CARDIOLIPIN AND CYTOCHROME $\underline{aa}_3$ IN INTACT LIVER MITOCHONDRIA OF RATS. EVIDENCE OF SUCCESSIVE FORMATION OF INNER MEMBRANE COMPONENTS

M. Hallman and P. Kankare

Department of Medical Chemistry, University of Helsinki, Helsinki, Finland

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Summary - Cardiolipin and cytochrome $\underline{aa}_3$ were assayed in three mitochondrial populations (M_1, M_2 and M_3) having respective mean densities of 1.172, 1.210 and 1.194 in a linear density gradient. In the rat at birth the development of the mitochondrial population appears to be synchronized. However, the mitochondria obtained from an adult animal form a heterogeneous population comprising different densities. In both systems the formation of cardiolipin seems to precede that of cytochrome $\underline{aa}_3$.

The synthesis of cardiolipin, a phospholipid characteristic of the mitochondrial inner membrane (1), has recently been demonstrated to occur in isolated hepatocyte mitochondria but not in microsomes (2). It is therefore a good marker of the mitochondrial membrane. The finding that cardiolipin is tightly bound to cytochrome oxidase (3) indicates the importance of this phospholipid as a structural component of the respiratory chain.

In the present report cardiolipin and cytochrome $\underline{aa}_3$ have been assayed in liver mitochondria obtained from both perinatal and adult rats. During the last few days of fetal life the sedimentation pattern of the mitochondria was found to change from M_1 to M_2 and after birth their density decreased to that of M_3. Neonatally, the content of cytochrome $\underline{aa}_3$ but not that of cardiolipin increased. In the adult rat M_1, M_2 and M_3, in that order, contained increasing concentrations of cytochrome $\underline{aa}_3$. M_2 and M_3 contained equal amounts of cardiolipin, but a lower concentration was observed in M_1. Five hours after intraperitoneal injection of $^{32}P_i$ into the adult, the specific activity of cardiolipin was highest in M_1 and lowest in M_3, but its radioactivity then changed, being lowest in M_1 and highest in M_3 both 84 and 168 hours after the injection.

MATERIALS AND METHODS

The adult rats used were females weighing from 190 to 220 g. The age of the fetal rats was determined as indicated before (4). The intra-

peritoneal dose of ^{32}P-orthophosphate ($^{32}P_i$) (Radiochemical Centre, Amersham), was 1.78 μCi/g body weight.

Liver mitochondria were obtained essentially as described earlier (4). Heavy mitochondria were sedimented at 7500 g for 10 min. The supernatant was carefully decanted and spun at 11000 g for 10 min in order to sediment the light mitochondria. Both heavy and light mitochondria were washed three times in isolation medium. During washing the heavy mitochondria were sedimented at 6000 g for 10 min. The mitochondria so obtained were laid on a 1.15 - 1.85 M linear sucrose density gradient containing 0.5 mM EDTA, pH 7.4. After centrifugation for 12 hrs at 25000 rpm in a Spinco SW-25.1 rotor, 1-ml fractions were collected through a hole in the bottom. The linearity of the gradient was checked by measuring the refractive index of each fraction. The samples used for the assays of cardiolipin and cytochrome aa_3 are shown in Fig. 2.

The lipids were extracted by the method of Folch et al. (5) and separated by means of two-dimensional thin-layer chromatography (TLC) essentially according to Renkonen et al. (6). Instead of commercial plates, washed silicagel H (Merck, Darmstadt) was used (7). The plates were dried for 0.5 hrs at 35° in a vacuum chamber between the two runs.

The lipid spots were coloured with iodine vapour and the amounts of phospholipids were measured by determining the phosphorus in the spots as described earlier (8). After the spectrophotometric recordings the same samples were used for measurement of radioactivity according to the method of St. C. Palmer (9). The specific activity of inorganic phosphate (P_i) was determined according to Ernster et al. (10). The radioactivity was measured in a Packard model 2002 liquid scintillation spectrometer. The efficiencies of counting were determined by internal standardization.

Cardiolipin was identified by comparing the R_f values with that of commercial cardiolipin. The possibility of contamination of the cardiolipin spot with its precursor was ruled out as follows: A reference compound phosphatidyl glycerol was prepared from E. coli by DEAE-cellulose column chromatography combined with TLC according to the method of Rouser et al. (11). Phosphatidyl glycerol was found to move behind phosphatidyl ethanolamine and thus could not contaminate the cardiolipin. Further, the fraction derived from light mitochondria with a density of less than 1.164 g x cm^{-3} contained neither measurable cardiolipin nor the radioactivity in cardiolipin spot after the in vivo ^{32}P labeling as shown in Table III. This was in spite of the adequate amounts of TLC input (10 to 17 μg of phospholipid phosphorus). Therefore, it is

unlikely that the cardiolipin spot contains minor amounts of impurity with ^{32}P labeling widely different from cardiolipin.

Cytochrome $\underline{aa}_3$ was measured with a dual wavelength spectrophotometer. The millimolar absorbance coefficient used was 14.0 (605-630 nm). Protein was measured by a modification of the biuret method (4).

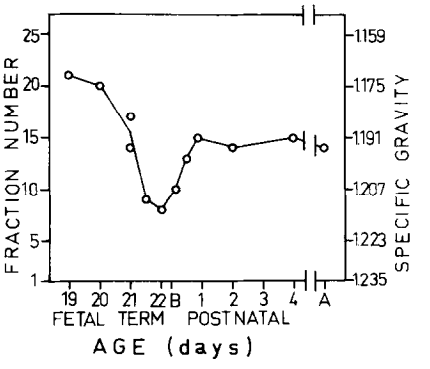

Fig. 1. Continuous density gradient sedimentation of mitochondria obtained from developing rat. 2 ml of mitochondrial suspension (14 to 30 mg protein) was laid on the top of the gradient which contained 28 ml of sucrose solution (see methods). After centrifugation (at 25000 rpm for 12 hrs in Spinco SW 25.1 rotor) 1-ml fractions were collected through a hole in the bottom and the absorbance at 550 nm was measured. The number of the fraction having the maximum turbidity in each age group is shown.

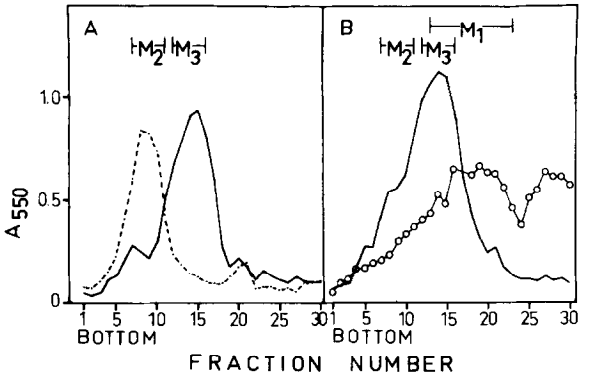

Fig. 2. Profile of mitochondria after centrifugation in a continuous sucrose density gradient. The conditions as shown in Fig. 1. A: heavy mitochondria from 22-day-old fetus (— · —), heavy mitochondria from 20 hours old newborn (——). B: heavy mitochondria from adult (——), light mitochondria from adult (—o—). M_1 (from 13th to 23rd fraction, the specific gravity from 1.199 to 1.164) was derived from the light mitochondria in contrast to M_2 (from 7th to 11th fraction, the specific gravity from 1.218 to 1.203) and M_3 (from 12th to 16th fraction, the specific gravity from 1.202 to 1.186), which were products of the heavy mitochondria.

RESULTS

The density of the mitochondria increased during the last few days of fetal life as shown in Fig. 1. Heavy mitochondria obtained from 19- to 21-day-old fetuses had several bands in the density gradient. The minor bands had a higher density than major band presented in Fig. 1 (data not shown). Not until the fetuses were 22 days old did the organelles sediment as a single band (Fig. 2). Shortly after the birth, there was a gradual shift of the mitochondrial band towards a lower density. Of the mitochondrial protein obtained from newborn animals 20 hours after birth 65 per cent sedimented as M_3. This change was independent of whether the fetuses were born at term by cesarean section or by the normal route. After this time period there was only a minor change in the sedimentation pattern of liver mitochondria from the developing rat.

Cardiolipin and cytochrome $\underline{aa}_3$ were assayed in heavy mitochondria obtained from fetal and newborn animals (Fig. 2) as shown in Table I. There was no difference in the cardiolipin content between the mitochondrial fractions. When the intraperitoneal injection of $^{32}P_i$ was given a few minutes after cesarean section the labeling of cardiolipin in 20-hour-old animals was 968 DPM/μg phosphorus in M_3 and 994 DPM/μg phosphorus in M_2 (the specific activity of P_i was 3510 DPM/μg phosphorus at 20 hr). Thus the labeling of cardiolipin during the first twenty neonatal hours resembled that of the adult (cf. Table III). Moreover, the absolute amount of cardiolipin assayed in the whole liver did not

Table I. The contents of cardiolipin and cytochrome $\underline{aa}_3$ in mitochondria of perinatal rat.

Mitochondrial population	Cardiolipin	Cytochrome $\underline{aa}_3$	Cyt $\underline{aa}_3$ / Cardiolipin
	μg phosphorus/mg prot. (% of total phospholipid)	nmoles/mg prot.	
M_2 from fetus, 22 days	0.71 ± 0.07 (3) (10.8 %)	99 ± 10 (3)	139
M_3 from newborn, 20 hrs	0.76 ± 0.04 (3) (11.0 %)	178 ± 11 (3)	234

M_2 and M_3 were the mitochondrial populations obtained in a density gradient as shown in Fig. 2A. Results are expressed as means ± S.E.M. Numbers in parentheses indicate the number of individual experiments.

significantly change during the first twenty neonatal hours (data not shown). However, at the same time there was a significant increase ($P < 0.05$) in the mitochondrial content of cytochrome $\underline{aa}_3$ concomitant with the decrease in the apparent density of mitochondria. The results did not differ greatly from those presented in Table I, if cytochrome $\underline{aa}_3$ (4) and cardiolipin were assayed in organelles obtained solely by differential centrifugation. The assays in M_3 and M_2 obtained from fetus and newborn, respectively, are not shown, because of the sparsity of material.

Cardiolipin and cytochrome $\underline{aa}_3$ were also measured in liver mitochondria from full-grown animals (Table II). There was a resemblance between the results and those obtained during the perinatal period: M_2 contained significantly less cytochrome $\underline{aa}_3$ than M_3 ($p < 0.05$) but no difference could be detected in the contents of cardiolipin between these two mitochondrial samples. In addition, assays were made on M_1 derived from light mitochondria as shown in Fig. 2. The total amount of cardiolipin in M_1 was only 4 to 7 % of that in M_3 and M_2 combined. Owing to lack of material assays could not be made on M_1 fraction from livers of perinatal rats. Although the use of a density gradient allowed some purification of light mitochondria (see methods), M_1 contained significant glucose-6-phosphatase activity (19 % of the specific activity of glucose-6-phosphatase in microsomes) that suggests the presence of nonmitochondrial

Table II. The contents of cardiolipin and cytochrome $\underline{aa}_3$ in adult mitochondria.

Mitochondrial population	Cardiolipin	Cytochrome $\underline{aa}_3$	Cyt $\underline{aa}_3$ / Cardiolipin
	μg phosphorus/mg prot. (% of total phospholipid)	nmoles/mg prot.	
M_1	0.29 ± 0.05 (3) (4.1 %)	93 ± 9 (3)	321
M_2	0.81 ± 0.06 (6) (13.4 %)	179 ± 11 (5)	221
M_3	0.79 ± 0.04 (6) (13.2 %)	265 ± 9 (5)	335

M_1, M_2 and M_3 were the mitochondrial populations obtained in a density gradient as shown in Fig. 2 B. Results are expressed as means ± S.E.M. Numbers in parentheses indicate the number of individual experiments.

contaminants. The contents of cytochrome $\underline{aa}_3$ and cardiolipin in M_1 were less than in M_2 and M_3, but the ratios between cytochrome $\underline{aa}_3$ and cardiolipin in M_1 and M_3 were rather similar (Table II).

Table III shows the specific activities of cardiolipin after intraperitoneal $^{32}P_i$. Five hours after injection the labeling was highest in M_1 and lowest in M_3. After this, the specific activity considerably increased in M_2 and M_3. Seven days after injection, M_3 contained cardiolipin with the highest specific activity. The labeling of P_i assayed in liver homogenate was considerably higher than that of cardiolipin in any mitochondrial populations shortly after intraperitoneal $^{32}P_i$, but 3.5 days after the injection the specific activity of cardiolipin phosphorus in M_3 had somewhat exceeded that of P_i. Attempts to measure the specific activity of phosphatidyl glycerol in mitochondrial populations were not successful because of the low content of this immediate precursor of cardiolipin. However, in mitochondria from growing heart the rate of $\underline{in\ vivo}$ ^{32}P labeling of cardiolipin coincided with the actual increase in the amount of this phospholipid (M. Hallman and P. Kankare, unpublished results). It is therefore a plausible assumption that the inner membrane matrix is synthesized more rapidly in M_1 than in M_2 or M_3.

Table III. The labeling of cardiolipin isolated from mitochondria after intraperitoneal injection of $^{32}P_i$.

Time after injection	DPM/μg phosphorus			
	M_1	M_2	M_3	P_i
5 hrs	1381	389	320	12061
20 hrs	1228	888	849	3105
3.5 days	1078	1100	1261	1087
7 days	960	1056	1342	923

M_1, M_2 and M_3 were the mitochondrial populations isolated from the adult rat, as shown in Fig. 2 B. The specific activity of inorganic phosphate (P_i) was assayed in liver homogenate.

DISCUSSION

In this study the formation of cardiolipin and cytochrome $\underline{aa}_3$ have been assessed in two physiological states of hepatocyte mitochondria. In the adult the formation of mitochondrial components is in a steady state.

On the other hand, shortly after birth hepatocyte mitochondria change in quality, although there seems to be no significant increase in the size of the mitochondrial population (4). The present results imply that the formation of mitochondria during the perinatal period is a more or less synchronous process, i.e. there is virtually one population of mitochondria. Shortly before the birth these organelles have a characteristic density, which gradually changes after the process of birth, with a concomitant increase in cytochrome aa_3 content, in contrast to cardiolipin. On the other hand, it seems that the adult liver contains a population of mitochondria, heterogeneous with respect to the contents of the inner membrane components (cf. ref. 12).

The characteristic change in the density of mitochondria during perinatal development (Fig. 1) and the pattern of cardiolipin labeling in the three mitochondrial fractions (Table III) suggest that M_1, M_2 and M_3 have precursor product relationships. We suggest that M_1, M_2 and M_3 contain organelles with increasing maturation of the inner membrane and that the phenomenon of the successive formation of cardiolipin and cytochrome aa_3 is not typical of the perinatal period but also occurs in the adult liver. The results obtained are not necessarily inconsistent with the view that mitochondria increase by growth and division (13).

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