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CHANGES IN MITOCHONDRIAL RESPIRATORY CHAIN PROTEINS DURING PERINATAL DEVELOPMENT. EVIDENCE OF THE IMPORTANCE OF ENVIRONMENTAL OXYGEN TENSION

MIKKO HALLMAN

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, Helsinki 17 (Finland)

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SUMMARY

1. Proteins associated with the respiratory chain were measured in heart and liver mitochondria during the perinatal period in the rat.

2. In heart and liver mitochondria cytochromes aa_3, b and $c + c_1$ increased during the first 48 h after birth. The increase of cytochromes $c + c_1$ preceded that of cytochrome aa_3.

3. In fetal liver mitochondria the uncoupler-stimulated ATPase had an absolute requirement of Mg^{2+}. This dependence was lost shortly after birth, parallel with the increase in ATPase activity. In fetal liver mitochondria albumin and ATP increased the respiratory control and the respiratory rate in the presence of succinate. The respiration was in proportion to the terminal succinate dehydrogenase activity only in the presence of albumin and ATP.

4. The incorporation of leucine into the protein of liver mitochondria isolated from 19-day-old fetal rats was about 3 times as high as in adult rats. The rate of incorporation decreased towards the term parallel to the decrease of liver growth. During the first postnatal day the rate increased again with the increase of the respiratory chain cytochromes.

5. When the environment of neonatal rats was made hypoxic, both the rate of amino acid incorporation into liver mitochondria and the content of cytochromes $c + c_1$ in the heart failed to increase. The present results suggest that a hypoxic uterine environment inhibits the formation of mitochondrial inner membrane proteins in the liver and heart.

INTRODUCTION

Biogenesis of mitochondria has been the subject of intensive research for the past ten years. However, our knowledge on the subject is still fragmentary and the mechanisms of the formation of mitochondria and the regulatory processes of mitochondrial biogenesis are still largely obscure[1,2]. One way to study these questions is to follow a system in which the mitochondrial population is in a changing state. Certain lower organisms, especially the facultative aerobic yeasts, are suitable for these studies.

In mammals the use of tissue cultures seems to be the most convenient, although least physiological, model for such studies[3]. Moreover, the steady state of mitochondrial formation may be altered by measures such as hepatectomy[4] or a change in the hormonal status[5,6].

It is known that in rat tissues the activities of some oxidative enzymes usually considered to reflect mitochondrial function are low in the fetus and newborn as compared to the adult. The organs in which circulatory changes occur at birth, namely the heart and liver, show relatively rapid increases in the tissue contents of cytochromes $c + c_1$, aa_3 and b (refs. 7, 8 and M. HALLMAN, P. MÄENPÄÄ AND I. HASSINEN, unpublished results) and in liver cytochrome oxidase[9,10], succinate oxidase[10] and succinate dehydrogenase[9,11] as compared with the more gradual changes in the brain and kidney (refs. 7, 8 and M. HALLMAN, P. MÄENPÄÄ AND I. HASSINEN, unpublished results) during the perinatal period. Recently, reports dealing with some developmental aspects of mitochondria in hepatocytes have appeared[12,13].

The aim of the present investigation was to study the formation of mitochondria during the perinatal period. In contrast to some matrix enzymes, the respiratory chain proteins, namely cytochromes, ATPase and succinate dehydrogenase, were found to increase in content or activity after birth. These changes were parallel with the increase in mitochondrial amino acid incorporation. The uterine environment either lacks a factor that stimulates or contains a factor that inhibits the formation of mitochondrial respiratory chain proteins.

MATERIALS

Albino rats of the Sprague–Dawley strain were used. The age of the fetal rats was calculated on the basis of either the gestation period ($\pm$12 h) or the mean fetal body weight of the litter as follows: 19 days old: 1.0–1.9 g; 20 days old: 2.0–3.1 g; 21 days old: 3.2–4.3 g; 22 days old: 4.4–5.4 g. The adults were males, 80–120 days old. The rats were fasted only when specifically indicated.

ADP, antimycin A, ATP, D(−)-chloramphenicol, creatine phosphate, creatine phosphokinase (EC 2.7.3.2.), *p*-iodonitrotetrazolium violet, phenazine methosulphate, puromycin and Tricine were supplied by the Sigma Chemical, St. Louis. Glucose 6-phosphate was obtained from Boehringer and Soehne, Mannheim, and Nagarse bacterial proteinase from the Enzyme Development Corp., New York. Bovine albumin (Fraction V) was a gift of the Armour Pharmaceutical Company, Eastbourne. L-[1-^{14}C]Leucine and DL-[1-^{14}C]valine were purchased from the Radiochemical Centre, Amersham. A mixture of L-amino acids (Sigma Chemical) was prepared according to KROON[14].

METHODS

Liver mitochondria were isolated in a medium consisting of 270 mM sucrose and 1 mM EDTA (pH 7.4). The liver was homogenized with a Potter–Elvehjem homogenizer (2–4 strokes). The nuclear fraction was spun down (750 $\times$ *g* for 10 min) and the mitochondria were sedimented at 7000 $\times$ *g* for 10 min. The supernatant was carefully decanted. The mitochondrial pellet was washed twice in the isolation medium, unless otherwise indicated. Heart mitochondria were isolated with Nagarse

bacterial proteinase as described earlier[15]. The time course between the isolation of the mitochondria and the assays was usually less than one hour. Microsomes were obtained as described by DALLNER *et al.*[16].

The activities of glutamate (EC 1.4.1.3), α-glycerophosphate (EC 1.1.2.1), β-hydroxybutyrate (EC 1.1.1.30), malate (EC 1.1.1.37) and succinate (EC 1.3.99.1) dehydrogenases were assayed by the method of LEE AND LARDY[17]. The absorbance coefficient used for formazan derivative of *p*-iodonitrotetrazolium violet was 8.0 $mM^{-1} \cdot cm^{-1}$ at 500 nm. ATPase (EC 3.6.1.3) and glucose-6-phosphatase (EC 3.1.3.9) were determined essentially according to the procedure of SIEKEVITZ *et al.*[18] and DALLNER *et al.*[19], respectively. Q_{O_2} was assayed in a Clark-type oxygen electrode, as described earlier[15].

The mitochondria used for the experiments on amino acid incorporation were washed 3 times in the isolation medium. Before use, the isolation and incubation media were filtered through millipore filters (0.25 μm pore size). The extent of bacterial contamination was checked as follows: 0.1 ml aliquots of the incubation medium with mitochondria were poured on blood–agar plates and the colonies were counted after 48 h at 38°. Amino acid incorporations were carried out at 30°, with a shaking rate of 60 cycles/min, in flat bottles having a diameter of 21 mm and each containing 0.5 ml sample unless otherwise indicated. After incubation the samples were treated essentially as described by ROODYN *et al.*[5]. The incubation medium with mitochondria was absorbed into Whatman no. 3 filter paper (6 cm × 4 cm) impregnated with either unlabelled leucine or valine. The papers were washed and the radioactivity determined in a Packard model 2002 liquid scintillation spectrometer at an efficiency of 60 %. In some experiments mitochondrial proteins were solubilized based on the procedure of WORK *et al.*[20] as follows: After labelling of mitochondria (4–5 mg protein/ml) in the presence of 9.0 μM L-[1-^{14}C]leucine (other conditions as in Fig. 5), an excess of unlabelled leucine was added. Subsequently, 0.1 % Triton X-100 and 5 % sodium dodecyl sulphate were added to separate samples, and the membranous protein was sedimented for 30 min at 105000 × *g*. The radioactivity and the protein content were measured in both the pellet and the supernatant.

The cytochrome assays were performed with a dual wavelength spectrophotometer, constructed in this laboratory by I. Hassinen, and a Beckman DK recording spectrometer, as described earlier[15]. The reduction of cytochromes was accomplished in the presence of cyanide or antimycin A. The absorbance coefficients used were 18.0 $mM^{-1} \cdot cm^{-1}$ (550–535 nm)[15] for cytochromes $c + c_1$, 14.0 $mM^{-1} \cdot cm^{-1}$ (605–630 nm)[21] for cytochrome aa_3 and 20.0 $mM^{-1} \cdot cm^{-1}$ (563–575 nm)[22] for cytochrome *b*.

Organs used for assays of ATP concentration[23] were placed in liquid nitrogen 1–4 sec after the first incision. Animals were killed under a stream of 10 % oxygen, when indicated.

Protein was determined after solubilization with 1.5 % deoxycholate by a modified biuret method[6]. When the solubility of mitochondrial proteins was measured according to WORK *et al.*[20], the protein contents were assayed by the method of LOWRY *et al.*[24]. Inorganic phosphate was measured as described by FISKE AND SUBBAROW[25].

Samples prepared for electron microscopy were initially fixed for 2 h in 3 % glutaraldehyde in 0.1 M phosphate, pH 7.4. The subsequent steps were carried out according to SABATINI *et al.*[26] and sections were viewed in a Philips EM 200 electron microscope.

The results are expressed as the mean ± S.E. of 4–11 assays. When the number of experiments was 2 or 3, S.E. was omitted.

RESULTS

The criteria of purity of mitochondrial fractions

Glucose-6-phosphatase is commonly used as a marker of microsomal membranes. On the basis of the protein content, the activity of this enzyme in liver microsomes of developing animals was 9.6 to 16.2 times higher than in the mitochondrial fraction. However, the activity of glucose-6-phosphatase is low in the fetus[19], which makes it difficult to estimate microsomal contamination.

As a further test of the degree of purity of the mitochondrial fractions, samples of liver and heart preparations from fetal (22nd day), 4-day-old and adult animals were studied by electron microscopy. The samples from each age group contained, in addition to well preserved mitochondria, only very small amounts of non-mitochondrial membranes (data not shown).

Fetal mitochondria isolated from both liver and heart contained relatively dense cristae that resembled those of the adult (*cf.* ref. 13).

The dehydrogenase activities in heart and liver mitochondria

The dehydrogenase enzymes assayed in this study were chosen on account of their location in mitochondria and their function in cellular energy metabolism. Succinate and α-glycerophosphate dehydrogenases are known to be components of the inner mitochondrial membrane, while glutamate and malate dehydrogenases are representatives of the matrix space[27].

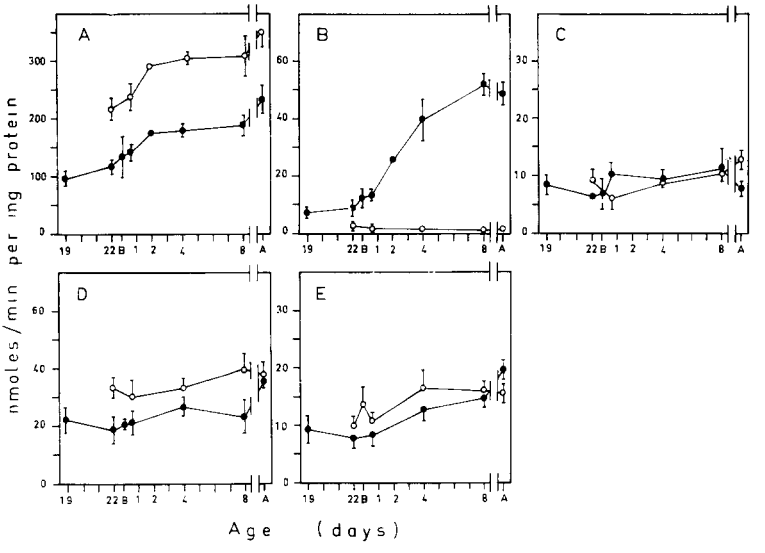

Fig. 1. The activities of dehydrogenases in mitochondria obtained from the liver (●—●) and heart (○—○) of developing rat. A. Succinate dehydrogenase. B. β-Hydroxybutyrate dehydrogenase. C. α-Glycerophosphate dehydrogenase. D. Malate dehydrogenase. E. Glutamate dehydrogenase. The vertical bars represent S.E.

The activities of glutamate and malate dehydrogenases did not change greatly during development (Figs. 1D, 1E). α-Glycerophosphate dehydrogenase activity was low throughout the developmental period (Fig. 1C). On the other hand, succinate dehydrogenase activity increased shortly after birth in both liver and heart mitochondria (Fig. 1A). The addition of ATP (2 mM) and oligomycin (1 μg/ml) stimulated the activity of succinate dehydrogenase in fetal (22-day-old) and postnatal (4-day-old) rats by 12 (15) and 8 (9) %, respectively. (The values are the results of assays in liver and heart mitochondria, the latter indicated in parentheses) (but see ref. 28). The trends in the activity of β-hydroxybutyrate dehydrogenase differed from those of the other activities assayed. In the liver, it was very low during the fetal period, but increased after birth, reaching the adult level around the 8th postnatal day (Fig. 1B). This is consistent with the finding of LEVY AND TOURY[12].

The respiratory chain cytochromes in heart and liver mitochondria

Cytochromes a, a_3, b and c_1 are localized in mitochondria, in contrast to cytochrome c, which is also found in small but significant amounts in other subcellular

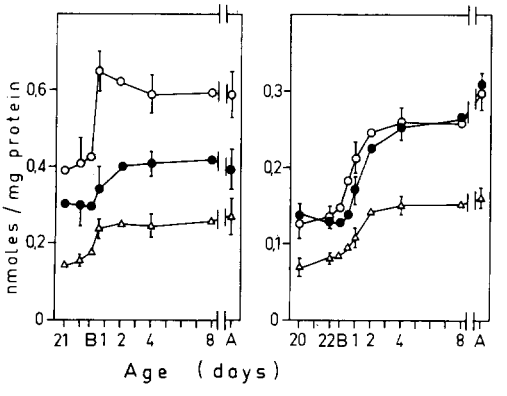

Fig. 2. The mitochondrial contents of cytochromes $c + c_1$ (○—○), aa_3 (●—●) and b (△—△) in developing rats. Left: heart mitochondria. Right: liver mitochondria. The vertical bars represent S.E.

TABLE I

THE RATIO BETWEEN TISSUE AND MITOCHONDRIAL CONTENTS OF CYTOCHROME aa_3

Cytochrome aa_3 concentrations were assayed as nmoles/mg protein. Mitochondrial protein calculated according to SCHOLLMAYER AND KLINGENBERG[21].

	Cytochrome aa_3 (tissue/mitochondria)		*Total protein (mg/g wet wt.)*		*Mitochondrial protein (mg/g wet wt.)*	
	Liver	*Heart*	*Liver*	*Heart*	*Liver*	*Heart*
Fetus, 20 days	0.22	—	180	—	40	—
22 days	0.27	0.19	171	185	46	35
Newborn, 1 day	0.28	0.20	176	190	49	38
4 days	0.28	0.27	198	189	55	51
8 days	0.30	0.30	218	206	65	62
Adult	0.34	0.40	249	219	85	88

fractions[29]. Fig. 2 shows the developmental pattern of cytochromes aa_3, b and $c + c_1$ in heart and liver mitochondria. In both tissues the cytochrome contents of the mitochondria were low in the late fetal period, but increased shortly after birth. The increase in cytochromes $c + c_1$ was more rapid than that of cytochrome aa_3.

As cytochrome aa_3 is a specific mitochondrial component, assays of this cytochrome can be used to evaluate the amount of mitochondria in tissue. Table I shows the ratio of the cytochrome aa_3 contents assayed in tissue and mitochondria, respectively. The results revealed that the amount of mitochondrial protein in tissue gradually increased during postnatal development. This was particularly evident in the heart.

The cytochrome spectra in liver mitochondria obtained from fetal (22-day-old), 1- and 4-day-old and adult rats confirmed the results presented in Fig. 2. However, when sodium dithionite was used as the reducing agent the absorbance minima at 541 and 576 nm found in the spectra of fetal and neonatal (1-day-old) mitochondria suggest the presence of hemoglobin. These deviations in the spectra diminished when the oxygen tension in the reference cuvette was lowered by a burst of respiration in the presence of succinate (3 mM) followed by an addition of malonate (0.3 mM). This is indicative of the presence of hemoglobin. The anomaly in the dithionite spectrum could not be avoided in spite of gentle homogenization of the tissue, careful removal of visible erythrocytes and repeated washing of the mitochondrial fraction.

Succinate oxidation in liver mitochondria

Recently it has been demonstrated that fetal mitochondria isolated from liver have a disproportionally low activity of succinate oxidase as compared to terminal succinate dehydrogenase[12]. In this study the poor respiration in the presence of succinate and the almost complete lack of response with either ADP or uncoupler was demonstrated in the oxygen electrode, as shown in Fig. 3A. This suggests that either the respiratory chain is incomplete or some co-factor necessary for respiration is lacking in the fetal mitochondria.

It is known that the respiration of adult mitochondria become loosely coupled unless the fatty layer that is present in the supernatant after sedimentation of the mitochondrial fraction is removed. Free fatty acids are known to uncouple oxidative

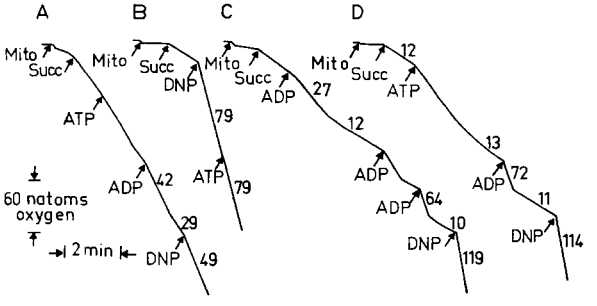

Fig. 3. Oxygen uptake of fetal (22 days) liver mitochondria. The assays were made at 30° (volume 0.9 ml) in 100 mM sucrose, 25 mM Tricine, 7 mM potassium phosphate, 5 mM $MgCl_2$, 30 mM KCl and 0.5 mM EDTA (pH 7.4). B, C and D: 0.4% bovine serum albumin was included. The additions were: 1.2 mg mitochondria (Mito), 9 μmoles succinate (Succ), 2 μmoles ATP, 0.06 μmole ADP and 0.15 μmole 2,4-dinitrophenol (DNP). The numbers above the curves indicate respiratory activity represented as natoms oxygen per min per mg protein.

TABLE II

SUCCINATE OXIDATION IN LIVER MITOCHONDRIA OF FETAL AND ADULT RATS

Q_{O_2} in the fetus was assayed in the presence of 0.4 % albumin, 0.17 mM ATP. Other conditions as in Figs. 3A and 1A. DNP = 2,4-dinitrophenol.

	Q_{O_2} (*natoms oxygen per min per mg protein*)		*ADP/O*	*Succinate dehydrogenase* (*nmoles formazan per min per mg protein*)	$\frac{Q_{O_2}\ (State\ 3)}{Succinate\ dehydrogenase}$	$\frac{Q_{O_2}\ (+DNP)}{Succinate\ dehydrogenase}$
	State 3	*+ DNP*				
Fetus, 22 days	71	115	1.79	92	0.77	1.25
Adult	192	301	1.85	242	0.79	1.24

phosphorylation, an effect that is abolished by addition of albumin, which binds fatty acids[30]. With the fetal mitochondria practically no visible fatty layer was formed during the isolation procedure. However, Fig. 3 shows the improvement of succinate oxidation in the presence of 0.4 % albumin. Moreover, state 3 respiration was stimulated only after addition of ATP, either from outside or through mitochondrial synthesis (Figs. 3C, 3D). In the adult, respiration was not affected by addition of albumin or ATP.

In Table II the oxidation of succinate in the hepatocyte mitochondria of fetal rats is compared with that of adults. Even in a suitable incubation medium the activities measured were lower in fetal than in adult mitochondria. However, the ratio of succinate oxidase to the terminal succinate dehydrogenase in the fetus was almost identical with the corresponding ratio in the adult (but see ref. 12).

ATPases in liver mitochondria

In fetal mitochondria, in the absence of Mg^{2+}, the uncoupler-stimulated ATPase activity was practically absent, but appeared rapidly during the first neonatal day, as shown in Fig. 4. In the presence of Mg^{2+} the 2,4-dinitrophenol-stimulated ATPase in fetal mitochondria was activated, although addition of this cation had little effect on the ATPase activity four days after birth.

If fetal (22-day-old) mitochondria were isolated in 0.4 % albumin, 2,4-dinitrophenol-stimulated ATPase in the absence of Mg^{2+} was 0.9 μmole/h per mg protein. The almost total inhibition of uncoupler-stimulated ATPase by oligomycin (5.0 μg/ml) in both fetal and adult mitochondria indicates that the activity measured is associated with the oxidative phosphorylation. Treatment of mitochondria with 0.1 % deoxycholate activated the Mg^{2+}-dependent ATPase as shown in Fig. 4. Both uncoupler- and deoxycholate-stimulated ATPase increased after birth, although in other respects the developmental patterns of these two activities did not resemble each other (but see ref. 31).

Amino acid incorporation into isolated liver mitochondria in vitro

Before the assays shown here, attempts were made to improve the medium used in the experiments. The rate of amino acid incorporation was dependent on the

concentrations of Mg^{2+}, phosphate, KCl and H^+. Moreover, in the presence of Tricine the rate of amino acid incorporation was higher than in the presence of Tris–HCl (M. HALLMAN, unpublished results). The medium adopted was slightly hypotonic (see Fig. 5). The variation in the protein concentration (from 0.9 to 1.8 mg protein per ml) did not affect the rate of leucine incorporation.

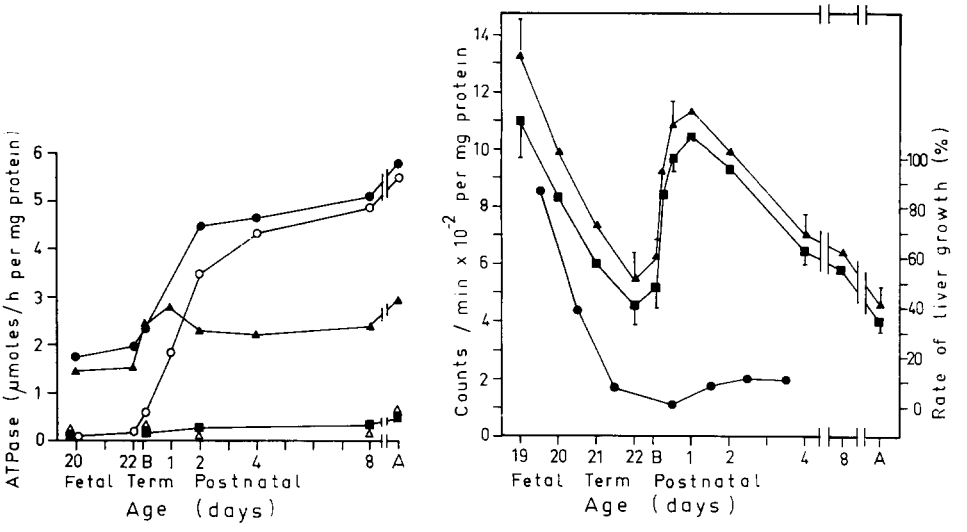

Fig. 4. ATPase in hepatocyte mitochondria during development. Incubation was carried out at 30° (volume 1.0 ml) for 10 min in 200 mM sucrose, 50 mM Tricine, 4 mM ATP (pH 7.0). ■—■, no additions; △—△, 5 mM $MgCl_2$; ▲—▲, 5 mM $MgCl_2$ after preincubation at 0° for 10 min in the presence of 0.1% deoxycholate; ○—○, 0.125 mM 2,4-dinitrophenol; ●—●, 0.125 mM 2,4-dinitrophenol and 5 mM $MgCl_2$.

Fig. 5. Incorporation of leucine into isolated liver mitochondria and the rate of liver growth. Incubation was carried out at 30° for 20 min in 30 mM sucrose, 30 mM Tricine, 10 mM potassium phosphate, 50 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 8 mM glutamate, 8 mM malate, 1 mM EDTA, 50 μg/ml of complete amino acid mixture *minus* leucine, 4.5 μM (■—■) or 9.0 μM (▲—▲) L-[1-^{14}C]leucine (62 mC/mmole) and 0.9–1.8 mg/ml mitochondrial protein (pH 7.4). The rate of liver growth is presented as a percentage increase of liver weight during 24 h (●—●). The vertical bars represent S.E.

In the tests on amino acid incorporation the mitochondria were washed three times in order to minimize the amount of microsomal contamination. Chloramphenicol (50 μg/ml) and cycloheximide (50 μg/ml) inhibited amino acid incorporation by 76–87% and −3–8%, respectively. No significant differences were found in the inhibition produced by these drugs at different stages of development. The numbers of bacteria present in the incubation medium were counted in blood–agar plates and ranged from 10 to 500 colonies per ml of incubation medium added with the mitochondria. This is far less than would be needed to account for a significant increase in the incorporation rate due to bacterial contamination[32]. Moreover, the inhibition caused by erythromycin (50 μg/ml) was only between 6 and 17% during the developmental period studied (*cf.* refs. 33, 15). The complete amino acid mixture (50 μg of amino acids per ml) was constantly present in the incubation medium. The relatively slight stimulation of leucine incorporation when twice the usual amount of L-[1-^{14}C]-leucine was used suggests that in the conditions used the supply of leucine was not

strictly rate-limiting. Moreover, the rate of amino acid incorporation was always linear during 20 min of incubation. In some experiments glutamate and malate were replaced by an extramitochondrial ATP regenerator, namely 50 μg/ml of creatine phosphokinase and 4 mM creatine phosphate, but, despite this addition, the rate of leucine incorporation was almost identical (data not shown).

The rate of leucine incorporation was about 3 times the adult value in the 19-day-old fetus, but decreased towards term. During the first 28 neonatal hours the rate of leucine incorporation increased in amount, but did not reach the activity observed during the 19th fetal day. The decreasing rate of incorporation during the fetal period paralleled the growth rate of the liver (Fig. 5). In contrast, during the neonatal period the rate of leucine incorporation coincided with the increase of proteins associated with the inner mitochondrial membrane (*cf.* Figs. 1A, 2 and 4).

In order to test the solubility of labelled mitochondrial proteins during development, mitochondria were subjected to treatments with 0.1 % Triton X-100 and 5 % sodium dodecylsulphate as described in METHODS. Organelles from fetal (20-day-old), newborn (12 h) and adult animals were used, *i.e.* the decreasing, increasing and steady-state developmental phases were chosen for observations. Triton X-100 solubilized 61–72 % of mitochondrial protein and 8–15 % of radioactivity, while the addition of sodium dodecylsulphate resulted in an almost quantitative solubilization of the *in vitro* labelled mitochondrial proteins. These experiments revealed no significant differences between the three experimental groups.

Effect of hypoxia on the synthesis of mitochondrial proteins during the development of the rat

Figs. 1, 2, 4 and 5 show that shortly after birth an increase occurred in the contents or activities of some mitochondrial proteins associated with the respiratory chain. In order to test the possible importance of external factors, the environment of perinatal rats was modified as shown in Table III and incorporation of amino acids into liver mitochondria and the content of cytochromes $c + c_1$ in the heart were measured.

Starvation of newborn rats somewhat inhibited but did not abolish the neonatal increases in the parameters assayed. The result was the same when the rats were delivered by cesarean section. The oxygen tension in the uterine environment[34] was simulated by keeping the neonatal rats in hypoxia, as shown in Table III. The environment was compatible with life to newborn rats kept without food for at least 15 h. After 4 h in hypoxia the concentrations of ATP (μmoles/g wet wt.) were 2.08 $\pm$ 0.14 (4) in heart and 1.82 $\pm$ 0.17 (7) in liver. In the control animals the values were 2.08 $\pm$ 0.27 (4) and 1.79 $\pm$ 0.14 (7), respectively (number of assays given in brackets). Thus, no significant differences were found between animals kept in normoxic and hypoxic environments.

Despite an adequate supply of ATP, no increase was found in the contents of cytochromes $c + c_1$ or in the rate of mitochondrial amino acid incorporation after 12 h in hypoxia, as compared to fetal rats. The difference in the rate of amino acid incorporation between hypoxic and control rats was evident even if the incubations *in vitro* were carried out under the stream of nitrogen. The increase in the content of cytochrome $c + c_1$ was also prevented, if the rats received puromycin intraperitoneally (60 μg/g body wt.), within a few minutes after cesarean section. On the other hand,

TABLE III

EFFECT OF ENVIRONMENT ON CYTOCHROME *c* CONCENTRATION IN HEART MITOCHONDRIA AND AMINO ACID INCORPORATION INTO PROTEIN OF ISOLATED LIVER MITOCHONDRIA

Starved newborn rats were kept at 37° in a humid atmosphere. The hypoxic environment contained 10–11 % O_2, 1 % CO_2 and 88–89 % N_2. In the measurements of amino acid incorporation the assay conditions were the same as in Fig. 5, except that in some cases 4.5 μM L-[1-^{14}C]leucine and 50 μg/ml complete amino acid mixture *minus* leucine were replaced by DL-[1-^{14}C]valine (32 mC/nmole) and 50 μg/ml complete amino acid mixture *minus* valine.

Age and environment	*Incorporation (counts/min per mg protein)*			*Cytochromes c + c_1 (nmoles/g protein)*
	Leucine	*Valine*	*Leucine/Valine*	
(A) Fetus, 22 days	470 ± 75 (7)	114 ± 8 (5)	4.01 ± 0.30 (5)	420 ± 61 (4)
(B) Fetus, 23 days	430 (2)	—	—	405 (2)
Newborn:				
(C) 1 h	525 ± 83 (7)	123 (3)	4.35 (3)	446 ± 59 (4)
(D) 12 h	977 ± 99 (4)*	187 ± 18 (4)**	5.22 ± 0.39 (4)§	675 ± 45 (4)**
(E) 12 h, starved	795 ± 78 (7)	—	—	673 (2)
(F) 12 h, cesarean section	856 ± 90 (4)	—	—	620 (3)
Starved, cesarean section:				
(G) 1 h, in hypoxia	460 (2)	—	—	—
(H) 12 h, the last 11 h in hypoxia	702 ± 56 (5)***	147 ± 17 (5)	4.77 ± 0.31 (5)***	652 ± 32 (4)**
(I) 12 h, in hypoxia	410 ± 62 (8)	121 ± 15 (7)	3.45 ± 0.31 (7)	338 (3)

* A significant difference ($P<0.05$) as compared with A and C.
** A significant difference ($P<0.05$) as compared with A.
*** A significant difference ($P<0.05$) as compared with I.
§ A significant difference ($P<0.05$) as compared with A and I.

when the rats spent only the first 60 postnatal minutes in a normal atmosphere and the following 11 h in reduced oxygen tension, the parameters assayed were somewhat increased, although no significant increase was detectable during the first 60 min after birth as compared to fetal rats (Table III, A, C, H and I).

Neonatally, the rate of incorporation of valine did not increase as rapidly as that of leucine. This was shown by the significant increase in the ratio of leucine to valine incorporation after birth (Table III, A and D). Moreover, this increase was prevented in a hypoxic environment (Table III, D and I). Several interpretations of this finding are possible. The phenomenon may reflect a neonatal change in (1) the mitochondrial amino acid pool, (2) the translocation of amino acids across the mitochondrial membrane and (3) the pattern of mitochondrially synthesized peptides (*cf.* ref. 35).

DISCUSSION

In this study the fates of protein components associated with the inner membranes of mitochondria were followed during the perinatal period in the rat. The contents/activities of these components (except α-glycerophosphate and β-hydroxybutyrate dehydrogenase) increased fairly synchronously shortly after birth. The repression of the hemopoietic cells in the liver may play a role in the changes observed.

However, these cells contain few mitochondria in relation to the parenchymal cells. In addition, the hemopoietic tissue uniformly decreases from 17 days of gestation to 6 days after birth[36]. Consequently, this is only of minor importance in the neonatal changes observed in the liver and certainly does not explain the parallel changes in the heart.

Role of mitochondrial protein synthesis

The synthesis of cytochromes *a*, a_3, *b*, c_1 and oligomycin-sensitive ATPase is selectively inhibited by chloramphenicol[2,15,37]. In addition, the neonatal acceleration in the rate of mitochondrial amino acid incorporation (Fig. 5) coincides with the increase in these parameters. Mitochondrial protein synthesis is therefore evidently an important mechanism that is responsible for the neonatal increase in the content of respiratory chain proteins. However, it is likely that the formation of some respiratory chain components (*e.g.* cytochrome oxidase and oligomycin-sensitive ATPase) is dependent on both intra- and extramitochondrial protein synthesis[2,37], whereas other components (*e.g.* cytochrome *c*, ref. 29) are solely extramitochondrial in origin. It is possible that the retarded increase in the content of cytochrome aa_3 as compared to cytochrome *c* (M. HALLMAN *et al.*, unpublished results) is due to the complex synthesis and assembly of cytochrome oxidase.

The importance of ambient oxygen tension

The present experiments indicate the importance of the first neonatal hour to the subsequent increase in some components of the mitochondrial inner membrane (Table III, C, H and I). It can be postulated that the change from the uterine environment to the normal atmosphere is of importance in triggering these changes in the inner membranes of the heart and liver mitochondria. From the assays of tissue ATP contents, it is evident that the inhibition by the hypoxic environment of the increase in the parameters assayed is not due to lack of an energy supply. It is suggested that the uterine environment either somehow represses the synthesis of certain mitochondrial proteins or does not contain some factor that is needed in the maturation of fetal mitochondria.

It is possible that the change in environmental oxygen tension after birth[34] initiates the events observed (Table III). This suggestion is strengthened by recent observations of mammalian fibroblasts in tissue culture[3]. Moreover, the influence of the ambient oxygen tension on the cytochrome content of mitochondria in obligate[38] and facultative[39] aerobic yeasts has been established. However, the mechanisms that control the formation of the respiratory chain are as yet rather unknown.

The formation of mitochondrial components during the perinatal period

The results presented here are mainly measurements of functionally active enzyme proteins. Therefore the possibility of stepwise synthesis and assembly of enzyme complexes such as cytochrome oxidase cannot be excluded. In fact, PORTER[40,41] has found large amounts of a copper-containing protein tightly bound to liver mitochondria of newborn mammals, including man. It is possible that this protein is a component of developing cytochrome oxidase.

The changes in cytochromes aa_3, *b* and $c + c_1$ as well as in the activities of succinate, malate and glutamate dehydrogenases after birth resemble those that

occur after injection of thyroxine[5,6]. However, the cytochrome content and the mitochondrial amino acid incorporation start to increase a few hours after birth (Figs. 2 and 5), in contrast to the lag phase of about 48 h after initiation of thyroxine treatment[5]. The different temporal patterns may be due to the developmental dissimilarity. However, the following data afford evidence of a basic difference in the two systems.

GROSS[42] has shown that mitochondrial DNA labelled before administration of thyroid hormone turns over, after the start of treatment, at a different rate from that of newly synthesized DNA. In addition, KATYARE *et al.*[43] have found that after thyroxine treatment the protein content and protein turnover rate increase in the fraction of "light" mitochondria. Recent evidence implies that a fraction derived from "light" mitochondria is the site of active synthesis of the mitochondrial membrane (M. HALLMAN AND P. KANKARE, unpublished results). Cardiolipin labelled *in vivo* with $^{32}P_i$ was used as a marker. These findings suggest that after injection of thyroxine mitochondria differing qualitatively from those present in the euthyroid state are formed from mitochondrial precursors.

The liver mitochondria present in the fetus at term are large as compared with those obtained from mature tissue and contain dense cristae[13,16] and relatively high activities of some matrix enzymes (ref. 12, Fig. 1). Moreover, the assays of cytochrome aa_3 in tissue homogenate and in isolated mitochondria do not give evidence of a significant increase in mitochondria (Table I, Fig. 5). The assays of cardiolipin during the perinatal period (M. HALLMAN AND P. KANKARE, unpublished results) provide additional support for the view that in the liver the increase in the respiratory chain components during the first day after birth is mainly preceded by prenatal formation of a mitochondrial inner membrane matrix. This suggestion does not exclude the possibility that the hepatocyte mitochondria divide (*cf.* refs. 17 and 44) shortly after birth.

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