THE BIOCHEMISTRY OF DEVELOPMENT

By JEAN BRACHET

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Foreword

THE present monograph does not aim to replace classical treatises of Chemical Embryology such as J. Needham's *Chemical Embryology* (1931) and *Biochemistry and Morphogenesis* (1942), or the author's *Embryologie chimique* (1944, 1945, 1950), but to introduce the reader to the recent advances made in the field. A full treatment of the main problems which now face the chemical embryologist is entirely outside the scope of the present small book: such a long and important task would mean an almost complete re-writing of our previous *Embryologie chimique*. Pending such an extensive revision, it was felt that a much shorter discussion of the main problems which now lie ahead might be useful for many advanced students and teachers, especially since there has been no text-book in Chemical Embryology for almost 10 years. In fact, the material presented in this monograph is essentially the subject of the author's lectures to advanced students who are already familiar with his *Embryologie chimique*; it is a brief summary of the many advances made in a field which is becoming more and more active again, as indicated by the recent publication of a very important Symposium which was held in Baltimore in 1958 (*The Chemical Basis of Development*, edited by Mc Elroy and Glass).

Since no attempt is made towards completeness, the importance of the different chapters varies a good deal; the general plan of the book, however, remains, in a broad sense, that of the *Embryologie chimique*: development will be followed, as is natural, from the formation of the gametes to the differentiation of specialized organs such as lens or muscle. The accent, of course, is always placed on the more biochemical aspects of embryonic development; however, the recent morphological findings, made with the electron microscope, have not been left out, and we have tried to present one integrated morphological and biochemical picture of development. In view of the limited length of the present monograph, morphogenesis in other organisms than developing eggs (unicellular organisms or plants) had to be left out, despite its obvious interest.

If this monograph owes much to *Embryologie chimique*, it is also a development of certain chapters of the author's more recent *Biochemical Cytology*: the reader of this book will find unavoidable repetitions; the emphasis, in the present monograph is, however, continuously on the egg and the embryo.

No attempt has been made to cover the whole of the literature in the field. Most of the papers cited in the present monograph are either review

articles or recent publications, in which references to older work can easily be found.

It is a great pleasure to express our warmest thanks to all those who helped us in preparing and writing this book: Drs. J. W. Legge (Melbourne), M. Nemer (Harvard University) and B. Sells (Montreal) kindly read the manuscripts and considerably improved the English. Drs. B. Afzelius, H. G. Callan, M. Durand, J. G. Gall, H. Gay, N. Kemp, D. Mazia, J. Pasteels, G. Reverberi, T. Yamada very kindly gave us a number of their photographs and allowed us to reproduce them; the book would have lost much without these beautiful illustrations. Invaluable help was obtained from Mrs. E. De Saedeleer and Y. Thomas for the preparation of the manuscript and from Professor H. De Saedeleer for that of many figures and the index. We are deeply grateful to all of them.

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CHAPTER I

Gametogenesis

A. INTRODUCTORY REMARKS

GAMETOGENESIS, the process which leads to the formation of eggs and spermatozoa, consists in a highly specialized series of events: for instance, eggs should not be considered as ordinary cells, simply inflated with the deutoplasmic reserves (yolk, fat droplets, glycogen) which the embryo requires for further development. Eggs are much more than that: during oogenesis, the genetic material of the oocyte (which is contained in the large nucleus, the so-called germinal vesicle) has been actively at work and has exerted its influence on cytoplasmic organization and synthetic processes. All the potentialities required for further development and differentiation, including the capacity to initiate such biochemical processes as synthesis of new, specific proteins, are already present in the unfertilized egg. Even though haploidy is, as a rule, lethal, parthenogenetic embryos can develop to a large extent, without the participation of sperm cells. In Amphibians, for instance, experimentally produced parthenogenetic embryos may undergo normal cleavage, gastrulation, neural induction and differentiation of highly specialized organs such as eyes, brain, heart or kidney.

Spermatogenesis is, obviously, a still more specialized process, since it leads to the formation of the haploid, freely swimming spermatozoa. These very peculiar cells are apparently devoid of the high potentialities for development which are so characteristic of the egg: so far, nobody has obtained morphogenesis starting from a spermatozoon, even when it is placed in a complex nutritive medium.

Nevertheless, the spermatozoon is, from a genetic viewpoint, as important as the much larger egg; its tiny nucleus, which is so simplified as to have the structure of a crystal, contains all the paternal genes, i.e. all the potentialities for the expression, in the adult, of paternal characters. It should also be kept in mind that the production of gametes (as well as that of spores in plants) represents a very complicated mechanism from the genetic viewpoint: meiosis, in ovaries and testes, leads to the formation of haploid cells and to genetic segregation, through elaborate mechanisms, which will not be described here since so little is known about their chemical nature; fertilization is accompanied by the recombination of genes, which had

been segregated at meiosis. The very fact that meiosis, a very specialized process, is always associated with gametogenesis in animals suffices to show that the haploid gametes are not cells in the usual sense of the term.

In the present chapter, oogenesis will be first studied, and we shall consider successivel the cytoplasm and the nucleus of the oocyte; the end of the chapter will be devoted to spermatogenesis, from both the morphological and the biochemical viewpoints.

B. OOGENESIS

1. General metabolism

The metabolism of growing oocytes is directed toward the synthesis of the various reserve materials which will later be used by the developing embryo: these materials primarily include glycogen, lipids and proteins; the latter, which are present in the form of the microscopically visible yolk platelets, will be discussed later on in some detail.

Concerning *glycogen*, it is enough to say that it is usually present in the form of small granules or macromolecules; when the egg is rich in yolk, as is the case for amphibian or avian eggs for instance, most of the glycogen is found at the animal pole; a definite gradient, decreasing from the animal to the vegetal pole, can often be easily demonstrated by cytochemical methods. In the frog eggs, Brachet and Needham (1935) found part of the glycogen bound to proteins, in the form of desmo-glycogen.

The situation is somewhat more complicated in the case of *lipid* droplets: according to Holtfreter (1946), such free droplets do not exist in amphibian eggs; the lipid inclusions are surrounded by a thin protein coat and the oocyte contains therefore 'lipochondria', rather than free lipid droplets. Treatments which produce the denaturation of proteins (marked shifts in the pH of the surrounding medium, for instance) break down the protein coat which surrounds the lipids and set these free.

It is a well-known fact that synthesis of glycogen, lipids and proteins requires energy, and that the latter is provided by the oxidative phosphorylations, which have their main site in the mitochondria (see Brachet, 1957, for a more detailed study of this question). It is very unfortunate that extremely little is known about energy production and utilization in growing oocytes; energy is probably stored in the energy-rich phosphate bounds of adenosine triphosphoric acid (ATP) during oogenesis. But no study of the ATP content during oogenesis has ever been made and all that we know is that, according to Metscherskaja (1935), the oxygen consumption of growing frog oocytes is highest at the stage when yolk platelets, glycogen and lipids are being synthesized at the highest rate (i.e. in middle-sized oocytes). A much more complete and recent study of respiration during oogenesis is that of Gonse (1955, 1957), who worked on a very favourable material, the oocytes of *Phascolosoma*, which grow freely in the coelomic

fluid of the maternal organism. He found that respiration, when measured in the coelomic plasma, shows two peaks, corresponding to periods of accumulation of ribonucleic acid (RNA) in the cytoplasm. He also studied in detail the effect of added substrates (succinate, pyruvate, glutamate, etc.) on the respiration of these oocytes and came to the conclusion that the Krebs (tricarboxylic acid) cycle functions normally in medium-sized cells.

While studies such as those of Metscherskaja (1935) and Gonse (1955, 1957) have obvious interest, it must be admitted that they will miss the most important point until they are concerned with ATP production and utilization, rather than oxygen consumption.

2. The role of follicle cells in yolk formation

There exists, at the present time, a good deal of evidence for the view that intact blood proteins can cross the barrier formed by follicle cells and directly get into the growing oocytes: the serological studies of Schechtman (1947), Nace (1953), Flickinger and Rounds (1956) have clearly shown that, in hen eggs, the yolk proteins bear very close resemblance with those of the maternal blood (review by Schechtman, 1956). In the rather different case of the Mammals also, the work of Brambell (1954, 1958) strongly suggests that the placental barrier is permeable to proteins and that the latter can be transferred as such from the blood to the embryo.

It is as yet not clear how this transfer of large molecules occurs; the most likely explanation is that the follicle cells, as well as the cell surface of the oocytes, play an active part in the protein transfer from blood to the egg cells. There is scattered evidence in favour of such a viewpoint: for instance, Brachet and Ficq (1956) have found that follicle cells, in amphibian ovaries, incorporate labelled amino acids very quickly, although it is unlikely that they are the site of extensive protein synthesis. In this respect, they strongly resemble kidney cells, which are also known to be capable of protein resorption (Oliver *et al.*, 1955).

It is very likely that the cell membrane of the oocyte itself is playing an essential part in the process of blood protein resorption: electron microscope studies by Kemp (1956a, b) on amphibian oocytes have clearly shown that the cell membrane of these oocytes forms 'micro-villi' (Fig. 1).* It is very probable, although not absolutely certain, that these micro-villi represent a mechanism of *pinocytosis*: the oocytes might, as amoebae or fibroblasts in cell cultures, expand pseudopodia and 'swallow' or 'drink' the protein-rich surrounding medium.

Such a process occurs on a grander scale in the ovaries of the insects, since, as shown by Schrader and Leuchtenberger (1952) and by Colombo

* Micro-villi have also been found in the oocytes of the Mammals (Sotelo and Porter, 1959, and Trujillo-Cenóz and Sotelo, 1959).

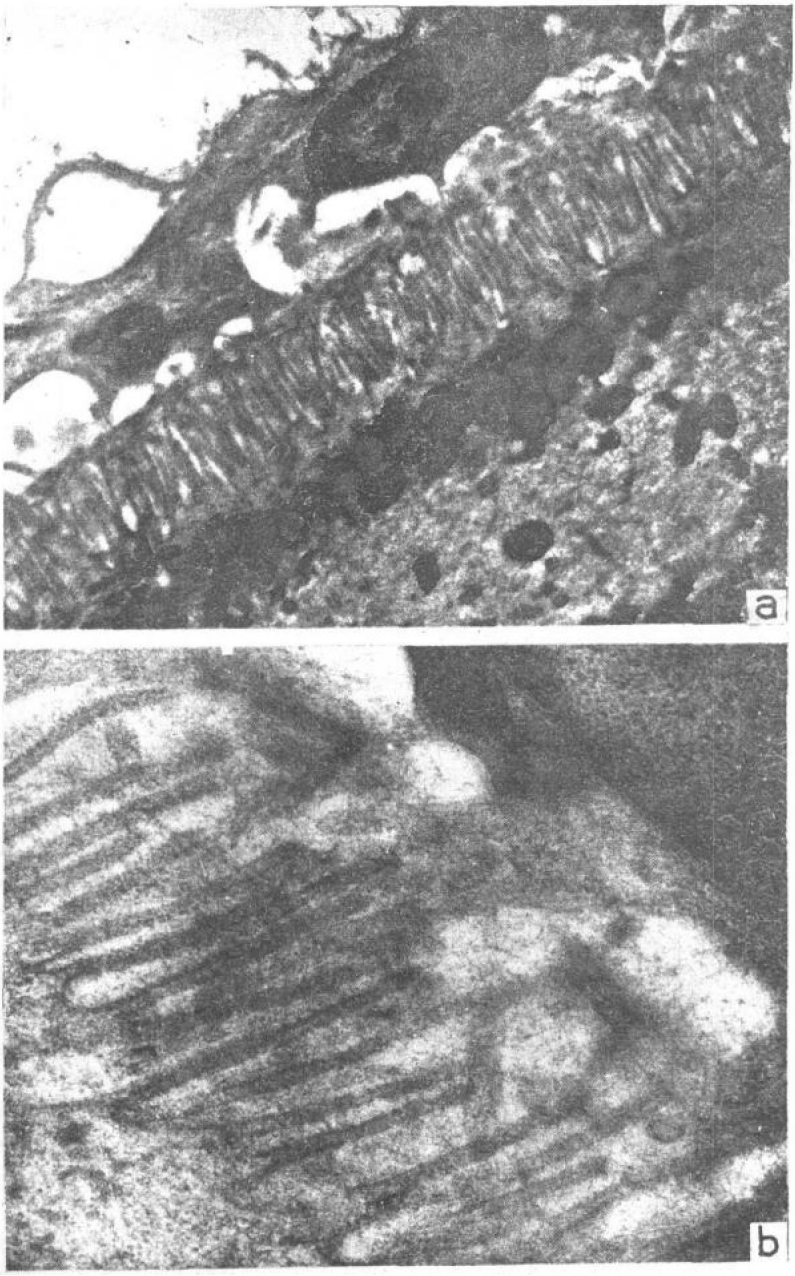

FIG. 1 (*a*). Surface of frog oocyte: below the follicle cells, the cortical layer contains the micro-villi and cortical granules (× 8750); (*b*). Micro-villi seen at a higher magnification (× 33,510) (courtesy of Dr. N. E. Kemp).

(1957) among others, whole follicle cells are engulfed into the growing oocytes and are assimilated by the egg cytoplasm.

3. The cytoplasm of the growing oocyte

(a) Ergastoplasm (Endoplasmic Reticulum)

The transparent, clear part of the cytoplasm (hyaloplasm) is known to be rich in *ribonucleic acid* (RNA) and to contain delicate and elaborate structures comparable to those known to electron microscopists as *ergastoplasm* or *endoplasmic reticulum*. It is impossible here to discuss adequately the significance and morphology of these RNA-containing structures; the interested reader will find an excellent summary of the question in the recent review by F. Haguenau (1958) and a less complete treatment of the question in the author's *Biochemical Cytology* (1957). In short, electron microscopy has shown that hyaloplasm, especially in gland cells, contains a network of double membranes of protein and phospholipid nature; small granules are imbedded in the membranes. This network, according to some electron microscopists, might play an essential part in the transfer of solutes from the cell membrane to the nucleus. There is good evidence for the view that the small granules (which are often called Palade's granules, since Palade first described them in 1955) are very rich in RNA; the latter, as shown many years ago by Caspersson (1941) and by the author (1942) and as repeatedly confirmed by many biochemists (see, for instance, Zamecnik *et al.*, 1956, or Brachet, 1957, for recent discussions of this question), plays a leading part in protein synthesis. The so-called 'microsomes' of the biochemists are nothing more than fragments of the ergastoplasm which has been broken down by extensive homogenization.

It is easy to detect RNA with cytochemical tests, either by combined staining with basic dyes and specific digestion with ribonuclease (Brachet, 1942), or by ultra-violet microspectrophotometry (Caspersson, 1941); it is also a relatively easy matter nowadays to study the ergastoplasm with the electron microscope in osmium-fixed ultra-thin sections.

In the following, we shall successively study the results obtained with the cytochemical and with the electron microscope techniques, restricting ourselves of course to the case of growing oocytes.

(α) *Cytochemical Studies on RNA Distribution With the Light Microscope.* It would be a worthless and almost impossible task to describe and discuss here *all* the papers which have dealt with RNA distribution in growing oocytes of all possible species. The main results have already been presented by us in 1942 and 1944 in the case of amphibian eggs; they can, *mutatis mutandis*, be extended to the ovaries of most animals.

The main findings can be briefly summarized as follows: young oocytes always contain large amounts of RNA in the cytoplasm and in the nucleoli.

During vitellogenesis, the yolk granules and the so-called yolk nucleus (*vide infra*) are poor in RNA: as a result, the observer often gathers the erroneous impression that there is a decrease in the RNA content during oogenesis (Fig. 2). In fact, there is no actual decrease in the RNA content, but a mere *dilution* of RNA in the oocyte which has tremendously increased in volume. A still more correct way to express things is to say that the RNA content increases during the whole course of oogenesis—but that, in late stages, the growth of the oocyte is faster than RNA synthesis. This can easily be demonstrated by two types of experiments (Brachet, 1941, 1942): (a) quantitative measurements of the RNA content of the oocyte at various stages of their growth clearly show that it increases continuously; (b) mild centrifugation of the ovary concentrates the RNA in the centripetal half of the oocytes: this part of the egg then becomes as basophilic as the young, yolk-free oocytes (Fig. 3). In many of the oocytes, however, an example being those of the Amphibians, part of the basophilic RNA-rich material is not displaced by centrifugation: a thin layer of this material is present, in the egg cortex, almost until the end of oogenesis; this is an interesting observation since it has often been assumed (but without experimental proof) by embryologists that substances which are not displaced by centrifugation are localized in the cortex (Brachet, 1947; Wittek, 1952). The role of this cortical, RNA-rich layer remains uncertain; it is likely that it plays a part in the synthesis of the egg proteins, at the expense of the amino acids present in the blood capillaries. The fact that this cortical layer of RNA disappears at the very end of oogenesis, when yolk formation stops (Wittek, 1952), supports such an interpretation. There is another RNA-rich region in the large oocytes: it is a thin perinuclear layer, which might represent a sign of nuclear intervention in cytoplasmic protein and RNA synthesis. Finally it should be pointed out that, in large oocytes such as those of the Amphibians, a very distinct polarity gradient in the RNA distribution can be observed: RNA is present in much larger amounts at the animal pole than at the vegetal end, with all the intermediaries in between.

Similar descriptions have been presented by many authors, working with very different materials; the interested reader will find many additional details in papers by Wittek (1952), Mulnard (1954), Urbani (1949, 1953), Bonhag (1955a, b), Yamamoto (1956), Cotronei and Urbani (1957), Fautrez-Firlefyn (1957), Colombo (1957), Cowden (1958), etc.; they deal mostly with oogenesis in Amphibians, Fishes, Insects and Crustaceans.

Since, as mentioned above, there is a close relationship between RNA content and protein synthesis, it is only to be expected that young oocytes, which are so rich in RNA, are important sites of protein synthesis. This expectation has been entirely fulfilled with the autoradiography studies of Brachet and Ficq (1956): they found that the incorporation of labelled

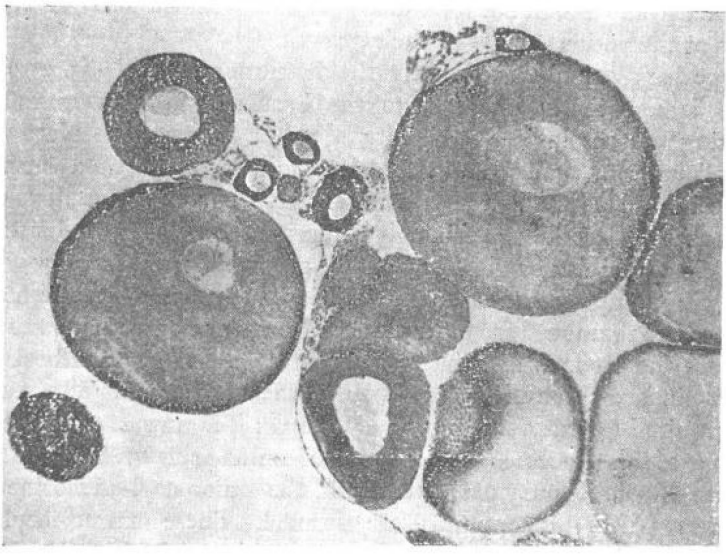

FIG 2. RNA distribution in amphibian oocytes (methyl green–pyronine staining method).

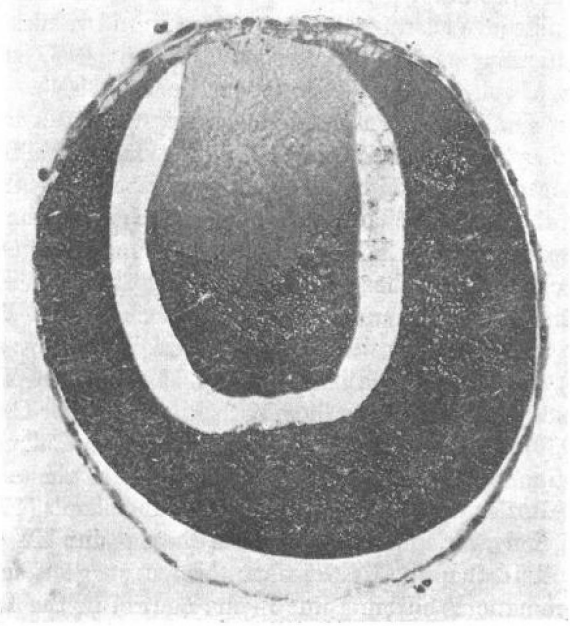

FIG. 3. Centrifuged oocyte of *Triturus*; sedimentation of the lampbrush chromosomes. Basophilia of the nucleus is due to the RNA present in the loops (Brachet and Ficq, 1956).

amino acids into proteins runs exactly parallel with the basophilia of the oocytes at the various stages of their growth.

It is perhaps a significant fact that the distribution of RNA in amphibian oocytes is apparently identical to the localization of macromolecules possessing the serological specificity of adult frog serum. Nace (1958) prepared antisera against adult female frog sera, conjugated them with fluorescein and used them as a stain for frog oocytes. The distribution pattern that he describes is strikingly similar to the well-known localization of RNA in amphibian ovaries. It would be of some interest to know whether the distribution pattern described by Nace (1958) remains the same when RNA has been removed from the sections by a digestion with ribonuclease.

Besides the obvious fact that growing oocytes actively synthesize yolk (i.e. proteins), there are reasons to believe that they contain all the equipment required for extensive protein synthesis: it is now accepted that the first stage of this process is the activation of amino acids by ATP, under the influence of soluble enzymes; as a result, the amino acyl-adenosinemonophosphate (AMP) compounds are formed. These amino acyl-AMP derivatives are, in a second stage, incorporated into soluble RNA and, in a third stage, into microsomal RNA (Hoagland *et al.*, 1958). Finally, the synthesis of specific proteins apparently occurs in the microsomes themselves (or, more accurately, in the ergastoplasm of the intact cell). It is perhaps a significant fact that eggs have been found to contain both the amino acid-activating enzymes (Scarano and Maggio, 1957) and unusually large amounts of soluble RNA (Brachet and Jeener, 1944).

A last point should be mentioned concerning the RNA content of the cytoplasm in oocytes: it has been claimed by Caspersson and Schultz (1939) that this content is controlled by the genetic composition of the nucleus, in particular by the amount of heterochromatin present in the latter. Thus oocytes of females having a XXY chromosomal composition would have a higher RNA content than those of females having the normal XX complement, since the Y chromosome is largely heterochromatic. This question has been the subject of considerable work and discussion: according to Callan (1948), chemical estimations of the RNA content of XX and XXY eggs show no difference between them both; the reason for Caspersson and Schultz's (1939) observations might be, according to Callan (1948), unequal resorption of the RNA-rich nurse cells into the oocytes. More recently, N. Altorfer (1953) essentially confirmed Callan's (1948) findings; she observed, however, that normal males (having the XY composition) contain more RNA than the XX females: this fact suggests, again, a role of the heterochromatic Y chromosome in the control of the RNA content. Similar observations have been made by Patterson *et al.* (1954), who worked with isolated *Drosophila* salivary glands. More recent and extensive work by Schultz (1956) and by Levenbook *et al.* (1958) has solved to a

large extent the previous discrepancies: the presence of a Y heterochromosome does not modify the actual RNA content of the oocytes, but it changes the composition (relative content in the various purine and pyrimidine bases) of their RNA; it also modifies the composition of the acid-soluble nucleotides, nucleosides and free bases pool. These very interesting studies of Schultz and his co-workers (1956, 1958) clearly show how delicate is the control exerted by the chromosomes (especially the heterochromosomes) on the RNA content and composition of the egg.

(β) *Studies with the Electron Microscope.* The number of papers describing the ultra-structure of oocytes is growing sensibly; mention has already been made of those of Kemp (1956 a, b) on amphibian eggs. Since the ultra-structure of these large oocytes is difficult to study, in view of its complexity, only smaller eggs, such as those of many marine Invertebrates, will be considered here.

The eggs of the sea urchin and those of *Spisula* (*Mactra*) have been studied in detail, with the electron microscope, respectively by Afzelius (1957) and Rebhun (1956). Their main conclusion, which is also shared by Pasteels *et al.* (1958, 1959a) and by Gross *et al.* (1958) is that the structure of the hyaloplasm is much simpler in these eggs than in cells of liver or pancreas. As shown in Fig. 4, no elaborate ergastoplasm or endoplasmic reticulum can be found in sea urchin eggs. The RNA-rich hyaloplasm only shows an abundance of granules with a diameter of about 150 Å, according to Afzelius (1957). They presumably contain RNA and can be compared to free Palade's small granules. Occasionally vesicles comparable to the microsomes obtained after mechanical destruction of ergastoplasmic lamellae in liver can be observed. There is no doubt that development of a typical ergastoplasm occurs at a rather late stage of development, as a result of a high order of structural, physiological and biochemical differentiation. It is interesting to note that a similar position exists in simple organisms, when the ultra-structure of amoebae, for instance, is studied with the electron microscope: like the eggs, they lack a well-defined ergastoplasm (Brachet, 1958).

(b) Mitochondria

Mitochondria, in eggs, have the same ultra-structure and functions as those of elsewhere (Fig. 4): morphologically, they are surrounded by a double membrane and *cristae mitochondriales* protrude in the interior; biochemically, they contain, as usual, a number of key respiratory enzymes, such as cytochrome oxidase. We shall see, later on, that there is some reason to believe that, in amphibian eggs at any rate, mitochondria undergo progressive complications from both the morphological and the biochemical viewpoints.

Many oocytes contain, at early stages of their development, a so-called

yolk nucleus; this microscopically visible structure is usually believed to be an accumulation of mitochondria; since the yolk platelets appear around this yolk nucleus, it is tempting to imagine that the mitochondria provide the energy required for the synthesis of some of the yolk proteins. Old work by Voss (1924) suggests that such an interpretation might well be correct for Amphibians: he found that the indophenoloxidase (or Nadi) reaction is given, in a specific way, by the yolk nucleus of young amphibian oocytes; there is little doubt that this reaction is a satisfactory index for cytochromeoxidase, a typical mitochrondrial enzyme.

In spiders, which contain a very elaborate yolk nucleus, Gabe (1956) has confirmed a previous finding of Jacquiert (1936) that —SH groups are abundant in this region of the oocyte; on the contrary, according to Gabe (1956), the yolk nucleus is poor in mucopolysaccharides, glycogen and RNA. Recent electron microscopy studies by André and Rouiller (1957) have confirmed that the yolk nucleus of spiders has a considerable degree of morphological complexity: it is formed of several layers, whose structure differs. Especially conspicuous is the abundance of mitochondria and Golgi elements in the outer layer, which is presumably the site of very active oxidative processes.*

A very different situation is found in sea urchin eggs, where the yolk nucleus is rich in RNA and is made of membrane pairs dotted with small granules or vesicles resembling microsomes (Afzelius, 1957).

It is obvious that very different structures, both morphologically and chemically, have been confused under the same name of yolk nuclei; a possible common denominator might, however, be their intervention in the synthesis of the yolk proteins, since protein synthesis requires the intervention of both microsomal RNA and ATP produced by the mitochondria.

(c) Golgi Elements—Heavy Bodies—Metachromatic Granules

If the situation regarding the yolk nuclei remains confuse, it is still worse in the case of other cell inclusions which are often found in oocytes: for instance, Dalcq (1957), Pasteels and Mulnard (1957) and Pasteels (1959b) have recently drawn attention to interesting granules or vacuoles which stain metachromatically with toluidine blue or brilliant cresyl blue. Immediately after the eggs are placed in contact with very dilute solutions of these dyes, small granules (the so-called α-granules) become visible; they soon enlarge and become transformed into 'β-granules'. Cytochemical tests show that they contain acid mucopolysaccharides and acid phosphatase. They have been found and studied in detail in the eggs of Ascidians, sea urchins and Molluscs. It is very likely that they have a still

* Comparable observations have been made by Millonig (1958) for the yolk nucleus of sea urchin oocytes.