Biochemical characterization of subcellular particles in fetal and neonatal rats

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ABSTRACT By a variety of methods, such as ultracentrifugation in different media, hypoosmotic activation and hydrostatic compression, subcellular particles were characterized at different perinatal ages and compared to the adult rats. Fetal mitochondria elicited a higher density, an increased osmotic space and a greater resistance to compression. The size of these particles was larger than in the adults. Mitochondria in the 1-day-old animals were freely permeable to sucrose and their external membrane was more resistant to hypoosmotic activation. Lyscsomes were shown to decrease their sucrose permeability and their resistance to hypoosmotic activation with development. Moreover, the size of the lysosomes increased with development.

KEY WORDS: *fetal, neonatal, mitochondria, lysosomes*

Introduction

In the last decade much information has accumulated on enzymic differentiation in mammalian liver during late gestation and early postnatal development. However, most biochemical preparations were whole liver homogenates and practically no attempts were made to further the investigations on subcellular particles.

Most of the reports in this field were morphological studies. Several authors have shown that fetal and early postnatal subcellular particles of the liver were qualitatively different from the adult rats. Some authors have stated that lysosomal particles were absent throughout prenatal life (Chedid and Nair, 1974) or were smaller in the fetal liver when compared with the adult rats (Kotoulas and Phillips, 1971). Fetal liver mitochondria were found to be larger (Jakovcic *et al.*, 1971) and swollen before birth when compared to adult animals (Dallner *et al.*, 1966a,b).

The biochemical modifications that the subcellular membranes undergo during late gestation and early postnatal life were not well documented and the more extensive observations were only related to the mitochondria. Pollak demonstrated the existence of three mitochondrial populations in late stages of development (Pollak and Munn, 1970). Biochemical data on lysosomes and peroxisomes were scarce or non-existent.

The aim of the present work was to contribute to the knowledge on subcellular membrane development during the perinatal period by monitoring parameters such as sedimentation, equilibrium density and membrane fragility, size and permeability (Beaufay and Berthet, 1963). In most of our experiments, a mitochondrial fraction was isolated by differential centrifugation from a postnuclear liver extract in fetal rats of 16, 19 and 21 days and postnatal animals of 1, 7, 14 and 30 days of age. Subsequently, various centrifugation methods were carried out on this fraction in order to obtain the different parameters characterizing the subcellular particles. In all these experiments, subcellular granules were identified with marker enzymes. This approach was based on the work of de Duve *et al.* (1955) stipulating that the percentage of a membrane marker enzyme found in a fraction is equal to the percentage with respect to the total tissue content of the membrane associated with the fraction.

Since in our analytical centrifugation experiments, dramatic modifications in the behavior of the subcellular particles were observed, to further characterize the fetal and neonatal mitochondrial and lysosomal membranes, other techniques like hypoosmotic activation and high-pressure treatment in a compression chamber were used.

Enzymic representativity in the subcellular fractions

The first centrifugation method used in this study was differential centrifugation in 0.25 M sucrose (de Duve *et al.*, 1955). Four fractions were isolated: a nuclear fraction N, a total mitochondrial fraction ML, a microsomal fraction P and a soluble fraction S. Since our experiments were mainly concerned with the mitochondrial

Abbreviations used in this paper: ML, mitochondrial fraction.

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0214-6282/92/$03.00

Printed in Spain

TABLE 1

INTRACELLULAR DISTRIBUTION OF PARTICLE-BOUND MARKER ENZYMES

Age	Enzyme	Nr. of experiments	Enzyme in fraction					
			E+N	E+N	N	ML	P	S	Recovery
			units/g	%					
Foetus (21 days)	cytochrome oxidase	9	9.12 ± 2.73	100	20.06 ± 9.63	65.80 ± 9.03	9.46 ± 5.52	-	94.00 ± 8.52
	ß-galactosidase	4	0.36 ± 0.06	100	15.60 ± 3.00	74.50 ± 1.60	8.10 ± 3.00	6.90 ± 1.40	104.30 ± 3.30
	catalase	5	18.68 ± 3.61	100	8.90 ± 3.70	75.30 ± 6.70	4.70 ± 2.90	11.30 ± 4.20	100.20 ± 7.80
	glucose-6-phosphatase	7	6.30 ± 0.80	100	14.24 ± 3.72	27.99 ± 3.80	59.73 ± 8.45	-	102.10 ± 4.40
	galactosyltransferase	4	603.70 ± 142.00	100	6.70 ± 6.20	19.50 ± 6.90	68.60 ± 13.30	8.40 ± 6.80	103.00 ± 9.40
	5' nucleotidase	4	3.63 ± 0.52	100	38.82 ± 4.70	13.55 ± 2.11	43.13 ± 3.94	7.37 ± 1.40	104.60 ± 10.30
Newborn (1 day old)	cytochrome oxydase	17	18.24 ± 5023	100	15.23 ± 7.02	69.17 ± 6.80	11.41 ± 7.87	-	95.68 ± 6.71
	ß-galactosidase	6	0.63 ± 0.05	100	15.23 ± 7.02	69.17 ± 6.80	9.30 ± 4.30	8.60 ± 2.10	98.80 ± 1.20
	catalase	4	28.43 ± 3.30	100	9.70 ± 1.40	69.20 ± 8.40	2.80 ± 1.20	21.70 ± 5.20	103.40 ± 7.40
	glucose-6-phosphatase	5	24.52 ± 5.01	100	16.83 ± 1.68	24.83 ± 3.79	59.28 ± 6.31	0.22 ± 0.35	101.30 ± 5.90
	galactosyltransferase	5	500.80 ± 67.70	100	5.80 ± 3.90	7.20 ± 1.20	84.80 ± 3.70	2.50 ± 2.00	100.60 ± 7.50
	5' nucleotidase	4	5.36 ± 0.52	100	36.98 ± 2.32	16.17 ± 3.53	47.08 ± 1.76	5.94 ± 1.26	109.70 ± 2.60
Newborn (7 days old)	cytochrome oxydase	4	31.92 ± 6.28	100	11.88 ± 3.39	73.58 ± 12.13	5.51 ± 4.48	-	91.02 ± 11.08
	ß-galactosidase	4	0.80 ± 0.13	100	14.00 ± 3.50	72.30 ± 5.90	4.60 ± 0.80	8.50 ± 1.10	99.40 ± 7.10
	catalase	7	30.61 ± 4.48	100	9.10 ± 1.90	63.20 ± 4.30	10.80 ± 7.40	11.40 ± 3.70	94.50 ± 10.40
	glucose-6-phosphatase	3	33.49 ± 4.56	100	10.57 ± 2.90	29.33 ± 8.13	65.26 ± 1.20	0.34 ± 0.19	105.52 ± 11.20
	galactosyltransferase	4	578.50 ± 61.20	100	5.00 ± 3.40	5.00 ± 0.90	76.60 ± 5.00	-	86.60 ± 6.50
	5' nucleotidase	6	12.17 ± 0.80	100	27.25 ± 3.74	20.43 ± 3.22	53.68 ± 5.68	2.84 ± 2.28	107.30 ± 4.13
Adult	cytochrome oxydase	16	33.62 ± 7.86	100	16.89 ± 6.41	70.63 ± 12.69	10.19 ± 5.41	-	96.81 ± 6.88
	ß-galactosidase	5	0.60 ± 0.07	100	11.40 ± 1.00	56.60 ± 4.50	6.50 ± 1.40	18.20 ± 2.30	93.30 ± 2.70
	catalase	9	44.88 ± 11.50	100	14.70 ± 6.20	53.50 ± 10.10	4.80 ± 3.00	19.90 ± 6.50	92.90 ± 6.00
	glucose-6-phosphatase	4	30.90 ± 4.70	100	10.04 ± 2.76	13.68 ± 5.10	63.62 ± 5.92	10.02 ± 2.44	9707 ± 4.42
	galactosyltransferase	5	936.40 ± 174.90	100	8.50 ± 3.70	5.50 ± 1.80	77.80 ± 13.60	4.00 ± 3.90	95.80 ± 9.30
	5' nucleotidase	5	17.22 ± 2.11	100	27.93 ± 3.94	18.64 ± 2.65	48.54 ± 2.82	5.84 ± 1.73	101.30 ± 7.20

Intracellular distribution of marker enzymes for mitochondria (cytochrome oxydase), lysosomes (ß-galactosidase), peroximes (catalase), endoplasmic reticulum (glucose-6-phosphatase). Golgi (galactosyltransferase) and for plasmic membranes (5' nucleotidase).
Absolute values are given in units/g fresh weight of liver. The results are given as means± standard error of the mean.

lysosomal and peroxisomal particles, it was mandatory to check upon the representativity of the ML fraction in the different ages studied. The distribution profile of mitochondria was illustrated by cytochrome oxidase, that of lysosomes by ß-galactosidase and the profile of peroxisomes by catalase. Glucose-6-phosphatase is associated with the endoplasmic reticulum. Galactosyltransferase is a marker of the Golgi apparatus and 5' nucleotidase stands for the plasma membrane. In this publication only one enzyme per particle is presented; however, other marker enzymes have been previously studied and published (Mertens-Strijthagen *et al.*, 1979).

For valid comparisons to be made between the different perinatal ML fractions and the adult one, a relative identical distribution in the different experimental conditions is necessary. In order to make certain that enzymic distributions at the different ages were not different from the adults in the ML fraction, we performed a variance analysis on the percentage contents of enzyme activity at the different ages (Snedecor, 1964). No differences were elicited in our experiments ($F=0.46$). For ß-galactosidase significant differences were observed between the perinatal and adult mitochondrial fractions($P<0.01$). The results from fetal 16-day and 19-day-old livers have been omitted for two reasons: first, the reduction of hematopoietic tissue during the last prenatal differentiation of liver (Greengard *et al.*, 1972) as well as the elevated nucleocytosolic ratio change the representativity of the distribution of the fractions as compared to the adults. The distributions of the subcellular particles of the neonatal, 14-day and 30-day-old rats were identical to the distributions in the adult animals. For the sake of clarity and in order to favor easy comparison, the results of the adults are mentioned in all the experiments.

Density and sucrose permeability of subcellular particles

In order to obtain further information about the physicochemical characterization of subcellular particles present in the ML fraction, we submitted this fraction to isopycnic centrifugation at different stages of development. Two types of density gradients were performed: first, a sucrose gradient was used and then a glycogen gradient with 0.25 M sucrose in water as solvent. Glycogen is a macromolecule devoid of osmotic properties.

In isopycnic gradient centrifugation, particles were centrifuged in a density gradient until they reached a position where their own density approached that of the medium and their rate of movement became infinitely slow. Thus, a separation was achieved according to the density of the particles. This density depended on the composition of the suspending medium (Beaufay and Berthet, 1963). So, from the distribution of the subcellular particles it was possible to obtain information about the osmotic behavior of these particles. As indicated before, the distributions of mitochondria,

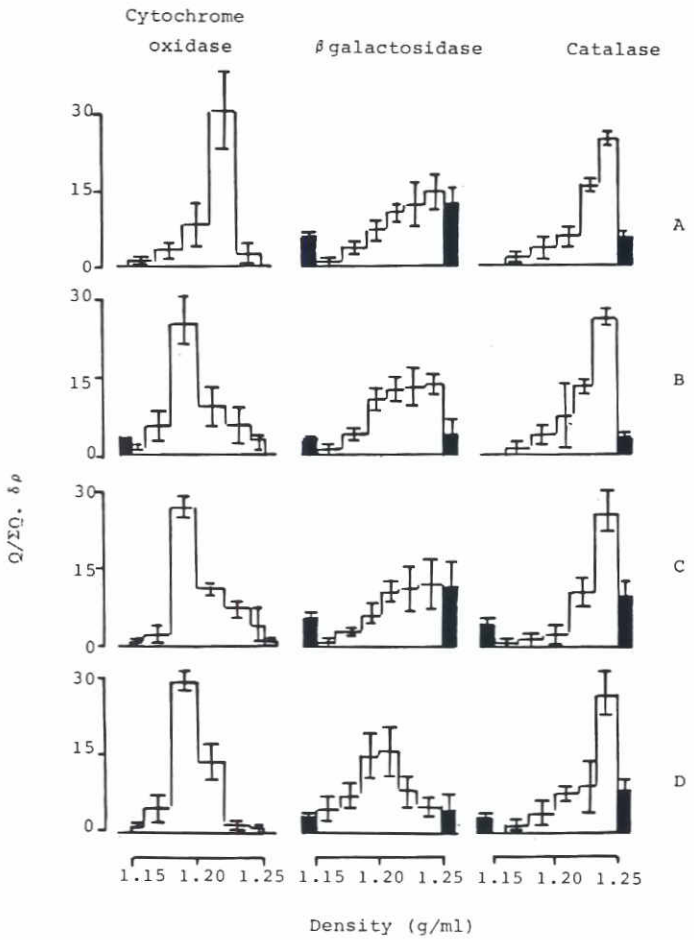

Fig. 1. Density distribution histograms of particle-bound enzymes after isopycnic centrifugation of rat-liver mitochondrial fractions in a sucrose gradient. *Time integral of the square angular velocity, 144 rad²/ns. Centrifugations were performed at 39,000 rev/min in the SW Spinco rotor. The sucrose gradient extended from 1.09 to 1.26 g/ml density. In ordinate: $Q/\Sigma Q.\delta p$ where Q represents the activity found in the fraction. ΣQ the total recovered activity and δp the increment of density from top to bottom of the fraction. To combine the results of different experiments, the base of the density distribution histograms were divided into arbitrary sections; the fractional areas covered by the same section in each histogram were averaged and a new histogram was drawn with these averaged values. Vertical lines through histogram bars correspond to standard errors. Shaded blocks (■) represent to scale material present in fractions of density inferior to 1.15 g/ml and superior to 1.25 g/ml; to facilitate comparison an identical abscissa value has been arbitrarily chosen in all cases.* **(A)** *21-day fetus;* **(B)** *1 day old;* **(C)** *7 days old;* **(D)** *adult. Results are shown as means± SEM.*

lysosomes and peroxisomes were established from the distribution patterns of the tracer enzymes.

Average recovery values for the enzymic activities ranged from 91% to 107%. Fig. 1 shows the distribution profile of enzymes in a sucrose gradient. The peroxisomes show an identical profile at the different ages studied. The distributions of ß-galactosidase are relatively flattened. However, the profile of different lysosomal enzymes (results not shown) did not strictly coincide, illustrating the heterogeneity of lysosomes, even in the late fetal liver.

The calculated median density of the fetal mitochondria was higher (1.218 g/cm³) than the neonatal and adult mitochondria (1.190 g/cm³). Our results indicating a unimodal profile of fetal mitochondria were not in accordance with the experiments of Pollak and Munn. According to Pollak and Munn (1970), mitochondria from adult and embryonic rat liver were separated into three bands. The ratio of the bands differs at the different stages of development. Nevertheless, like Pollak and Munn, we observed also a shift of fetal mitochondria to a higher density.

In order to investigate whether the higher fetal mitochondrial density was related to an increased sucrose permeability in the fetal as compared to adult mitochondria, a centrifugation was performed with the ML fraction in an isoosmotic medium with glycogen as macromolecule. As illustrated in Fig. 2, the pattern of distribution

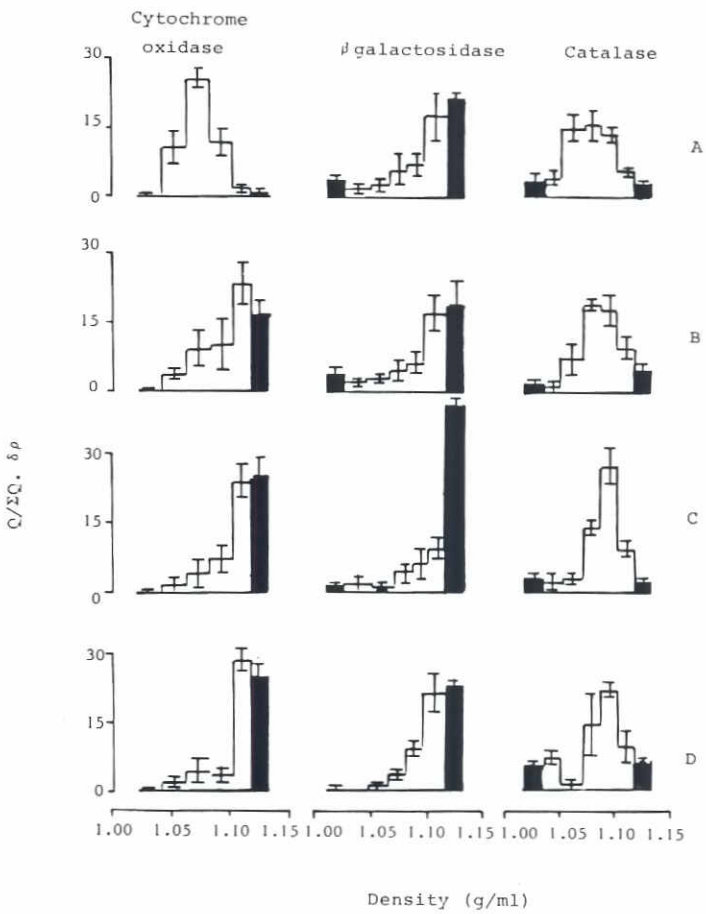

Fig. 2. Density distribution histograms of particle-bound enzymes after isopycnic centrifugation of rat-liver mitochondrial fractions in 5 to 20% (W/W) glycogen gradient with 0.25 M sucrose in water as solvent. *Time integral of the square angular velocity, 144 rad²/ns. Centrifugation was performed at 39,000 rev/min in the SW 65 Spinco rotor. Particles suspended in 0.25 M sucrose were initially layered at the top of the gradient. For explanation of the graph see legend of Fig. 1. In this case, the shaded area represents material recovered in the top and the bottom subfractions, beyond the limits of the gradient.* **(A)** *21-day fetus;* **(B)** *1 day old;* **(C)** *7 days old;* **(D)** *adult.*

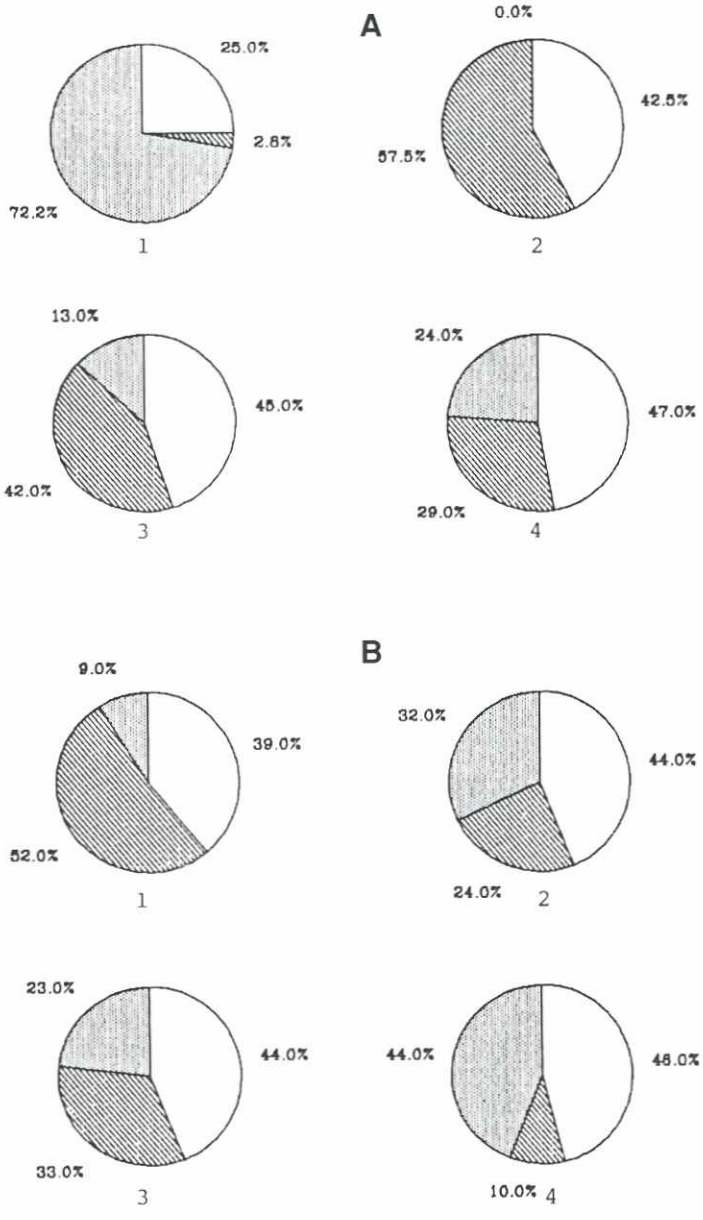

Fig. 3. Mitochondrial and lysosomal subcellular particles. Schematic presentation of the relative volumes in subcellular particles: the osmotic space, the matrix space and the space permeable to sucrose. *The total space of the particles is set at 100%. (□) stands for matrix space, (■) for osmotic space and (▨) for the space permeable to sucrose.* **(A)** *represents the mitochondrial and* **(B)** *the lysosomal particles in the fetal (1), the 1-day-old (2), the 7-day-old (3) and adult (4) rats.*

of fetal cytochrome oxidase was entirely different from the neonatal and adult. The median density of fetal mitochondria was 1.07 g/cm^3 and about 1.10 g/cm^3 for the mitochondria of other ages.

Beaufay and Berthet (1963) have demonstrated that the density of subcellular particles changes with the sucrose concentration of the medium. In order to check this behavior, experiments were carried out in glycogen gradients extending from 0 to 20% (W/W) with as solvent 1.273, 1.000, 0.561 and 0.264 molal sucrose in H_2O. If the membrane is permeable to sucrose, the particle will behave like an osmometer. If the membrane is freely permeable to sucrose the granule will be loaded with a sucrose solution with a density equal to that of the medium. In their theoretical model, Beaufay and Berthet showed that rat liver mitochondria contain two water compartments, one permeable to sucrose and characterized by a parameter ß and one impermeable to sucrose characterized by α. This model contains a third parameter, p_d which represents the density of the matrix of the granule.

Fetal mitochondria were characterized by a high osmotic space (α), a small sucrose space (ß) and a high hydrated matrix (Fig. 3). At birth, dramatic changes of the mitochondrial osmotic behavior occurred. Neonatal mitochondria were devoid of an osmotic space and the density of the hydrated matrix was lower than that of fetal mitochondria (α) became larger in the mitochondria of 7-day-old animals and the density *p*d similar to that found for adult liver mitochondria). These results also suggested that the inner membrane of neonatal mitochondria became freely permeable to sucrose.

Pollak (1975) has shown that the permeability of mitochondria to sucrose decreases with increasing developmental age. Our results are not in agreement with these observations.

When the theoretical model of particles was applied to lysosomes, the quantitative values were less valid than those computed for mitochondria, owing to the heterogeneity of lysosomes. Nevertheless, the behavior of ß-galactosidase in media of increasing concentrations of sucrose suggested that fetal lysosomes were endowed with a very small osmotic space. Peroxisomes do not exhibit osmotic behavior at all. The membrane of these granules seemed freely permeable to sucrose at all stages of development.

Membrane stability and permeability at different perinatal ages

In order to characterize further the membranes of the subcellular particles and to estimate the fragility or stability of those membranes, several techniques, such as hypoosmotic activations and high pressure produced by centrifugation or in a hydraulic compression chamber, were used.

In order to obtain more information about the osmotic behavior of the subcellular particles, a ML fraction was submitted to hypoosmotic conditions for 10 min at 0°C. Then, a concentrated sucrose solution was added to restore isoosmoticity. Free activities of the mitochondrial marker enzymes, malate dehydrogenase (matrix) and sulfite cytochrome c reductase (inter membranous space) were measured in 0.25 M sucrose and the total activities were assayed in the presence of 1 mg. ml^{-1} Triton X-100. A similar procedure was used to estimate the fragility of the lysosomes using ß-galactosidase as marker enzyme. When maintained in hypotonic solutions, the mitochondrial swelling led first to the disruption of the outer membrane, followed at still lower tonicities by a rupture of the inner membrane. This sequence of events could be monitored by measuring the release of malate dehydrogenase and sulfite cytochrome c reductase as illustrated in Fig. 4 A, B. An increase in the free activities of the enzymes was observed at all stages studied when the sucrose concentration decreased. However, the curve representing the release of sulfite cytochrome c reductase in the 1-day-old preparation shifted toward lower sugar concentrations. The results were in accordance with our previous centrifugation experiments which suggested that the 1-day-old rat liver mitochondria

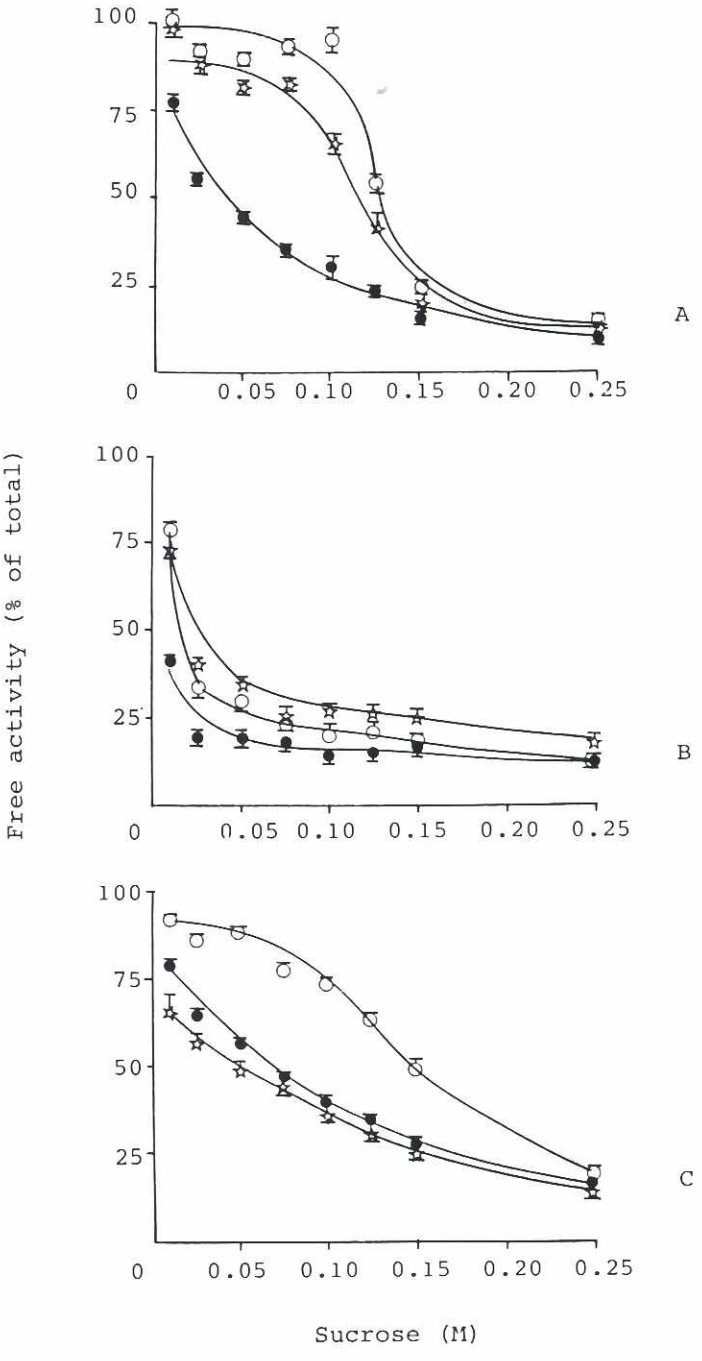

Fig. 4. Hypoosmotic activation experiments on mitochondria and lysosomes. Sulfite cytochrome c reductase **(A)**, *malate dehydrogenase* **(B)** *and ß-galactosidase* **(C)** *in fetal ☆, 1 day old ●, adult animals ○. Samples of a liver mitochondrial fraction prepared in 0.25 M sucrose were diluted with water to give the indicated sucrose concentrations and kept for 10 min at 0° C. After that, a concentrated sucrose solution was added to restore isoosmoticity. Free activity was determined in 0.25 M sucrose, and total activity in 1 mg ml^{-1} Triton X-100. The results are shown as means ± SEM.*

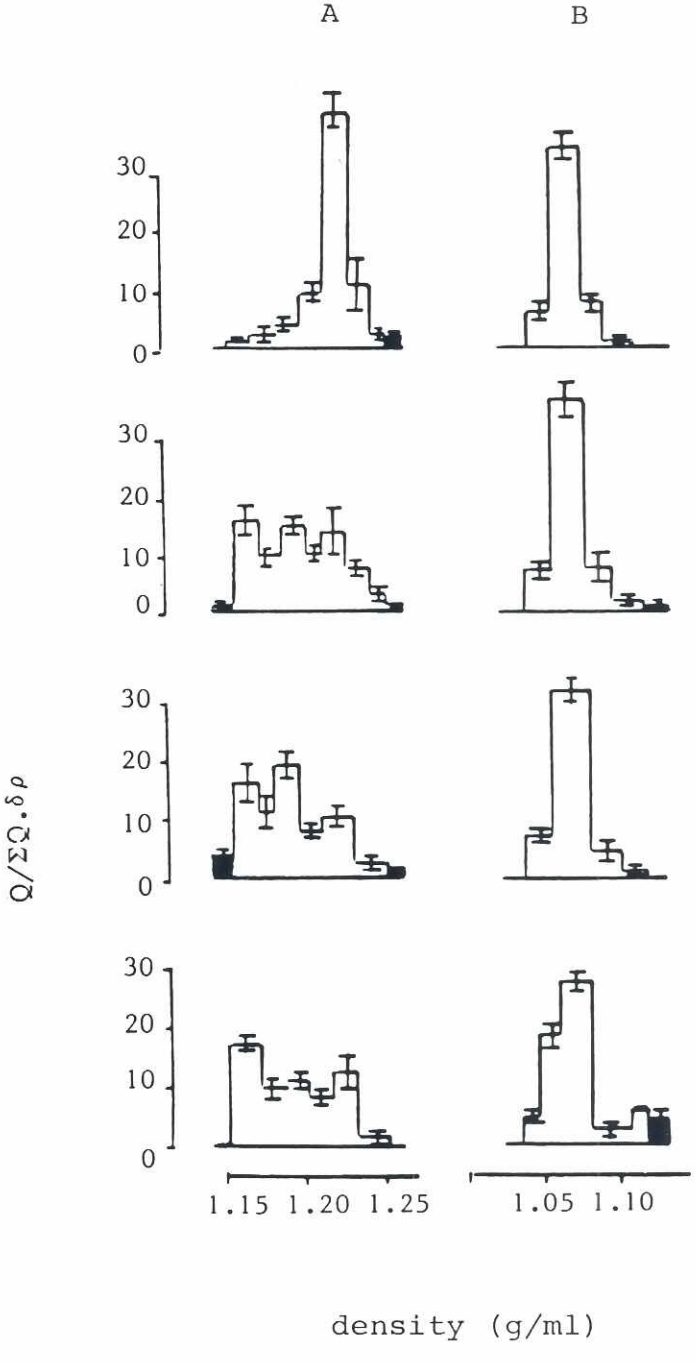

Fig. 5. Density distribution histograms of mitochondria bound enzymes after isopycnic centrifugation of rat-liver mitochondrial fractions at high speed centrifugation. *Density distribution histograms of cytochrome oxidase after isopycnic centrifugation of rat-liver mitochondrial fractions in a sucrose* **(A)** *and a glycogen* **(B)** *gradient. The time integral of the square velocity was 144 rad^2/ns. Centrifugations were performed at 65,000 rpm at 0° C in a Beckman SW 65 Ti rotor. For explanation of the graph see legend of Fig. 1 and Fig. 2.*

were devoid of osmotic space. According to the calculated relative volume of the different spaces of the liver mitochondria (Fig. 3), one would expect that the fetal liver mitochondria which were found to have a larger osmotic space than the 1-day, 7-day and adult rat mitochondria would be more sensitive to hypotonic media. This might be explained by the assumption that the mathematical three compartment model used for calculating the different mitochondrial spaces from the centrifugation results did not take into account

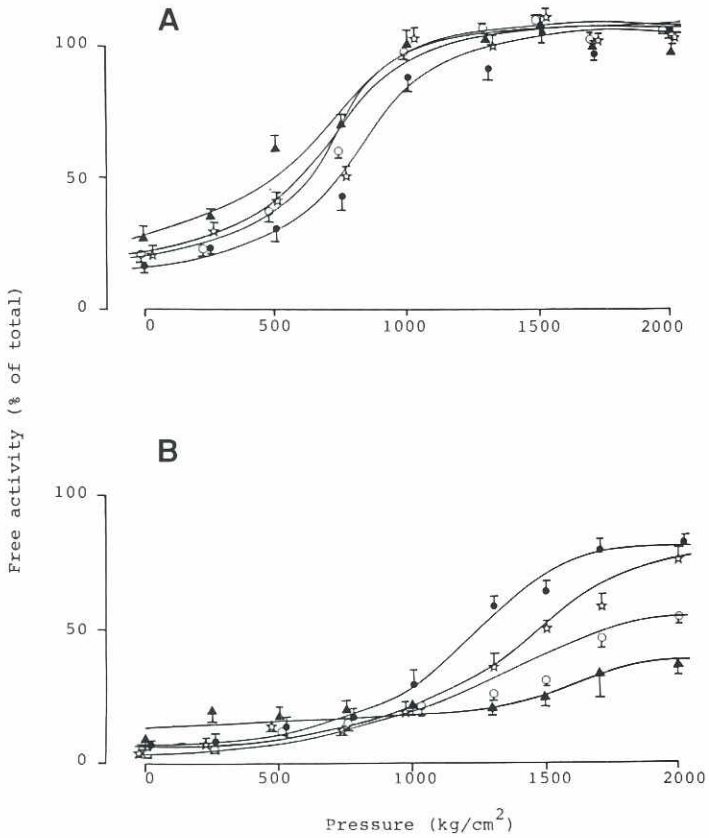

Fig. 6. Free activity of enzymes of liver mitochondria exposed to compression. *Sulfite cytochrome c reductase* **(A)** *and malate dehydrogenase* **(B)** *free activity of liver mitochondria exposed to increasing pressure for 1 h at 0°C; ▲ fetus, ○ 1-day-old, ☆ 7-day-old rat and ● adult. Vertical lines indicate the SEM.*

other factors related to the biophysical nature of the membranes of the particle, such as viscoelasticity (Kapitulnik *et al.*, 1979) which may determine a greater resistance to enzyme leakage in hypotonic media. The fetal lysosomes appeared to be relatively more resistant to hypoosmoticity (Fig. 4C) (Mertens-Strijthagen *et al.*, 1977). These results are in agreement with the centrifugation experiments and also with the observations performed on guinea pigs by Turnbull and Neil (1969).

In order to further characterize the fragility of the fetal and neonatal mitochondrial membrane it seemed appropriate to submit a mitochondrial fraction to high-speed centrifugation in different types of gradients. Wattiaux *et al.* (1971) demonstrated that rat liver mitochondria were damaged when subjected to high pressure. Fig. 5A shows the distribution of a reference enzyme, in a sucrose gradient after an isopycnic centrifugation. Centrifugations were performed in a Beckman SW 65 Ti rotor at 65,000 rpm. The time integral of the square velocity ($W = \int_0^t \omega^2 dt$) was 144 rad^2/ns. The calculated hydrostatic pressure at this centrifugation speed amounted to 1,040 kg/cm^3. Only the distribution patterns of the fetal mitochondrial reference enzymes were unimodal at a density of 1.22 g/cm^3. The previous experiments in a sucrose gradient showed that when centrifuging at 39,000 rpm, in the same rotor with the same integral of the square velocity, the equilibrium density of fetal mitochondria was the same. Under these conditions the hydrostatic pressure was of 310 kg/cm^2. It seemed obvious that the hydrostatic pressure did not produce major alterations in the fetal mitochondrial membranes. However, it was not excluded that the higher buoyant density at which the fetal mitochondria equilibrated in a sucrose gradient may indicate an inner membrane more permeable to sucrose as suggested by Packer (1960). Fig. 5B illustrates the distribution of mitochondria in a glycogen gradient after high speed centrifugation. In this type of gradient, the structures remained in isotonic conditions. When one compares the enzyme distributions of the mitochondria in the neonatal animals at 65,000 rpm with previous experiments (Fig. 2), one can see that, as in the adults, the mitochondria had shifted towards a lower density. The enzyme distributions of the fetal mitochondria were similar at 65,000 and 39,000 rpm. Moreover, Wattiaux-De Coninck *et al.* (1973) previously showed that an increase in the temperature to 15°C for a high-speed centrifugation experiment could neutralize the hydrostatic pressure effect on adult rat liver mitochondria.

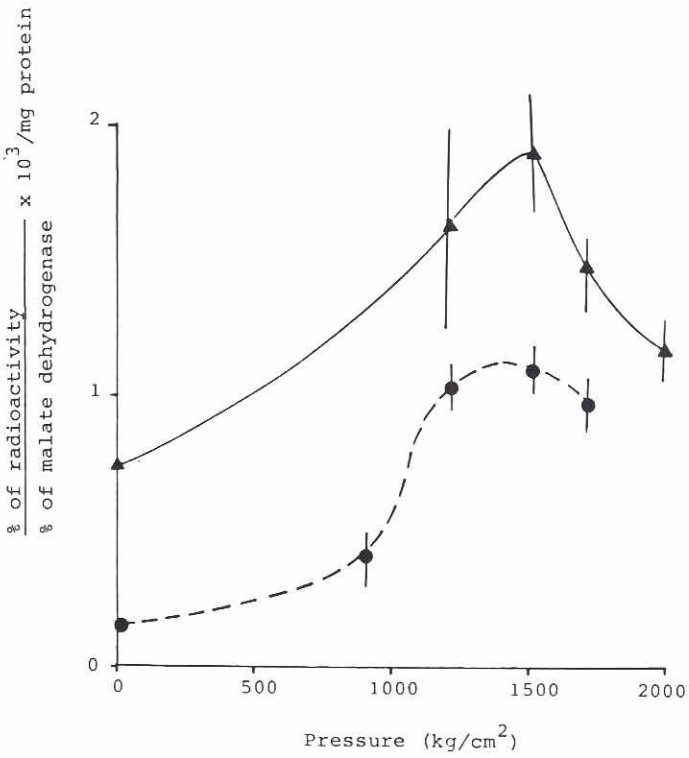

Fig. 7. Penetration of sucrose in rat liver mitochondria submitted to increasing pressure for 1 h. *The amount of sucrose associated with mitochondria is expressed as the ratio of the percentage of radioactivity found in the first six fractions after chromatography on sepharose 4 B to the percentage of malate dehydrogenase measured in these fractions. Moreover, taking into account the differences in the yield of the mitochondria recovered in the mitochondrial fractions, this ratio has been divided by the amount of proteins present in the compressed mitochondrial fraction assuming that the proteins present in this fraction essentially originate from the mitochondria. ▲ stands for fetal rats and ● for adults. Vertical lines correspond to standard deviation.*

TABLE 2

MEDIAN SEDIMENTATION COEFFICIENTS (SVEDBERG)

Enzymes	Median sedimentation coefficients (10^3 sec)		
	Fetus	Newborn	Adult
Cytochrome oxydase	18.700± 0.500* n= 11	12.900±0.640 n= 6	13.550± 0.450 n= 10
ß-galactosidase	5.090± 0.460* n= 11	6.600±0.300* n= 8	10.430± 0.877 n= 10

Mean sedimentation coefficients were calculated according to the equation: $1n\ X/X_0 = S\int_{t=0}^{t} \omega^2 dt$. X_0 is the radial distance to the meniscus (cm), X= mean radial distance of the analyzed fraction (cm), ω= angular velocity (rad sec^{-1}), t= centrifugation time in seconds, S= Svedberg unit (10^{-13} sec). The X values were estimated from boundary curves at a relative concentration midway between 1.0 and the baseline of unsedimentable activity. Results are given as means±standard error of the mean, n stands for the number of experiments. *Significantly different (P<0.01).

Whether the centrifugation speed was 39,000 or 65,000 rpm, the distribution of the enzymes of fetal mitochondria did not change by setting the experimental temperature at 15°C (Mertens-Strijthagen *et al.*, 1985).

In the high speed glycogen gradient centrifugation experiments, the particles were continuously in an isotonic environment; but, as suggested by Collot *et al.* (1975), the down and up migration movements of the mitochondria during the centrifugation experiments were considerable. According to their position in the column, the particles were subjected to variable hydrostatic pressures during a time period difficult to estimate.

Hydraulic compression experiments were mandatory because in this experimental set-up, the particles remain in an isoosmotic medium and the amount of pressure exerted can be accurately timed. In this type of experiment, two marker enzymes chosen for their known locations in different submitochondrial compartments were assayed: sulfite cytochrome c reductase (intermembrane space) and malate dehydrogenase (matrix). The structural integrity of the mitochondrial membranes was estimated by assays of the free and total activities of the enzymes. In Fig. 6A one can see that the outer mitochondrial membrane seemed to be less resistant to compression in the fetal than in the adult animals. As observed by Bronfman and Beaufay (1973), it was necessary to proceed to higher pressure increments in order to obtain an activity of malate dehydrogenase comparable to the activity of sulfite cytochrome c reductase. The evolution of free activity of malate dehydrogenase (Fig. 6B) suggested that the inner membrane of the fetal mitochondria was more resistant to compression when compared to the inner membrane of adult animals. As in the centrifugation experiments, the two types of high pressure experiments indicated a greater resistance to rupture of fetal mitochondria. Nevertheless, high-pressure centrifugation and hydraulic compression chamber experiments have suggested that the permeability to sucrose and the rupture of the internal mitochondrial membrane are probably two unrelated phenomena (Wattiaux-De Coninck *et al.*, 1980). In order to evaluate the changes in permeability of fetal mitochondria in high pressure conditions, we designed a complementary experiment. A ML fraction of fetal mitochondria was compressed in the presence of 2 µCi of [^{14}C] sucrose in 0.25 M sucrose. After compression, the granules were filtered through a sepharose 4 B column. Fractions were collected and analyzed for radioactivity and malate dehydrogenase activity. Considering their size, mitochondrial membranes (ghosts) and undisrupted mitochondria were recovered together in the first fractions. A matricial enzyme-like malate dehydrogenase recovered in these fractions corresponded to the undisrupted mitochondria, except for some readsorption on membranes after release (which was minimal in the case of this enzyme). The radioactivity associated with the first fractions corresponded to the sucrose which migrated with the mitochondria. Indeed, free sucrose was found with the soluble components at the end of the elution. Thus, the ratio of the percentage of radioactivity found in the first fractions to the percentage of malate dehydrogenase measured in these fractions gave us the amount of sucrose associated with mitochondria and an estimate of the penetration of sucrose in these granules. In Fig. 7, one can see that the permeability to sucrose in fetal liver mitochondria at different pressures was shown to be similar to that of the adult rats.

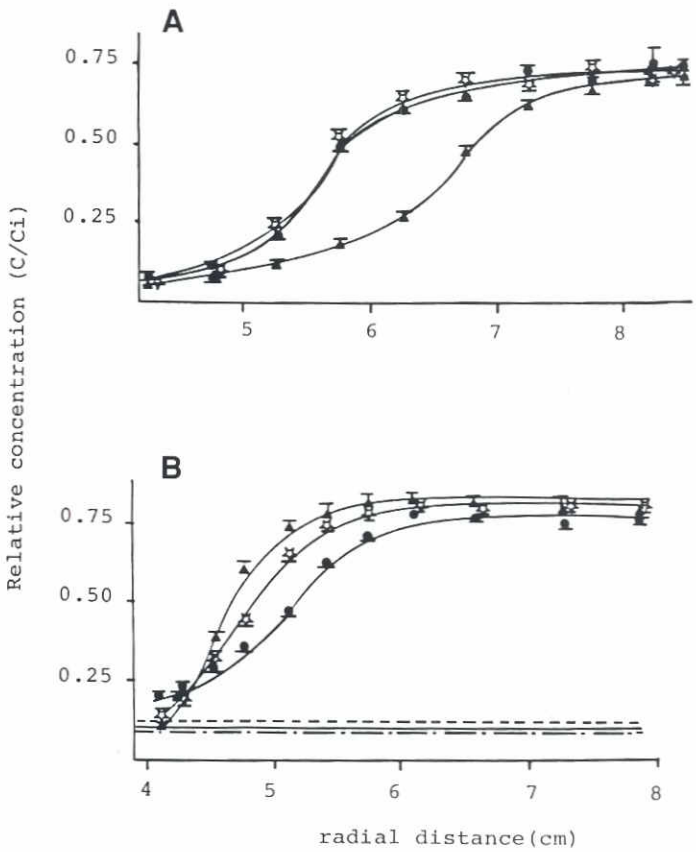

Fig. 8. Mean sedimentation boundary of particle-bound enzymes. *Mean sedimentation boundary of particle-bound cytochrome oxidase* **(A)** *and ß-galactosidase* **(B)** *in fetal (▲), 1 day old (☆) and adult (●). The experiments were carried out on a linear gradient of 0.25-0.5 M sucrose over a radial distance of 3.8-8.9 cm with W= 2.8x10^8 rad^{2}sec^{-1}. The diagram represents the radial distance on the X axis and the relative concentration on the Y axis. Ci and C refer respectively to enzyme concentration before and after centrifugation. The vertical bars indicate the SEM. For ß-galactosidase, the unsedimentable activities assayed on the high speed supernatant are plotted as dashed lines (fetal -----, 1 day old · – · – ·) and solid lines (——— adult).*

TABLE 3

MEDIAN EQUILIBRIUM DENSITIES OF MITOCHONDRIAL AND LYSOSOMAL ENZYMES FROM PERCOLL GRADIENTS ON A POSTNUCLEAR FRACTION

	Median equilibrium densities (g/cm^3)		
Enzymes	Fetus	Newborn	Adult
Cytochrome oxydase	1.060±0.001* n= 4	1.092±0.001** n= 4	1.097±0.001 n= 4
ß-galactosidase	1.078±0.002 n= 4	1.080±0.001 n= 4	1.081±0.002 n= 6

Results are given as means (g/cm^3)± standard error of the mean. The mean densities of particle-bound enzymes were calculated after isopycnic centrifugation of a postnuclear fraction in a Percoll gradient (d= 1.040-1.140 g/cm^3) with 0.25 M sucrose solution as solvent. The centrifugation was performed at 25,000 rpm for 1 h in a Beckman SW 65 Ti rotor at 4°C. Particles suspended in 0.25 M sucrose were layered on top of the gradient. The mean densities were analysed by an analysis of variance. n stands for the number of experiments. *Significantly different (P<0.01); ** significantly different (P<0.005).

Thus, since the fetal mitochondria equilibrate with the same density at 0°C or 15°C in high speed centrifugation, it is likely that the increase in density, as reported in the sucrose gradient experiments compared to adults, may have other causes than a greater sucrose permeation of the inner membrane as suggested before (Mertens-Strijthagen *et al.*, 1979).

Size of the subcellular particles in fetal and neonatal rats

In order to obtain information about the sedimentation coefficient of the subcellular particles, another type of centrifugation experiment was designed. Centrifugations were carried out on a sucrose gradient produced from two samples of a postnuclear extract containing 0.25 M and 0.5 M sucrose (Deter and de Duve 1967). The densities at all levels of the gradient were lower than densities of the subcellular particles and the centrifugation should be incomplete. The purpose of the gradient was to stabilize the preparation, which was centrifuged at low speed (6,000 rpm) for a short time. The mean sedimentation coefficients in the sucrose medium were calculated according to Deter and de Duve (1967).

Table 2 shows that values of the sedimentation coefficient of fetal mitochondria are larger as compared to the other ages studied. For the lysosomes, there is an increase in the sedimentation coefficient with development. In order to evaluate the importance of the density factor in the displacement of the boundary curves (Fig. 8), isopycnic centrifugation experiments were performed on the total nuclear extract with an isoosmotic gradient. Table 3 shows lower densities for fetal mitochondria and fetal lysosomes.

In order to make a rough estimate of the size of mitochondria and lysosomes, we assumed that all these particles were spherical. From the sedimentation coefficients, and taking into account the change in densities, we calculated that the mitochondria were larger and the lysosomes smaller in the fetal animals as compared to the adults. For the mitochondria, this result was in accordance with some morphological observations which describe «*in situ*» enlarged (Jakovcic *et al.*, 1971) or swollen (Dallner *et al.*, 1966a,b) mitochondria. However, our results did not confirm the morphometric *in situ* studies of Rohr *et al.* (1971), who reported an increase in the size of the mitochondria from the 21-day old fetus to the 1-day-old neonatal stage of development. Some authors have also shown that isolated fetal mitochondria were subjected to a spontaneous swelling (Pollak 1975; Mintz *et al.*, 1967). This suggests that the substantial increase in size of our isolated fetal mitochondria may have a double origin: the enlarged mitochondria as evaluated by *in situ* morphological studies and also a possible spontaneous swelling secondary to the isolation of mitochondria. This swelling may be related to the increased osmotic space shown to exist in fetal mitochondria.

Besides the well-known lysosomal heterogeneity evidenced by sedimentation coefficients and density measurements obtained from other lysosomal tracer enzymes (Mertens-Strijthagen *et al.*, 1991), the lysosomes as a population do increase their size with development especially after birth. This confirmed morphological studies showing a very small number of lysosomes closely related to the Golgi apparatus in fetal animals (Dvorak and Konecna, 1969) and an increased volume density (Rohr *et al.*, 1971) as well as larger autophagic vacuoles in the postnatal period (Kotoulas and Phillips 1971; Jezequel *et al.*, 1965). In our study, peroxisomes at all stages of development showed the same physicochemical characteristics as the adults (results not published).

Conclusion

Our results showed a dramatic change in the physicochemical characteristics of mitochondrial and lysosomal particles after birth as revealed by modifications in the mean equilibrium densities, the sedimentation coefficients and the osmotic properties.

Calculation of the volume of the particles allowed us to estimate a decrease in size of the mitochondria after birth. This decrease may be related in part to the frequent mitochondrial divisions observed at this stage of development (Rohr *et al.*, 1971).

Acknowledgments

The authors thank Mrs. C. Daelman-Fautre and J. Lacremans-Pirsoul for excellent technical assistance.

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