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Differentiation of liver peroxisomes in the foetal and newborn rat. Cytochemistry of catalase and D-aminoacid oxidase

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SUMMARY

Organules containing cytochemically detectable amounts of catalase and D-aminoacid oxidase activities are observed between the 14th and 21st day of development in the parenchymal cells of the foetal rat liver and in the liver of newborn rats.

As early as 14 to 15 days, a limited number of small microperoxisomes, scattered in the cytoplasm of very few hepatocytes, can be found. These are roundish shaped, have a granulous matrix and contain very low, hardly detectable levels of the above mentioned enzymes.

In later development both the size and the enzymatic content of the organules gradually increase, approaching adult levels at the end of foetal development. Starting from the 18th to 19th day of intrauterine life nucleoids can be seen in many peroxisomes.

The morphological and biochemical maturation from microperoxisomes to peroxisomes is accompanied by a gradual increase in the number of stainable organules, both per individual cell and per tissue area.

INTRODUCTION

Following the first description of peroxisomes in rat hepatocytes (Rouiller & Bernhard, 1956), the electron microscopic features and the enzymatic complement of these organules have been reported in detail in a number of animal species and different tissues.

While the peroxisomal population has been extensively studied in the adult tissues, limited information is available concerning the embryogenesis of these organules. Data on peroxisome development have been obtained on rat (Tsukada, Mochizuki & Konishi, 1968), mouse (Essner, 1969) and chick (Essner, 1970) liver, on guinea pig adrenocortical cells (Black & Bogart, 1973) and on mouse enterocytes (Pipan & Pšeničnik, 1975; Calvert & Menard, 1978).

In particular, in foetal rat liver the morphological, morphometrical and biochemical study of Tsukada *et al.* (1968) described organules identifiable as

Key words: cytochemistry, catalase, D-aminoacid oxidase, liver peroxisomes, foetal rat, newborn rat.

'microbodies' starting from 15 days of intrauterine life and reported the increases in the total number of organules and in the number of nucleoid-containing peroxisomes during pre- and postnatal development; in this study the morphological and morphometrical results were correlated with the biochemical data on catalase, D-aminoacid oxidase (D-AAO) and urate oxidase.

At present, however, cytochemical studies on peroxisomes in foetal rat hepatocytes are still lacking, although specific and sensitive methods, such as the 3,3'-diaminobenzidine (DAB) technique for catalase (Graham & Karnovsky, 1966; Fahimi, 1969; Novikoff & Goldfisher, 1969) and the cerium method for peroxisomal oxidases (Briggs, Drath, Karnovsky & Karnovsky, 1975; Veenhuis & Wendelaar Bonga, 1977) have been introduced and extensively applied to the study of peroxisomes in various tissues, mostly adult.

It seemed worthwhile, therefore, to study the pre- and perinatal development of rat liver peroxisomes through their responsiveness to catalase and D-AAO cytochemical reactions. The application of these methods could reveal nascent organules more specifically and at earlier times with respect to morphological or biochemical techniques, thus allowing, for the first time, a cytochemical characterization of the peroxisome population in the developing hepatocyte.

The appearance, morphology, distribution and size of catalase and D-AAO containing organules have been studied as from 13th day of intrauterine life to birth. For each developmental stage the number of peroxisomes per thin section of cell was also determined; values were examined by keeping in mind data reported by Greengard, Federman & Knox (1972) and by Herzfeld, Federman & Greengard (1973) on the volumetric variations of foetal hepatocytes and on the differentiation of other organules.

MATERIAL AND METHODS

Albino Wistar rats were commercially obtained and fed a standard laboratory diet and tap water 'ad libitum'. The females were placed with males overnight and examined the following morning for presence of sperm in the vaginal smear. The day of sperm observation was considered day 1 of pregnancy. Foetal age was further confirmed using the tables of Stotsenburg (1915).

Foetuses were quickly removed from the anaesthetized female. Livers were excised, cut into small pieces and fixed by immersion in cold fixative. Samples of the liver taken from the newborn and adult animals were processed in the same way as the foetal ones.

Catalase cytochemistry

Experiments were carried out according to the technique introduced by Graham & Karnovsky (1966), with some modifications (LeHir, Herzog & Fahimi, 1979).

Specimens were fixed in 2 % glutaraldehyde in 0·1 M-cacodylate buffer pH 7·4, containing 5 % sucrose and 0·05 % $CaCl_2$ for 30 min at 4 °C and rinsed in the same buffer for approximately 1 h. Following a preincubation period of 30 min at 37 °C in 0·1 M-Tris-HCl buffer pH 8·5, these were incubated at the same temperature and for different time intervals (30 min–3 h) in the same buffer containing 0·2 % 3,3'-diaminobenzidine tetra-HCl (DAB) (Serva, Feinbiochemica-Heidelberg) and 0·2 % H_2O_2 (freshly added).

In control experiments, preincubation and incubation media were supplemented with 0·1–0·2 M-3-amino-1,2,4-triazole as an inhibitor of the peroxidatic activity of catalase.

At the end of the incubation period, specimens were rinsed twice in distilled water for 10 min, three times in 0·1 M-cacodylate buffer pH 7·4 with 5 % sucrose for about 1 h and finally postfixed in 1 % OsO_4 in 0·1 M-cacodylate buffer pH 7·4 for 1 h at 4°C.

D-aminoacid oxidase cytochemistry

Experiments were carried out according to the cerium technique introduced by Briggs *et al.* (1975) and adapted to the detection of peroxisomal oxidases in animal tissues by Veenhuis & Wendelaar Bonga (1977).

Specimens, cut in blocks thinner than 1 mm to allow for the slow penetration rate of the cerous ions, were fixed by immersion in 1 % glutaraldehyde in 0·1 M-cacodylate buffer pH 7·4 containing 5 % sucrose, 1 mM-$CaCl_2$ and 1 mM-$MgCl_2$ for 30 min at 4°C. They were then rinsed three times in the same buffer for about 1 h.

The blocks were preincubated for 30 min at 37°C in 0·1 M-Tris-maleate buffer pH 7·5 containing 5 mM-cerium chloride and 0·1 M-3-amino-1,2,4-triazole and then incubated at 37°C for different time intervals (2–6 h) in the same medium containing 50 mM-D-alanine or D-proline (Fluka, Buchs, Switzerland).

Controls were performed by incubating tissue in medium lacking substrate or by adding 10 mM-kojic acid (D-AAO competitive inhibitor) both to the preincubation and incubation media.

At the end of incubation, specimens were washed for 10 min in 0·1 M-cacodylate buffer pH 6 to remove any cerium hydroxyde precipitate formed during incubation, then in 0·1 M-cacodylate buffer pH 7·4 for about 1 h and postfixed in 1 % OsO_4 in 0·1 M-cacodylate buffer pH 7·4 for 1 h at 4°C.

After postfixation, all the specimens were dehydrated, embedded in Epon 812 and sectioned with a LKB Ultrotome III. These sections, unstained or stained with lead citrate and uranyl acetate, were observed under a Philips 400 T electron microscope.

Morphometry

To obtain a rough idea of the developmental pattern of peroxisomal population in differentiating rat liver, we counted the number of catalase-positive structures in electron micrographs of cells enlarged ×8000. Counts were made in at least 50 hepatocytes from each developmental stage.

Concerning the size of the microperoxisomes and the peroxisomes during liver development, we measured the diameters of at least 40–50 organules from each developmental stage in electron micrographs enlarged from ×8000 to ×22 000.

Only peroxisomes with a well-defined limiting membrane were chosen, supposedly cut not far from their maximum diameter. The size of oval or irregular peroxisomes is expressed as the mean between the different diameters.

Enzyme and chemical assays

Biochemical assays were performed on total homogenates prepared from foetal, newborn and adult livers in 0·25 M-sucrose. Proteins were determined according to Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin (Fluka, Buchs, Switzerland) as a standard. Catalase activity was measured according to the spectrophotometric method of Lück (1963). D-AAO activity was determined according to Hayashi, Suga & Niinobe, 1971. One unit of D-AAO activity is defined as the amount of enzyme forming 1 μmole of pyruvate h^{-1}, under the conditions employed.

RESULTS

Catalase cytochemistry

In all the specimens observed the reaction product was limited to organules morphologically identifiable as peroxisomes or microperoxisomes, according to

the criteria of Hicks & Fahimi (1977). Diffusion of the reaction product outside of the organules was never observed. In the control specimens incubated in the presence of the catalase inhibitor, no reaction product was formed.

Figs 1 and 2 show catalase-positive organules in the rat liver during foetal and neonatal development.

On the 13th day of intrauterine life, no microperoxisome can be identified in any hepatocyte, not even after 3 h of incubation.

On the 14th day (Fig. 1A), following 2 h of incubation, a few organules with a moderately DAB-positive content (never more than five for each cell section) may be observed in only a few hepatocytes. They have the appearance of small roundish vesicles delimited by a single membrane that often looks corrugated and detached from the matrix. Their distribution in the cytoplasm does not present preferential localizations, as regards the differentiating regions of the hepatocyte; however, they are often in close proximity to the endoplasmic reticulum, at this stage essentially of the rough type.

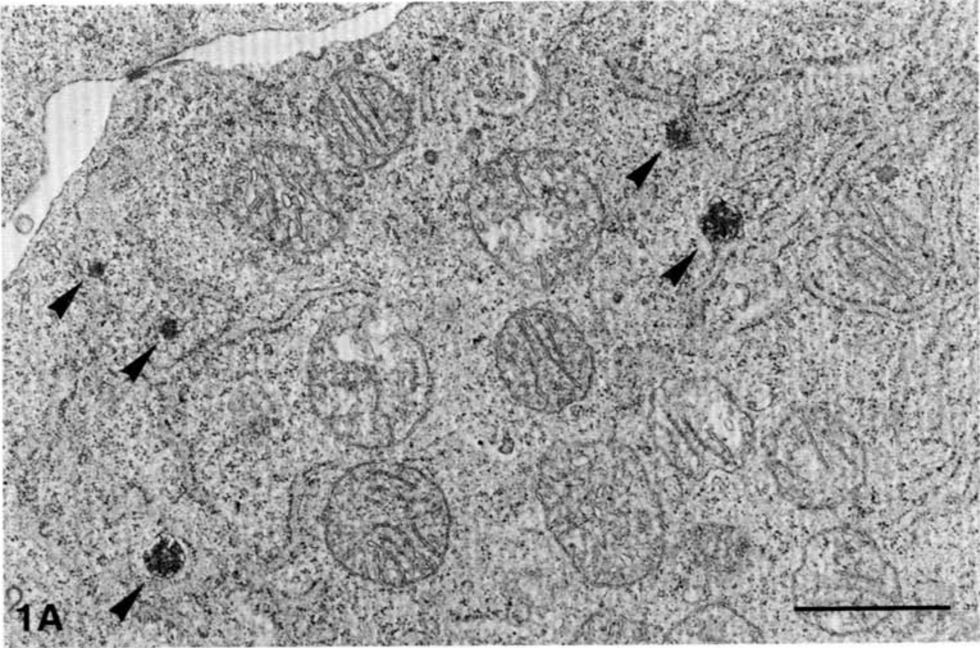
In later development (16th to 17th day) more numerous DAB-positive structures may be easily identified in many, but not in all, the hepatocytes.

Fig. 1B,C shows some microperoxisomes in hepatocytes on the 16th day of development. The organules generally have a roundish shape and a larger size than at the preceding stages. The limiting membrane hardly ever has corrugations, but often does not adhere to the matrix. The organules are closely connected to ribosome-free portions of the rough endoplasmic reticulum.

On the 19th day, all hepatocytes contain quite a number of organules with a strongly DAB-positive granulous matrix, even after 1 h of incubation. They are often seen at the periphery of the glycogen deposits, which appear in the hepatocyte at this stage of development (Fig. 1D). At the same age, nucleoids may be seen in some organules that can therefore be considered real peroxisomes (Fig. 1E).

On the 21st day and in the newborn all the hepatocytes have many DAB-positive organules that are closely connected to cisternae of the rough reticulum, most often with ribosome-free portions (Fig. 2A,C), and with glycogen deposits (Fig. 2B). The nucleoids are particularly visible when the peroxisomal matrix is slightly positive and not very packed (Fig. 2A).

Fig. 1. Foetal rat liver. Catalase cytochemistry. (A) 14-day-old foetus; 2 h incubation. Few microperoxisomes are present in the hepatocyte (arrowheads). The organules are small, roundish, and show a DAB-positive, loosely packed content and a slightly corrugated limiting membrane. (B) 16-day-old foetus; 2 h incubation. Some microperoxisomes are present in the hepatocyte. The organules are roundish and show a strongly positive, amorphous matrix. (C) 16-day-old foetus; 2 h incubation. A DAB-positive roundish microperoxisome. The close proximity to elements of endoplasmic reticulum should be noted. (D) 19-day-old foetus; 1 h incubation. Some peroxisomes are visible in proximity of an unstained glycogen deposit (*g*). The organules are round and show a strongly DAB-positive matrix. (E) 19-day-old foetus; 1 h incubation. A DAB-positive peroxisome, showing a nucleoid with the typical microtubular arrangement. Scale bar (A,B,D) equals 1 μm; (C,E) equals 0·2 μm.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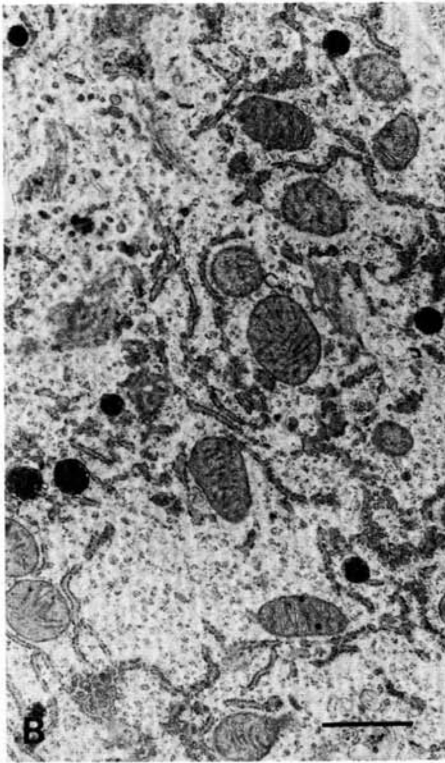
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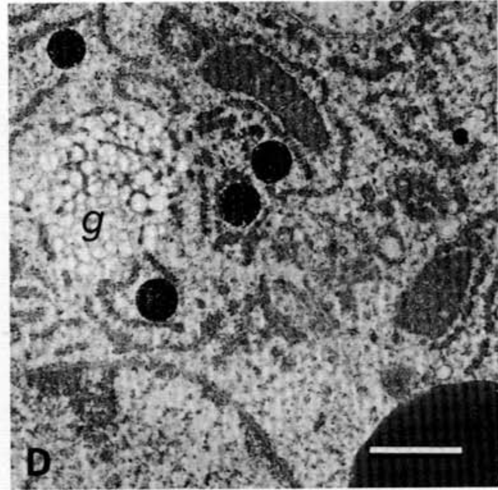
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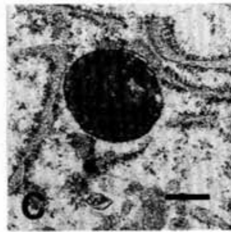
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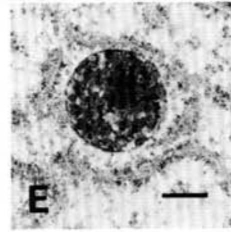
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D-aminoacid oxidase cytochemistry

In preliminary experiments employing D-alanine and D-proline as substrates, we found that only the latter gave a specific reaction product; this product has a granulous appearance and is restricted to microperoxisomes and peroxisomes. No trace of diffusion outside of the organules is ever found. In control specimens no specific staining is observed when the substrate is omitted from the incubation medium; a relevant inhibition of the reaction is noted in specimens incubated in the presence of the D-AAO-specific inhibitor.

Besides the above described reaction product, deposits of electron-dense amorphous material are observed on some mitochondrial and endoplasmic reticulum membranes, in the specimens incubated either with D-alanine or D-proline and in the controls. The presence of this staining even in specimens incubated in D-AAO

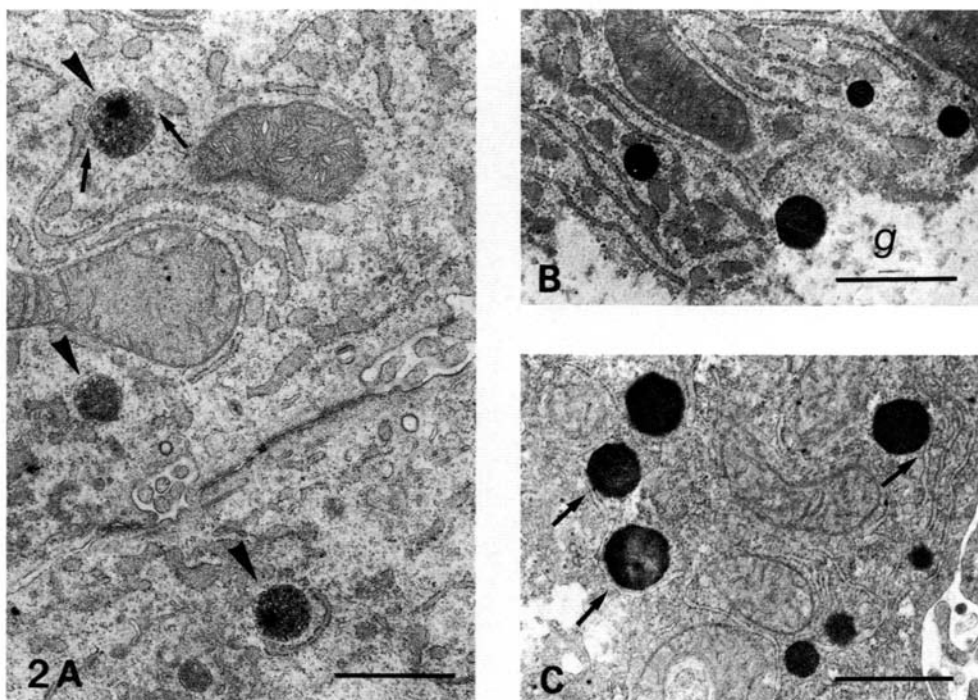


Fig. 2. Foetal and newborn rat liver. Catalase cytochemistry. (A) 21-day-old foetus; 1 h incubation. Some peroxisomes with a well detectable but not very heavy DAB reaction product (arrowheads). Two show a nucleoid definitely darker than the matrix. The close proximity of one peroxisome to some ribosome-free areas of rough endoplasmic reticulum should be noted (arrows). (B) 21-day-old foetus; 1 h incubation. Some peroxisomes with heavily osmiophylic, amorphous DAB reaction product. One of them is on the boundary of an area occupied by unstained glycogen (*g*). (C) Newborn rat; 30 min incubation. Some peroxisomes with heavy osmiophylic amorphous DAB reaction product. The close proximity of some organules to endoplasmic reticulum elements should be noted (arrows). Scale bar equals 1 μm.

plus kojic acid medium means that this is not caused by D-AAO activity (Arnold, Liscum & Holtzman, 1979).

Fig. 3 shows the cytochemical localization of D-AAO in some selected stages of foetal rat liver development and, as a comparison, in the adult hepatocyte.

Up to the 14th day of intrauterine life, no trace of specific staining can be found in any hepatocyte. On the 15th day a limited number of positive organules may be observed in very few hepatocytes; even after 6 h of incubation the staining presents different degrees of intensity in the various organules within the same cell (Fig. 3A).

On the 16–17th day of development positive microperoxisomes can be found in a greater percentage of cells. The organules show marked differences in staining intensity and close connections to elements of rough endoplasmic reticulum, that at this developmental stage is frequently arranged to form ‘fingerprints’ (Fig. 3B,C).

Later in foetal development, beginning on the 19th day, 2 h of incubation are enough to put into evidence a gradually increasing number of peroxisomes, that reaches a maximum of 10 per thin section of cell at the end of pregnancy; at all these stages, peroxisomes with markedly different intensity of staining coexist within the same cell.

At 19 days of development a certain number of peroxisomes irregularly shaped can be found (Fig. 3D), together with organules showing bleb-like protrusions, generally filled with a material identical to the rest of the matrix (Fig. 3D, inset).

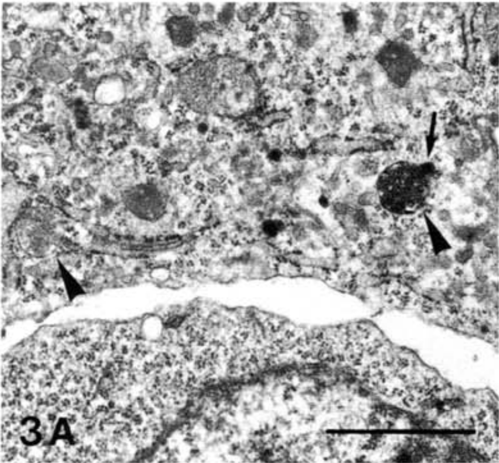
In the adult liver, all hepatocytes contain many peroxisomes with a great variability in staining intensity (Fig. 3E); negative organules are still observed, though in a lower number than in the foetus. Nucleoids are more easily visible in the strongly positive peroxisomes, where they appear to be much less electron dense than the matrix; when the staining intensity is not very strong, the reaction product appears to be condensed close to the limiting membrane.

Morphometry and biochemistry

Table 1 reports morphometrical and biochemical data on peroxisomes during rat liver development. The mean diameter significatively increases throughout intrauterine life to birth, at which time it almost attains the adult size. The rate of peroxisomal growth, measured by diameter, is greater during early development (14–16 days), decreases during late embryogenesis and at birth reaches a plateau.

The mean number of catalase-positive organules per thin section of cell increases during foetal development and continues increasing after birth as well to reach adult values. As negative peroxisomes were never seen in the specimens incubated for catalase, it may be assumed that the number of catalase-positive peroxisomes coincides with that of the total peroxisomes.

The relevant scattering of data reported in Table 1 regarding the number of peroxisomes can be explained on the basis of the extremely high variability of morphometric data. This can be better understood from data reported in Fig. 4, where the frequency distributions of peroxisomes in hepatocytes of selected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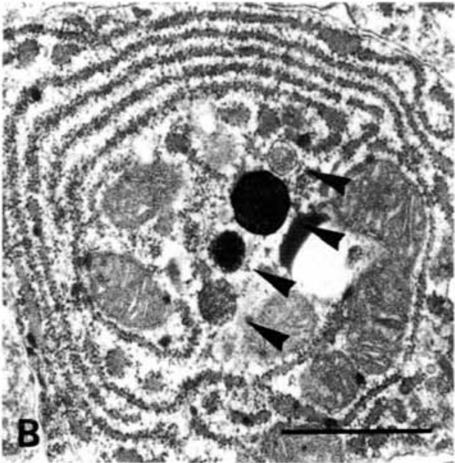
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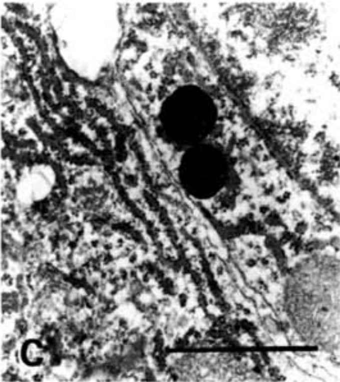
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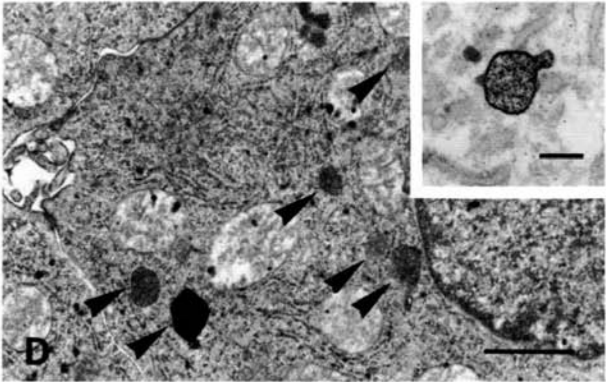
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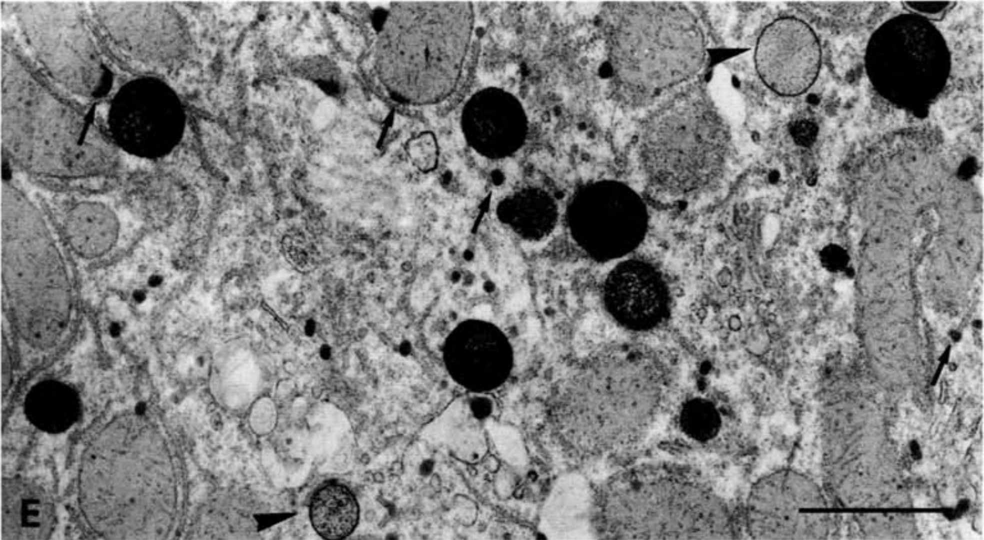
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developmental stages are shown. Analysis of histograms obtained on the 16th, 19th and 21st days of foetal development clearly indicates that the distribution becomes progressively symmetrical around the mean value.

Table 1 also reports the protein content and the catalase and D-AAO-specific activities of rat liver homogenates measured at different developmental stages and in the adult.

Liver protein content increases up to the 19th day when it approaches the adult level. The somewhat lower value obtained immediately before birth is probably due to the conspicuous intracellular deposition of glycogen and lipids during this period (Greengard *et al.* 1972).

Catalase activity is at first measurable at 14 days of foetal development and increases during pre- and postnatal life, as already reported (Tsukada *et al.* 1968; Mavelli *et al.* 1981). Between the 14th and 19th day of foetal life the enzyme activity shows a more than four-fold increase; later in development and after birth the enzyme activity is still increasing, but to a lower extent than before.

D-AAO activity is measurable only at very late stages (21 days) of intrauterine life, according to Tsukada *et al.* (1968), and increases after birth to reach maximum levels in the adult stage.

DISCUSSION

In the present report we have studied the appearance and differentiation of catalase and D-AAO-positive organules during rat liver development.

The presence of organules morphologically interpreted as 'microbodies' has been already reported by Tsukada *et al.* (1968) in rat liver starting on the 15th day of foetal development.

Our morphological results are strictly comparable to those reported by Tsukada *et al.* (1968) as far as the shape, size and cytoplasmic distribution of peroxisomes

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Fig. 3. Foetal and adult rat liver. D-aminoacid oxidase cytochemistry. (A) 15-day-old foetus; 6 h incubation. A weakly positive and a negative microperoxisome (arrowheads) are visible. The organules are round and have a granulous matrix; an aspecific deposit of electron-dense material, contiguous with the membrane of a microperoxisome, is evident (arrow). (B) 16-day-old foetus; 6 h incubation. Four microperoxisomes with marked differences in staining intensity (between ++ and −) (arrowheads) are visible in a cytoplasmic area delimited by fingerprints of rough endoplasmic reticulum. (C) 17-day-old foetus; 6 h incubation. Two heavily stained round microperoxisomes; their close proximity to endoplasmic reticulum elements should be observed. (D) 19-day-old foetus; 2 h incubation. Some irregularly shaped peroxisomes showing different degrees of positivity (+, +− and −) (arrowheads). Inset: a weakly positive peroxisome with a bleb-like protrusion; the reaction product is particularly evident on the limiting membrane. Unstained. (E) Adult liver; 2 h incubation. Many round peroxisomes with marked differences in staining intensity. Nucleoids, notably less stained than the matrix, can be easily seen within more positive organules. The reaction product appears to be more concentrated on the limiting membrane in the weakly stained peroxisomes (arrowheads); small deposits of aspecific product are visible on the mitochondrial and endoplasmic reticulum membranes (arrows). Unstained. Scale bar equals 1 μm (equals 0·2 μm in inset).

Table 1. *Morphometrical and biochemical data on peroxisomes during pre- and postnatal rat liver development*

Age of development days	Mean diameter* μm	Mean number/thin section of cell†	Protein§ mg g^{-1}	Catalase activity§ i.u. mg^{-1} protein	D-AAO activity§ i.u. mg^{-1} protein
14	0·23 ± 0·03	n.a.	71·62 ± 9·54	0·62 ± 0·07	n.a.
15	0·28 ± 0·02	n.a.	113·22 ± 7·76	1·13 ± 0·07	n.a.
16	0·34 ± 0·03	2·47 ± 2·23 (0–9)	122·81 ± 26·23	1·20 ± 0·16	n.a.
17	0·36 ± 0·03	3·35 ± 2·51 (0–11)	116·42 ± 14·30	2·52 ± 0·25	n.d.
19	0·41 ± 0·04	4·52 ± 2·47 (0–12)	187·13 ± 23·41	2·70 ± 0·32	n.d.
20	0·44 ± 0·03	7·03 ± 3·64 (1–14)	n.a.	n.a.	n.a.
21	0·47 ± 0·03	7·81 ± 3·69 (1–17)	169·73 ± 10·23	4·69 ± 0·36	0·012 ± 0·002
newborn	0·50 ± 0·05	17·30 ± 7·15 (8–33)	186·81 ± 16·15	7·65 ± 0·69	0·029 ± 0·003
adult	0·52 ± 0·04	30·05 ± 12·88 (11–59)	199·00 ± 18·13	19·12 ± 1·46	0·287 ± 0·031

* Measurements were taken in at least 40–50 organules from each developmental stage.

† Counts were made in at least 50 thin sections of different hepatocytes from each developmental stage, in specimens incubated for catalase cytochemistry.

§ Protein and enzyme activity are the mean ± S.D. of three different determinations.

n.a.: not assayed.

n.d.: not detectable.

Numbers in parentheses: minimum and maximum values.

Data concerning mean diameter, protein and catalase activity from 14th day to newborn were analysed by F test, being $P < 0{\cdot}01$ in all cases.

are concerned; data on the presence and morphology of nucleoids are also in good agreement. However, at variance with their results, we never found continuities between microperoxisomes and/or peroxisomes and endoplasmic reticulum elements; however, a close spatial proximity with ribosome-free areas of rough cisternae was frequently seen.

Concerning our cytochemical results, the DAB method for catalase allows us to demonstrate the presence of microperoxisomes as early as at 14 days of intrauterine life. At this age the few microperoxisomes do not contain cytochemically detectable amounts of D-AAO activity, which can be revealed only a day later in very few organules.

The staining intensity of both catalase and D-AAO gradually increases throughout the development. Catalase positivity appears uniform in the different organules at all stages examined and attains a staining intensity comparable to the adult one already at 19 days of foetal development.

With regard to D-AAO cytochemistry, on the other hand, organules with marked differences in staining intensity and negative ones as well are always found, even in the adult liver.

D-AAO activity, therefore, seems to be present at a lower concentration and less ubiquitously than catalase in peroxisomes of the foetal, perinatal and adult rat liver. This conclusion is in agreement with our biochemical data and those reported by Tsukada *et al.* (1968).

As far as increases in mean number and size of peroxisomes during prenatal development are concerned, some conclusions can be drawn, keeping in mind the reported variations of hepatocyte volume (Greengard *et al.* 1972) and the intracellular dimensions of other organules (Herzfeld *et al.* 1973).

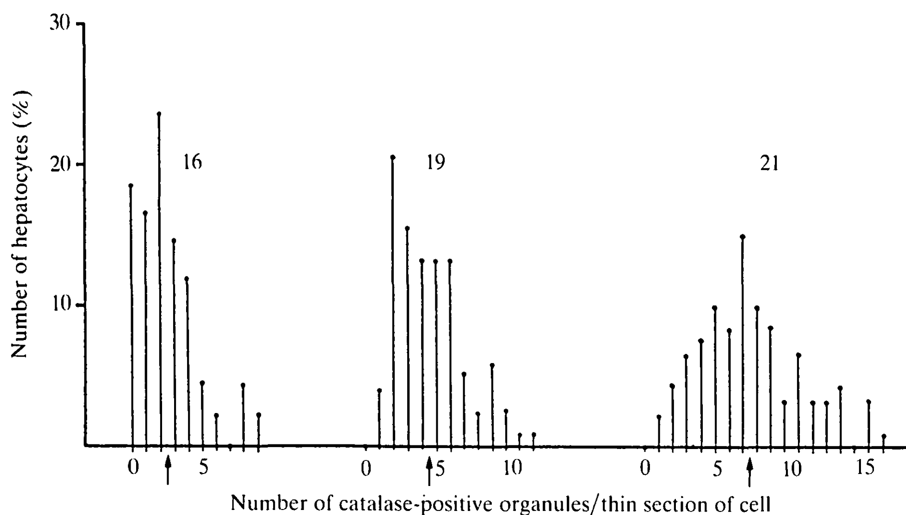

Fig. 4. Frequency distribution of catalase-positive organules per thin section of cell in foetal rat hepatocytes. Arrows indicate the mean numbers of catalase-positive organules per thin section of cell. 16, 19 and 21: days of development. Counts were made on at least 50 hepatocytes from each developmental stage.

In fact, the almost doubling of both the mean diameter and the mean number of organules observed between the 14th and 19th day means a significant and specific intracellular proliferation of the peroxisomal population, because, during the same period, neither the hepatocyte volume nor the intracellular extension of other organules increases. This conclusion is also supported by our biochemical data on catalase activity, which show a relevant increase of specific activity between days 14 and 19, when the percentage of liver volume occupied by the hepatocytes does not change at all (Greengard *et al.* 1972).

In later development (19–21 days), the growth rate of peroxisomal population decreases; in fact, the mean diameter increases more slowly than before and the doubling of the mean number is balanced by changes in the hepatocyte volume. It may be assumed, therefore, that during this period the peroxisomes occupy a relatively constant volume in the cytoplasm of the hepatocyte, similar to that occurring in other organules. This conclusion agrees with the results reported by Tsukada *et al.* (1968), who described a doubling in the number of microbodies per unit of cytoplasmic area between the 15th and 18th day and no variation between the 18th and 21st day of foetal development.

Finally, the increase observed in the catalase activity of the liver homogenate from the 19th day of foetal life to birth can be attributed only in part to the increase in number and size of the single organules. This increased activity is probably also due to the fact that during this phase of development the hepatocytes occupy a progressively increasing volume in the liver (Greengard *et al.* 1972). Of course, although the staining intensity of the peroxisomes remains unchanged during this period, the possibility that a further concentration of the enzyme occurs within the peroxisomes cannot be excluded.

From the data collected it may be stated that, in the foetal development of the rat liver, microperoxisomes containing cytochemically significant catalase activity appear at an early stage and significantly increase in size and number until the 19th day of intrauterine life. In subsequent development, the further increase which takes place appears to be balanced by the increasing cell volume.

Between the 14th and 19th day, the enzymatic content is also enriched and differentiated in all organules; in fact, they all contain catalase but only a few contain D-AAO.

Finally, around the 18th to 19th day of development a nucleoid may be observed in many of the organules. The peroxisomal population of the hepatocyte at this stage of intrauterine life appears, at least qualitatively, like that present in the adult cell. The hepatocyte itself, however, at this time of development, already presents the fundamental morphological characteristics of the adult cell (Ciofi Luzzatto, 1981).

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