitochondria always appear as minute grey granules under the phase-contrast, whereas the vacuoles completely escape detection, probably because the cytoplasm is choked with the lipid bodies. Moreover, the out-of-focus images of the lipid bodies also appear as vacuoles; thus it is not easy to differentiate them from the real vacuoles.

When oocytes are studied under the interference microscope and the analyser is so set as to give a pinkish-yellow coloration to the cytoplasm, the larger spheres (L_2) show a deep violet cortex surrounding a blue medulla. A general diffused, though patchy, violet coloration is also present throughout the cytoplasm; this might be due to the presence of mitochondria. Some vacuoles are also visible. If a blue-green filter is now used in front of the light source, the picture presented by these spheres is quite similar to that under the phase-contrast microscope. But when the analyser of the interference microscope is so set that the colour of the cytoplasm is yellow, the colour of both the L_1 and L_2 spheres is pinkish violet, and the L_2 spheres do not exhibit a duplex or ringed structure at all.

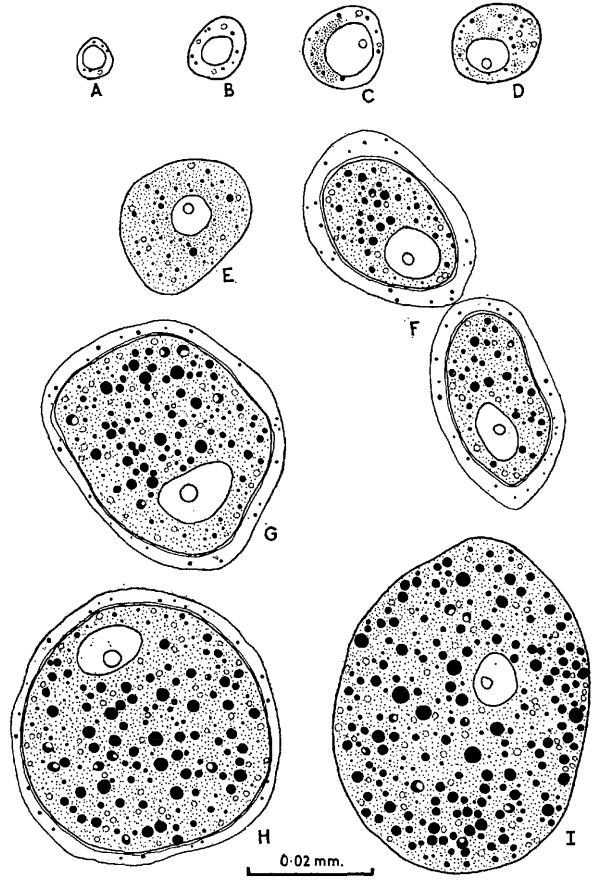

FIG. 1. Camera lucida drawings of the oocytes of *P. posthuma*, showing the distribution of the lipid bodies, vacuoles, and mitochondria during oogenesis, and their reaction to Sudan black B after fixation in formaldehyde-calcium and postchroming. A and B, very young oocytes showing a few lipid bodies and vacuoles but no mitochondria. C and D, oocytes showing the appearance and gradual dispersal of the mitochondria. E–H, oocytes showing the growth of the cell and increase in the number of lipid bodies, mitochondria, and vacuoles. I, largest oocyte studied. Note the increase in the size of the lipid bodies, some of which (L_2) have a ringed or crescentic appearance.

The above observations suggest that the duplex or ringed appearance of the L_2 spheres under the phase-contrast and interference microscopes is an optical artifact produced by a great difference in the phase displacement of light caused by the lipid bodies in comparison with the ground cytoplasm. This difference, when reduced by changing the analyser of the interference microscope and thus reducing the over-all contrast, results in the disappearance of the duplex spheres, which now appear homogeneous.

The homogeneous nature of the lipid bodies is stressed in the histochemical section of this paper.

Centrifuged oocytes reveal the following stratification under the phase-contrast microscope, as well as by lipid tests (figs. 2, E, F; 3, C).

(i) The vacuoles go to the centrifugal pole and generally collapse.

(ii) The mitochondria as well as the small lipid bodies (L_1) form a distinct stratum, next to the heavy, vacuolar substance.

(iii) The third stratum is formed by the hyaloplasm containing the nucleus with its nucleolus.

(iv) The centripetal pole is occupied by the larger lipid spheres (L_2), which give the usual ringed appearance under the phase-contrast microscope (fig. 2, F).

Histochemical reactions

The various histochemical reactions tried and their results are summarized in table 1 on page 477.

Mitochondria. The mitochondria have the usual phospholipid-protein nature which remains unchanged throughout the course of oogenesis.

The PFAS for unsaturated bonds (HC=CH) as given by Lillie (1952) and Pearse (1951) invariably gave a negative reaction with the mitochondria, although natural phospholipids are usually highly unsaturated. The very small size of these granules may be the cause of this failure.

Lipid bodies. Some of the L_2 bodies are not homogeneously coloured by Sudan colouring agents but appear as crescents or rings. This is due to the presence of some lipids which are solid at room temperature (12° C to 40° C). Almost all of them lose their crescentic appearance when coloured with Sudan black B in propylene glycol at 60° C. In this respect they are comparable to some of the L_3 bodies described in cockroach by Nath and others (1958).

FIG. 2 (plate). Photomicrographs of the living oocytes of *P. posthuma*, taken by positive phase-contrast microscopy.

A, a very young oocyte showing the optically homogeneous L_1 bodies in the cytoplasm.

B–D, oocytes showing a gradual increase in the size of the cell as well as in number and size of the lipid bodies; the larger lipid bodies (L_2) have a ringed appearance.

E, a large centrifuged oocyte, showing four distinct strata of the cell inclusions (further explanation in text).

F, an oocyte which is only partially centrifuged. Note the L_2 bodies, which appear as distinct rings; they have moved towards the centripetal pole. The heavy substance has just started accumulating at the centrifugal pole.

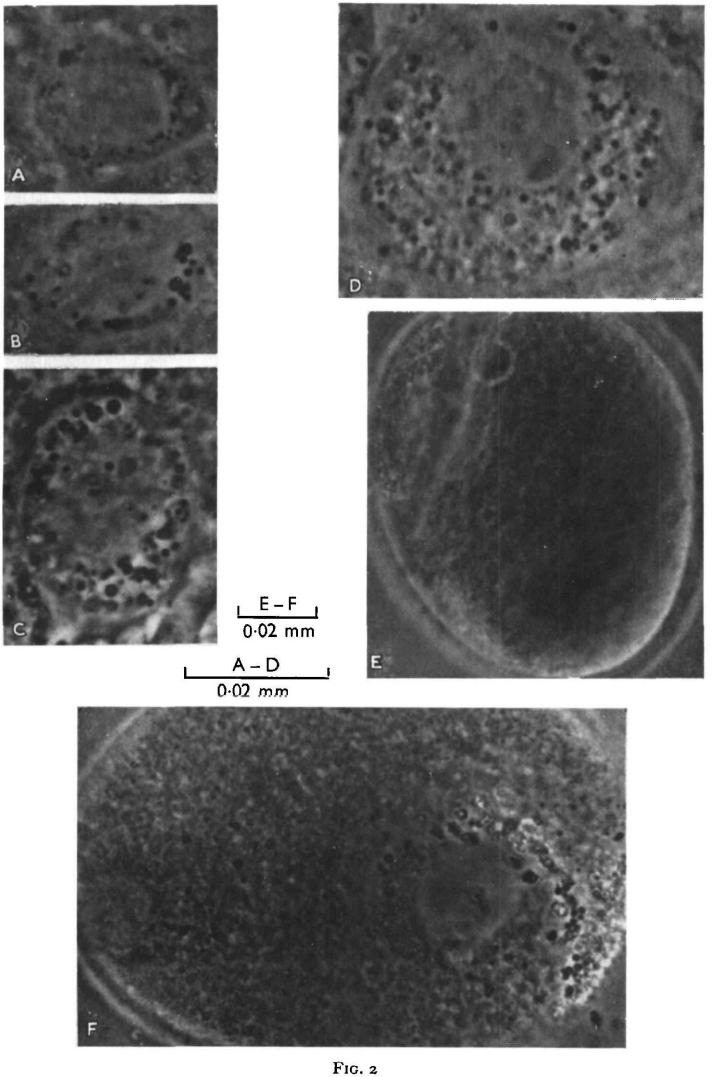

FIG. 2

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The results of the acid-haematein (AH) test of Baker (1946) with its pyridine extraction (PE) control require special mention. The L_1 bodies give a uniform, intense, blue-black coloration in the AH test; this indicates the presence of phospholipids and/or phosphatidic acids (Casselman and George, 1952). The absence of the latter is shown by the negative reaction which both

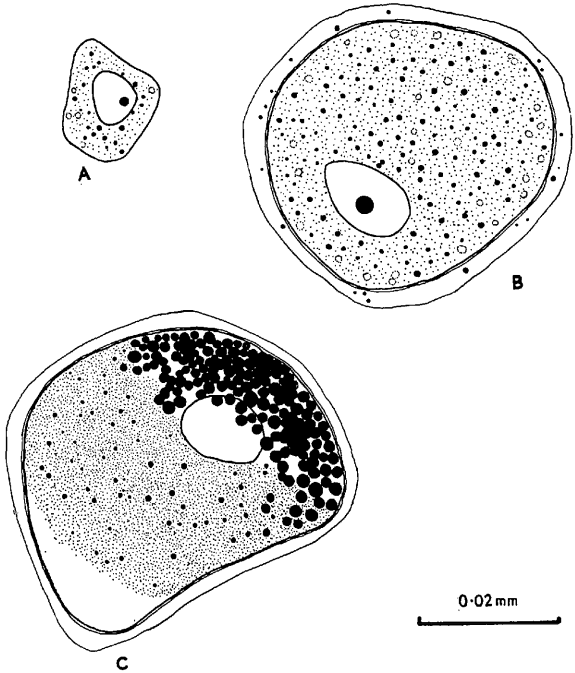

FIG. 3. Camera lucida drawings of the oocytes of *P. posthuma*. A and B, a young and an old oocyte from an acid-haematein preparation. Note the reduction in the size of L_2 bodies in B (compare fig. 1, H), which appear almost as small as L_1 bodies. The single nucleolus is also AH-positive. C, an oocyte from a centrifuged ovary fixed in formaldehyde-calcium, post-chromed, the sections coloured subsequently with Sudan black B. Note four distinct strata. (Further explanation in text.)

the L_1 and L_2 bodies give to Fischler's test for fatty acids (Pearse, 1954) as well as by various extractions. Thus the L_1 bodies contain phospholipids. Now, their reaction to the PE test is not completely negative: the L_1 bodies in these preparations appear as blue granules, though they are much reduced in size. The appearance of the L_1 bodies in PE preparations is very similar to their appearance in Hg–BPB, where also they are coloured blue. This clearly

indicates that the L_1 bodies have a core rich in proteins, surrounded by a thick sheath of phospholipids. The core also seems to contain phospholipids in addition to proteins, as L_1 bodies do not appear as rings either in Sudan or AH preparations (fig. 1, D; 3, A).

The L_2 bodies, on the contrary, do not have any proteins in them, as they give a completely negative reaction in Hg–BPB and PE control. They appear much reduced in the AH test. A similar picture is given by the L_2 bodies when the acetone-extracted material is coloured with Sudan black B. This shows that they have a phospholipid core which is surrounded by a sheath of some neutral lipids (acetone-soluble) (compare fig. 3, B with fig. 1, H, I).

This duplex nature of the L_2 bodies is further elucidated when AH preparations are coloured with either Sudan III, IV, or Fettrot 7B. In such preparations the L_2 bodies show a blue-black core (AH positive) surrounded by a brilliant pink sheath (Fettrot- or Sudan-positive).

The reactions of the L_2 bodies show that they have a neutral lipid sheath, which in the absence of other lipids, presumably consists of triglycerides.

Thus it appears that the L_1 bodies contain a core rich in proteins mixed with phospholipids, surrounded by a thick sheath of phospholipids only; whereas the L_2 bodies have a core of phospholipids surrounded by a thick sheath of triglycerides. The lipids of the L_1 bodies are comparatively saturated, whereas the lipids of L_2 bodies are highly unsaturated (PFAS positive).

The cytoplasm of the syncytial follicular epithelium also contains lipid spheres, which contain phospholipid but no protein.

Vacuoles. The vacuoles present in the cytoplasm do not give a positive reaction to any of the histochemical tests tried. Thus their contents appear to be devoid of any lipids, proteins, or carbohydrates. They remain unchanged even in the oldest oocyte studied.

Discussion

The egg of the earthworm is alecithal, since as no yolk globules, consisting of proteins or carbohydrates or both, have been described in it. The egg of *P. posthuma* contains three types of well-defined cytoplasmic inclusions, viz. mitochondria, lipid bodies, and vacuoles; the latter are thrown towards the centrifugal pole when the eggs are centrifuged.

Mitochondria. We confirm the observations of Nath and Bhatia (1944) that the mitochondria in the oocytes of *P. posthuma* are granular in form, and remain so throughout oogenesis. Our studies under the phase-contrast and interference microscopes have very clearly shown that the mitochondria, in this material, are not filamentous in form. They appear for the first time in the form of a mass near the nucleus. This fragments into several patches and soon the mitochondria get distributed uniformly in the cytoplasm.

Lipid bodies. Nath and Bhatia (1944) described two kinds of lipid granules in the oocytes of *P. posthuma*: the small so-called Golgi granules not stainable with Sudan IV, and spherules colouring with Sudan IV. By employing various Golgi techniques, these authors took great pains to show that the

so-called Golgi granules grow into the spherules colouring with Sudan IV. We have also concluded that there are two types of sudanophil lipid spheres in the oocytes of the earthworm, viz. L_1 and L_2 bodies, which respectively correspond to the Golgi granules and Sudan IV spherules of Nath and Bhatia.

Nath and Bhatia (1944) made some observations on the chemical composition of these lipid bodies, which are not fully warranted by the inadequate Golgi techniques that they used. Nevertheless, with the techniques they employed, these authors showed: 'To begin with the Golgi granule seems to be made up of a thick cortical part consisting of phospholipides linked with proteins and a very small core consisting of fats or lipoids or both. On account of these proteins in the thick cortical part, the Golgi granule resists solubility in acetic acid and alcohol, and does not stain with Sudan IV. With the growth of the oocyte the Golgi granules also grow, and during their growth they get shorn of their protein constituents. Consequently, the cortex of the Golgi vesicle is greatly attenuated. The large spherule thus formed differs markedly from the initial Golgi granule as shown by its stainability with Sudan IV and its almost complete solubility in acetic acid.'

By employing various modern histochemical techniques we have shown that the L_1 bodies (Golgi granules of Nath and Bhatia) have, on the contrary, a core of proteins and phospholipids and a cortex of phospholipids only. The L_2 bodies, which are derived from the L_1 bodies by growth and change in chemical composition, have a phospholipid core surrounded by a triglyceride sheath. The L_1 bodies resist extraction in pyridine after fixation in weak Bouin (Baker, 1946) owing to their protein constituents; on the contrary, the L_2 bodies are completely dissolved out.

It may be pointed out that in the cockroach egg (Nath and others, 1958) the L_2 bodies contain a core of triglycerides and a sheath of phospholipids. In both cases the appearance of the L_2 bodies is ringed in osmium techniques (Nath and Mohan, 1929, in the cockroach; Nath, 1930, and Nath and Bhatia, 1944, in the earthworm). As pointed out by Nath and others (1958) in the cockroach egg, this ringed appearance of the lipid bodies is due to an incomplete reduction of osmium tetroxide.

We have shown that the ringed appearance of the L_2 bodies of the egg of the earthworm under the phase-contrast microscope and also under the interference microscope at a particular setting of the analyser is an optical artifact.

It will be clear from this discussion that, since the lipid bodies in the egg of the earthworm have neither a reticular structure nor a fixed chemical composition, the classical reticular Golgi apparatus is not homologous with these lipid bodies.

Vacuoles. Norminton (1937) described in the egg of *Lumbricus* a heavy fluid substance, which goes to the centrifugal pole in the ultracentrifuged eggs. Nath and Bhatia (1944), by employing an ordinary electric centrifuge, confirmed this observation of Norminton. It is significant that Nath and Bhatia (1944) figure and describe this heavy substance as honey-combed in Da Fano, Kolatchev, and even in Bouin preparations. Indeed, in some of the figures

(e.g., text-fig. 6, D) of these authors, delicate, clear vacuoles have been shown in the centrifugal pole occupied by the heavy substance.

Nath and Bhatia (1944) considered these vacuoles as artifacts. We have seen clear delicate vacuoles uniformly distributed in the living oocytes studied under the interference microscope, distinct from the other cytoplasmic inclusions. It has already been stated that under phase-contrast these vacuoles are mixed up with the out-of-focus images of the lipid bodies, which also appear as vacuoles. We have further shown that in all the histochemical preparations, these vacuoles are distinct from the mitochondria and the lipid bodies; they do not contain any lipids or carbohydrates or proteins.

O'Brien and Gatenby (1930) described in the egg of *Lumbricus* vacuoles staining vitally with neutral red, distinct from the mitochondria and the Golgi elements (our lipid bodies). The vacuoles described by us are homologous with the 'vacuolar system' of these authors. It is also significant that Norminton (1937) suggested that the heavy substance is present in the form of droplets in uncentrifuged eggs.

Nucleolar extrusions. Srivastava (1952, 1953) is the only worker on the earthworm egg who has described nucleolar extrusions in *Lumbricus*. The nucleolar extrusions of Srivastava are in fact the protein cores of the L_1 bodies which resist solubility in Bouin and Carnoy.

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