MITOCHONDRIAL DIFFERENTIATION DURING THE EARLY DEVELOPMENT OF THE AMPHIBIAN EMBRYO

by

Lennart Nelson

AKADEMISK AVHANDLING som med tillstånd av rektors-ämbetet vid Umeå universitet för erhållande av filosofie doktorsexamen, framlägges till offentlig granskning torsdagen den 17 december 1981 kl 10.00 vid Fysiologi-Botanik Hufo, seminarierum B.

Examinator: Professor Sören Lövtrup, Umeå
Opponent: Docent Barbara Cannon, Stockholm
Title: Mitochondrial differentiation during the early development of the amphibian embryo.

Author: Lennart Nelson.

Abstract: Mitochondria from Xenopus laevis and Ambystoma mexicanum embryos between fertilization and the beginning of feeding were studied: the former with respect to metabolic behaviour, enzyme pattern and carrier activity, and the latter with respect to morphological parameters.

The metabolic behaviour of mitochondria was studied by assessing the rates of oxygen uptake in presence of various substrates. The rates of oxidation of most substrates change during development. The only substrate to be readily metabolized is glutamate (in presence of malate), whose rate of oxidation presents a peak during gastrulation and declines during larval development. The high rate of oxidation of glutamate and a high aspartate aminotransferase activity indicate that the glutamate-aspartate cycle may be predominant in early embryonic mitochondria.

The activity of enzymes from the matrix, the inner membrane and the outer membrane were studied. During early development activities of enzymes in the various compartments change independently of each other. Furthermore, enzymes within one compartment may vary independently. Measurements of carrier activity reveal that the carrier for dicarboxylic acids displays a high activity before gastrulation and decreases thereafter, while the tricarboxylic acid, pyruvate and glutamate/OH carriers show the opposite pattern of change, their activities being low or undetectable during early development.

This implies that a mitochondrial differentiation takes place during development, beginning at gastrulation when the first differentiated cells appear. In order to correlate mitochondrial and cellular differentiation, morphological parameters of mitochondria from undifferentiated and differentiated cells - Ruffini cells and epidermal cells - were analyzed. Mitochondria from the differentiated cells are significantly different from those in undifferentiated cells. Thus the processes of cell differentiation are accompanied by morphological and biochemical differentiation of the mitochondria.

Key words: Amphibia, Xenopus laevis, Ambystoma mexicanum, mitochondria, differentiation, enzymes, carriers, oxidation, development.

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LIST OF PUBLICATIONS

The present thesis is based on the following publications:


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The papers will be referred to by their Roman numerals given above.
INTRODUCTION

In the course of embryonic development the fertilized egg, a morphologically simple entity, is transformed into a larva which usually possesses the main features, morphological as well as physiological, of the body distinguishing the members of some major animal taxon. This marvellous transformation has been the subject of inquisition for several centuries.

From observations made on the development of vertebrate embryos, particularly those of various amphibia, it has been possible to subdivide the embryonic development into three phases: blastulation, morphogenesis and larval development. This subdivision was originally based on visible criteria. Thus, blastulation is characterized by rapid mitoses leading to a fragmentation of the egg in a number of cells, without other obvious morphological changes. Blastulation ends when morphogenesis begins, as evidenced by the appearance of the blastopore. As implied by the name, the embryonic body is constructed during the phase of morphogenesis. Larval development is the phase during which various physiological functions start. It is difficult to give an exact morphological definition of the transition between these two phases, but the beating of the heart (stage 33/34) may be taken as evidence that larval development has begun.

It is possible by means of chemical indices to characterize three phases almost coincident with blastula-
tion, morphogenesis and embryogenesis. Thus there are three phases in the protein synthesis, distinct both qualitatively and quantitatively (Løvtrup, 1974). But still more characteristic are the differences obtaining with respect to the synthesis of informational RNA: mRNA is synthesized at low and constant rate during cleavage and blastulation (Bachvarova, Davidson, Allfrey and Mirsky, 1966; Brown and Littna, 1966). But this synthesis seems to be independent of the nucleus, and unnecessary for mitoses since actinomycin D is without effect on development up to the late blastula stage (Brachet and Denis, 1963; Wallace and Elsdale, 1963). The protein synthesis taking place must therefore depend upon maternal mRNA. This means that no cell differentiation can take place in the early embryo; all the cells arising through division must belong to the same type, and they may in principle be characterized as "undifferentiated" cells.

The blastopore is formed by a new kind of cells, the Ruffini cells, which pull the embryonic cells into the interior, thereby intitiating gastrulation. This phenomenon must therefore be preceded by and be dependent upon a process of cell differentiation. In agreement with this inference it is found that the synthesis of mRNA increases in the late blastula some time before gastrulation sets in (Bachvarova, Davidson, Allfrey and Mirsky, 1966; Brown and Littna, 1966). Furthermore, during this period a new stage-specific population of unstable mRNA molecules appears which can no longer be detected at later stages of development (Denis, 1968).
The synthesis of mRNA which takes place at the onset of larval development is both quantitatively and qualitatively different from that going on in the preceding phase; first now is the information residing in the genome extensively exploited, resulting in the formation of stable adult mRNA molecules (Brown and Litton, 1966. Denis, 1968). In the following text the attention has been focused mainly on the two first phases. For the sake of simplicity the following discussion will mostly operate with the concepts "pregastrulation" and "post-gastrulation".
CARBOHYDRATE METABOLISM

Energy sources

The nature of the energy sources consumed during amphibian development has been studied since the beginning of the century. Brachet and Needham (1935), Gregg (1948), and Barbieri and Salomón (1963) have shown that in Rana and Bufo no glycogen seems to disappear before gastrulation, though at the time of hatching about 50 per cent has been consumed. Gregg's results show that the loss in total reducing carbohydrate is nearly identical with the decrease in glycogen, the latter thus being the main energy source.

The two other common energy sources, lipid and protein, have also been investigated (Wills, 1936; Gregg and Ballentine, 1946; Mes-Hartree and Armstrong, 1980). No measurable changes could be observed until late during development. It appears that the energy sources are used in the succession carbohydrates, lipids, proteins; proteins may be combusted only when no food is supplied (Løvtrup, 1974).

The chemical analyses have been complemented by measurements of the respiratory quotient (RQ). The value of RQ was found to be low during early development, reaching the value of unity only after gastrulation (Barth and Barth, 1954; Brachet, 1934; Petrucci, 1961; Legname and Barbieri, 1962). This suggests that glycogen is not combusted to any greater extent before
gastrulation, thus in agreement with the carbohydrate determinations.

The low RQ values may be interpreted as an indication of (a) lipid or protein combustion, (b) an incomplete carbohydrate oxidation or (c) combustion of other metabolites. The first alternative does not conform with the chemical analyses. The second possibility, if true, makes the customary interpretation of the RQ determinations void of sense, since they are based on the assumption that the oxidation is complete. The third alternative will be discussed later on (page 11).

Glycolytic pathway

The fact that total carbohydrate remains roughly constant before gastrulation does not necessarily imply that there is no turnover of carbohydrates during the earliest stages of development, carbohydrates may be involved in various synthetic reactions. One way to study this question involves the application of inhibitors. It has been found that sodium fluoride and iodoacetate, inhibitors of glycolysis, do not affect either oxygen consumption or development up to the late blastula stage (Barth and Barth, 1954; Salomón and Barbieri, 1964).

Among the glycolytic enzymes, hexokinase (Petrucci and Miranda, 1972; Wesolowski and Lyerla, 1979), phosphoglucomutase, phosphoglyceromutase and enolase (Salomón de Legname, Sánchez Riera and Sánchez, 1971) and lactate dehydrogenase (Adams and Finnegan, 1965)
have been studied. Hexokinase is the only enzyme claimed to be undetectable until metamorphosis. However, this is not in agreement with the result of Salomón de Legname et al (1971), Thoman and Gerhart (1979) and our own (I), since the determinations of the "shunt index" show that even at the earliest stages about 15 per cent of the glucose is metabolized in the glycolytic pathway, and the share increases continually. We may therefore conclude that although the embryo possesses the enzymes required for glycolysis, this pathway is hardly exploited at all before gastrulation, and early development is independent of glycolysis.

The reason for the low activity of this pathway during early development is not known. However, it is known that the phosphorylation of fructose-6-phosphate is the most important control point in glycolysis. Phosphofructokinase, the enzyme catalysing this reaction step is inhibited by high concentrations of ATP (Lehninger, 1975). The early amphibian embryo contains high levels of ATP (Løvtrup-Rein, Nelson and Løvtrup, 1974; Salomón de Legname, Fernandez, Miceli, Mariano and Legname, 1977) with an energy charge of about 0.95 (Thoman and Gerhart, 1979). As appears from figure 1 the \(^{14}\text{CO}_2\) production from \([6-^{14}\text{C}]-\text{glucose}\) increases with time whereas the ATP level decreases, suggesting a possible mechanism to explain the low initial rate of glycolysis (I).
Fig. 1. Changes in the glycolytic activity (I) and the level of ATP (Løvtrup-Rein et al., 1974) in the Xenopus embryo.
Pentose phosphate pathway

The importance of the pentose phosphate pathway for embryonic development was first demonstrated by Lindberg and Ernster (1948) in the sea urchin embryo. Later it has been found in many other embryos, among which those of amphibian species (Hermann and Tootle, 1964; Broyles and Strittmatter, 1973).

Confirming previous observations by Salomón de Legname et al. (1971) we have established that there is a turnover of glucose during development before gastrulation, but that most of the glucose passes through the pentose phosphate pathway (I). Since there is no net loss of reducing carbohydrates it may be presumed that this pathway is engaged in the production of ribose-5-phosphate for the synthesis of nucleic acids.
AMINO ACID METABOLISM

The main substance oxidized in the early embryo must be looked for outside the range of traditional energy sources. Various kinds of evidence suggest that the amino acids glutamic and aspartic acid are involved in the oxidative metabolism. Thus, it has been observed repeatedly that the egg contains large amounts of these amino acids which decrease during the early part of development (Deuchar, 1956; Metafora, 1967).

Isotope experiments show that glutamate is the preferred substrate during early development, the oxidation of glutamate being highest during gastrulation (I). Furthermore, until the late gastrula (II), glutamate, in the presence of malate, is oxidized faster than any other substrate by isolated mitochondria. Salomón de Legname, Sánchez Riera and Sánchez (1975) have shown that when homogenates of Bufo blastulae are incubated with radioactive glutamate, the isotope is found in aspartate and in uridine mono-, di- and tri-phosphates.

These observations suggest that glutamate is transformed to aspartate in the glutamate-aspartate cycle (Fig. 2). This cycle has an RQ value of 0.67 (Salomón de Legname, 1969), which corresponds well with that actually observed.
Fig. 2. The glutamate-aspartate cycle and the production of pyruvate.
PROPERTIES OF EMBRYONIC MITOCHONDRIA

Oxygen consumption

Since Godlewski's observations (1900) it is known that the rate of respiration increases during the course of amphibian development. This has been confirmed later innumerable times (e.g. Legname and Barbieri, 1962; Landström and Løvtrup, 1974).

The ratio between ATP and ADP + P_i is known to be essential for the regulation of the respiratory chain (Holian, Owen and Wilson, 1977). As mentioned earlier, the ATP level in *Xenopus* is high during cleavage stages (Løvtrup-Rein et al., 1974). By comparing the shape of the ATP curve with that established by Landström and Løvtrup (1974) for the oxygen uptake during early development of *Xenopus* it is obvious that a reversed relation exists between respiration and the amount of ATP per embryo (Fig. 3). A similar relation has been established for *Bufo* by Salomón de Legname et al. (1977). The pregastrula stages have the lowest respiration, in agreement with the high level of ATP. When the amount of ATP decreases the respiration increases.

Studies of the effects on development of the respiratory inhibitors, cyanide and azide, have been conflicting (Brachet, 1934; Barnes, 1944; Spiegelman and Moog, 1945; Crawford and Wilde, 1966; Lamy and Melton, 1972). However, cyanide and azide do not seem to inhibit oxygen uptake in the same way. Cyanide strongly in-
Fig. 3. Changes in oxygen consumption (Landström and Løvtrup, 1974) and ATP level (Løvtrup-Rein et al., 1974) in the Xenopus embryo.
hibits oxygen consumption in intact embryos at all stages of development, whereas the inhibitory effect of azide is low during early development and increases gradually (Brachet, 1934; Spiegelman and Steinbach, 1945; Crawford and Wilde, 1966). These results may be explained by the sensitivity of the mitochondrial respiratory chain towards the inhibitors. Cyanide inhibits mitochondrial oxygen uptake during the entire embryonic development, while the sensitivity towards azide increases (II).

Furthermore, dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, stimulates the increase in oxygen uptake to a larger extent before gastrulation than in later developmental stages (Gregg, 1960; Legname, 1968a; Legname, Fernandez and Miceli, 1971), indicating a "respiratory potential" exceeding the oxygen uptake which is exhibited by embryos under normal conditions. Since it is known that DNP has ATP:ase activity, and further that the ratio of ATP and ADP+P\(_i\) exerts a regulatory function on respiration, the effect mentioned here may be explained.

A number of observations indicate that mitochondria in the early embryo are metabolically different from those in later development. Our results concerning the oxidation of glutamate (+ malate) bear out this difference. As regards the citric acid cycle, Legname (1968b) has shown that arsenite stimulates mitochondrial oxygen uptake up to the blastula stage, but inhibits it during later stages. This double behaviour could be ascribed, on the one hand to its role as uncoupler and ATP:ase
stimulator effect, on the other hand, to its capacity to inhibit oxidative decarboxylation (Salomón de Legname et al., 1977).

During early development the level of ATP is high and the activity of the tricarboxylic acid cycle is low (Thoman and Gerhart, 1979). Thus, the main effect of arsenite is probably related to the ATP:ase effect which lowers the $\text{ATP}/(\text{ADP}+P_i)$ ratio which in turn stimulates respiration. However, the later phase of development is characterized by relatively low ATP levels and an active Krebs cycle (Salomón de Legname et al., 1977). The effect of arsenite may then be related to its action on oxidative decarboxylation, which is expressed as an inhibition of respiration.

Mitochondrial enzymes

The change in mitochondrial metabolism may be looked for both at the regulatory level and at the level of mitochondrial changes. Various findings indicate that mitochondria in the early embryo are different from those in the later embryo, quantitatively as well as qualitatively. For example, quantitative analyses of mitochondrial fatty acids reveal that their composition changes during early development (Bonini de Romanelli, Alonso and Bazán, 1981).

Wallace (1961) suggested that isocitrate is oxidized by the $\text{NADP}^+$-dependent enzyme during early amphibian development and Petrucci, Amicarelli, Di Cola and Papo-
Netti (1975) were unable to detect NAD\(^+\)-dependent isocitrate dehydrogenase. In the present studies these results could not be corroborated, NAD\(^+\)-dependent isocitrate dehydrogenase is present, although the activity is quite low, the lowest among the enzymes investigated (III).

In many other respects early mitochondria are indeed different (II; III and IV). Thus, malate dehydrogenase and aspartate aminotransferase, enzymes involved in the glutamate-aspartate cycle, are both very active before gastrulation, but decrease later on. The "rotenone-insensitive" NADH reductase decreases drastically after gastrulation, and begins to increase only during larval development. Monoamine oxidase is absent in early mitochondria, and becomes detectable only at the tailbud stage. Cytochrome oxidase and succinate dehydrogenase decrease slightly, but significantly, during development.

Mitochondrial carriers

A similar picture obtains with respect to the carriers (IV). Thus the activity of the malate carrier is high in the early embryo, and decreases gradually. A fumarate carrier, absent in mitochondria from the embryo of later developmental stages, and from the adult liver, is present in mitochondria isolated from the early embryo. On the contrary, the glutamate/hydroxyl carrier is absent in early mitochondria, and becomes detectable only at the tailbud stage. The activity of the tricarboxylic acid carrier is relatively low in early mitochondria, and increases subsequently.
Mitochondrial morphology

It was mentioned above that cell differentiation begins in the gastrula and it is thus a reasonable assumption that the observed mitochondrial differentiation is correlated with cell differentiation. It has been possible to confirm this conjecture as far as the mitochondrial morphology is concerned. Working with three different cell types, representing the cell differentiation patterns that arise spontaneously in the amphibian embryo, it was shown that in undifferentiated cells the mitochondria are small, almost spherical and with a condensed conformation. In differentiated cells, representing Ruffini cells and epidermal cells, the mitochondria are much larger and elongated, and with an orthodox conformation. The mitochondria are significantly different in the two types of differentiated cells (V).

Since it is know that mitochondria are capable of changing their shape and size by movement, fusion and fragmentation these observations raise the issue of whether true differentiation or temporary morphological change is being observed. In our case there is no reason to doubt that a real differentiation occurs, since the change in morphology is in good correlation with the functional changes.

Condensed mitochondria and high levels of ATP prevail in the pregastrula embryo (Løvtrup-Rein et al., 1974; Salomón de Legname et al., 1977). It is tempting to suggest that the high level of ATP is responsible for
this conformation. This assumption would receive some support from Pollak (1975) and Sutton and Pollak (1980), who reported a condensed conformation in foetal rat liver mitochondria in correlation with a transient high concentration of ATP.
MITOCHONDRIAL DIFFERENTIATION

The results discussed so far demonstrate that mitochondria undergo a distinct differentiation in the course of development, and much of the evidence suggests that it is possible to distinguish two phases with respect to mitochondrial differentiation.

The first phase, depending on an embryonic type of mitochondria occurring in undifferentiated cells, is characterized by a particular metabolism directed mainly towards synthetic activities. The tricarboxylic acid cycle is coupled to transaminative reactions catalyzed by aspartate transaminase. This atypical cycle, which represents the main oxidative pathway during early development (I), produces aspartate while some of the intermediates of the glutamate-aspartate cycle such as malate seem to be channeled toward the synthesis of pyruvic acid (Petrucci, Amicarelli and Paponetti, 1977). Both aspartate and pyruvate are used as precursors in the pathways leading to the formation of purine and pyrimidine bases (Fig. 2; page 12).

The second phase, initiated by metabolic changes occurring during gastrulation, is characterized by the acquisition of an "adult" type of mitochondria, more involved in energy production (Salomón de Legname, 1969; I and II). As cell differentiation proceeds the "embryonic" type of mitochondria is gradually replaced by an "adult" type.
The changes in morphology and biochemical properties of mitochondria during early development may be caused either by changes in preexisting mitochondria in the undifferentiated cells or by the replacement of this population of mitochondria by another one with different properties.

These alternatives represent actually two of the hypotheses put forward to explain the mechanism of mitochondrial biogenesis in all living cells. As yet we have no indication of which type of mechanism is involved. Some information, however, is worth mentioning which may favour the second alternative.

As we have seen, the mitochondria of differentiated cells are substantially larger than those of undifferentiated cells (V), so the first alternative would require that mitochondrial protein per embryo increases significantly. In fact, it decreases by thirty per cent from the gastrula to the tailbud stage (II). Therefore, the number of mitochondria ought to decrease, and if some of the early mitochondria are bound to degenerate, it seems likely that they all do so, being replaced by a new population.

The curve representing the changes in protein content per embryo (II) may be resolved into two curves (Fig. 4), which make it possible to distinguish two mitochondrial populations: one which begins to decline and the other to rise around gastrulation. The decreasing population represents the "embryonic" type, while the
Fig. 4. Changes in mitochondrial protein content (II) and mtDNA synthesis (Chase and David, 1972) during the development of Xenopus. The dashed lines represent the suggested resolution of the protein curve.
increasing population represents the "adult" type of mitochondria.

Studies of cytochrome oxidase activity in amphibian nucleocytoplasmic hybrids reveal that the activity of this enzyme fails to increase during post-neurul development as it does in control embryos (Liepins and Hennen, 1977). This result suggests that the maternally inherited mitochondria are able to maintain a functional population of mitochondria up to neurulation. This population may constitute the "embryonic" mitochondria.

Mitochondrial DNA is synthesized in correlation with the increasing "adult" population (Chase and Dawid, 1972). The synthetic activity is too low to explain the increase in the "adult" population, but the method yields a minimum estimate of the synthetic rate. Replication of mitochondrial DNA is known to take place during early development of sea urchin (Bresch, 1973), as well as of fish embryos (Mikhailov and Gause, 1974), with a doubling time of seven hours, although the mitochondrial DNA content remains constant.

The two mitochondrial populations have different properties. The swelling in ammonium-fumarate (IV) is a good marker for the "embryonic" population, while monoamine oxidase activity (III) and glutamate/OH carrier activity (IV) may be used for the "adult" type of mitochondria.
Replacement of mitochondria occurs concomitantly with the process of cellular differentiation and may thus be causally related to it. This assumption receives some support from Landström, Løvtrup-Rein and Løvtrup (1976), who reported that an inhibitor of mitochondrial protein synthesis, chloramphenicol, may affect the differentiation of Ruffini cells, and from Pritchard (1981), who reported that disturbances of mitochondrial metabolism may affect the differentiation of chicken embryo neural retina into pigment epithelium.
REFERENCES


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