SUBCELLULAR MORPHOMETRIC AND BIOCHEMICAL ANALYSES OF DEVELOPING RAT HEPATOCYTES

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ABSTRACT

Livers of rats between the 16th gestational and 100th postnatal day of age were subjected to quantitative biochemical and electron microscope, morphometric analyses. The amount of total mitochondrial protein per gram of liver remained at 34% of the adult level throughout the last 4 days of gestation but this was the period of rapid rise in the levels of cytochrome c oxidase, aspartate aminotransferase, and glutamate dehydrogenase in mitochondria; the nuclear fraction also acquired some glutamate dehydrogenase but lost most of it during postnatal development. During early postnatal life the amount of mitochondrial protein rose in parallel with the levels of cytochrome c oxidase and glutamate dehydrogenase but the upsurges of glutaminase and, later, of ornithine aminotransferase were accompanied by relatively little change in total mitochondrial protein. The surface area of rough endoplasmic reticulum per unit volume of hepatocyte cytoplasm (S_v^{RER}) did not change significantly throughout the period of development studied. From the 16th day of gestation to term the surface area of smooth ER (S_v^{SER}), the volume occupied by mitochondria (V_v^{MT}) and their number (N_v^{MT}) remained at 30, 66, and 45% of their adult values, respectively. V_v^{MT} and N_v^{MT} attained their maximal levels by the 2nd postnatal day and S_v^{SER} between days 2 and 12. Mitochondria of adult liver are thus smaller and contain more protein per unit volume than do those of fetal liver. After the 12th postnatal day, hepatocytes treble their size; they acquire more cytoplasm with additional enzymes but without further change in organelle concentration. The data reveal several distinct phases in the differentiation of hepatocytes. Each phase can be characterized by the extent to which the quantity and composition of various subcellular compartments evolve.

INTRODUCTION

In order to correlate enzymic and morphological aspects of differentiation, quantitative information has recently been obtained about the distribution of cell types in rat liver as a function of prenatal and postnatal age (1). The present investigations concentrated on subcellular structures in developing hepatocytes. The amounts of mitochondria (2), of smooth endoplasmic reticulum (3), and of several enzymes located in these organelles (4) are known to increase with age; however, the exact timing and magnitude of these changes in relation to each other have not been investigated systematically. Using a method of electron microscope stereology, we quantified these structures in thin sections of rat liver at different fetal and postnatal ages; the levels of marker enzymes and of mito-

chondrial protein were determined after homogenization and fractionation of such livers. The results describe the differentiation of hepatocytes in terms of stepwise changes in subcellular morphology and biochemical composition.

MATERIALS AND METHODS

Rats were of the inbred Kx strain. Estimations of fetal ages were based on timed matings and the correlations between body weight and age (5). Adult rats were 90–100 days old.

10% homogenates in 0.25 M sucrose were separated into nuclear and mitochondrial fractions by differential centrifugation in a discontinuous sucrose gradient by a modification (6) of the procedure of Hogeboom (7). (Contamination of nuclei with mitochondria was higher when homogenates in 0.88 M sucrose were separated as described by Schneider and Hogeboom [8].) Nuclei and mitochondria were washed and resuspended in the original volume in 0.25 M sucrose. Aliquots were examined microscopically to determine the purity of the subcellular fractions and were analyzed for protein content by the procedure of Lowry et al. (9). For enzyme determinations, the particulate fractions or whole homogenates were treated with Triton X-100 at a final concentration of 0.5%, sonicated for 3 × 10 s, and assayed after further dilution in water.

The assay of glutamate dehydrogenase (E.C. 1.4.1.3), based on the procedure of Wergedal and Harper (10), has been described previously (11). The conversion of NADH to NAD was followed spectrophotometrically at 340 nm in a reaction mixture containing 350 mM NH_4Cl, 3 mM α-ketoglutarate, 0.2 mM NADH, 100 mM PO_4 buffer (pH 7.5), and 0.05 and 0.1 ml of 0.5–1% solubilized tissue preparations. Blank rates, obtained in the absence of α-ketoglutarate (0–5% of the experimental rates), were subtracted from the experimental values.

Aspartate aminotransferase (E.C. 6.2.1.1) activities were determined as previously described (6, 12). Cytochrome c oxidase (E.C. 1.9.3.1) activity was determined according to Cooperstein and Lazarow (12) at 25°C in the presence of 20 μM reduced cytochrome c and expressed in k per minute units where k is a first order rate constant calculated in common logarithms. For all other enzymes, "unit" refers to 1 μmol of substrate utilized per minute at 25°C.

For the morphometric studies livers were excised and embedded as previously described (1). Electron micrographs were taken of cytoplasmic areas of parenchymal cells (hepatocytes) at a magnification of 25,000. Randomly chosen electron micrographs (20–50 per liver, derived from four different tissue blocks) were analyzed with a superimposed test lattice of 24 lines. The surface area of a membrane, according to Weibel et al. (13), is given by $2I/L$, where I is

the number of intersections and L is the sum of the lengths of the individual test lines (in our case, 24 μm). We determined by this method the surface area per unit volume of cytoplasm of rough and smooth endoplasmic reticulum (S_v^{RER} and S_v^{SER}).

The 24 lines of the above test lattice provide 48 points; the fraction of these points falling on mitochondria is a measure of the volume occupied by mitochondria per unit volume of cytoplasm (V_v^{MT}) (13–14). The number of mitochondria per unit volume of cytoplasm is:

$$N_v^{MT} = \frac{1 \times N_A^{MT^{3/2}}}{\beta_m \times V_v^{MT^{1/2}}} \times K,$$

where N_A^{MT} represents the number of mitochondria counted in a test area, divided by the test area (in our case, 50×10^{-8} cm^2); β_m is the shape-dependent coefficient for which we accepted Weibel's value of 2.25 (13, 15). For factor K, referring to size distribution of particles, we assumed, as Weibel et al. (13), the value of 1.0.

RESULTS

The first set of experiments to be described were designed to quantify developmental changes in the levels of mitochondrial enzymes in liver. Their distribution among fractions isolated from homogenates of livers at different stages of development also served to check the recovery and purity of the mitochondrial fraction. One of the enzymes chosen, aspartate aminotransferase, exists in two isozymic variants, particulate (to be referred to as m) and soluble (s); their separate developmental formations and their different responses to regulators were studied previously (6). Since only isozyme m was relevant to the present investigations, activities in the supernatant fractions are not given; the glutamate dehydrogenase and cytochrome c oxidase activities in the supernatant fractions were nil or very low (see Table I). The cytochrome c oxidase and aspartate aminotransferase activities of the nuclear fraction throughout development represented a very low and constant percentage of those of the mitochondrial fraction (Table I); they probably reflected a small degree of contamination of nuclei with mitochondria. However, the subcellular distribution of glutamate dehydrogenase assayed in the same preparations changed significantly with age. In fetal and neonatal liver the nuclear and the mitochondrial glutamate dehydrogenase activities (per gram) were of similar magnitude, whereas in adult liver the latter clearly predomi-

TABLE I

The Subcellular Distribution of Three Enzymes in Fetal and Postnatal Liver

The same preparations were used for the determinations of the three enzyme activities. The values given are means (±SD) of determinations on three to four postnatal livers or four to nine pools of fetal liver (each pool was from a different litter and comprised five to eight livers). Cytosol refers to postmitochondrial supernatants obtained after centrifuging down the mitochondria at 25,000 g for 15 min. The s isozyme of aspartate aminotransferase in the cytosol was not assayed. Fetal: 19–21 days of gestation; neonatal: 1 day after birth.

		Enzyme activities, U/g liver		
Age	Fractions	Cytochrome c oxidase	Glutamate dehydrogenase	Aspartate aminotransferase m
Fetal	Nuclei	14 ± 3	10 ± 3	8 ± 4
	Mitochondria	89 ± 6	12 ± 5	36 ± 5
	Cytosol	<3	0.3 ± 0.3	—
Neonatal	Nuclei	35 ± 5	22 ± 1	7 ± 3
	Mitochondria	151 ± 36	26 ± 1	39 ± 4
	Cytosol	<3	0.2 ± 0.1	—
Adult	Nuclei	22 ± 4	7 ± 2	6 ± 2
	Mitochondria	225 ± 12	47 ± 6	64 ± 8
	Cytosol	<3	3.3 ± 0.8	—

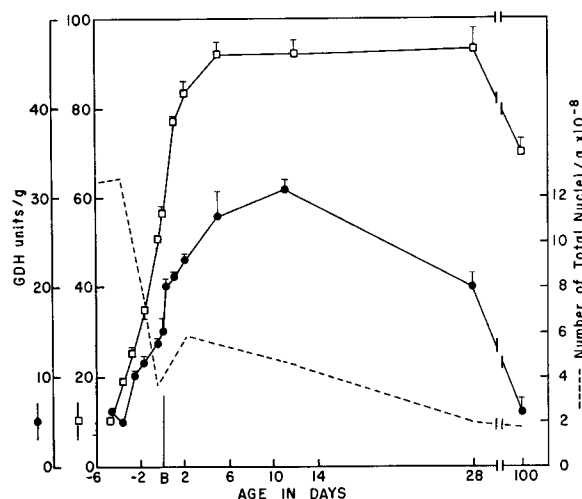FIGURE 1 Changes in total and nuclear glutamate dehydrogenase (*GDH*) activity with age. Total (□——□) and nuclear (●——●); (NB: double scale) activities are expressed as units per gram of liver (left ordinate). Each prenatal point is an average from two to four pools from a single litter or the mean from three to four litters. Postnatal points are means from three to six animals. Bars = 1 SD. Number of nuclei per gram (---) (right ordinate) are transcribed from Greengard et al. (1).

nated. (The small amount of activity detected in adult liver nuclei confirms previous observations [16].)

As previously reported (1) and shown in Fig. 1, the number of nuclei per gram of liver decreases during late fetal life (largely due to the disappearance of the small hematopoietic cells) and decreases again after the 12th postnatal day

(as a result of the enlargement of the hepatocytes [1]). The glutamate dehydrogenase activity of the nuclear fraction did not parallel these changes in cellularity: it began to rise during late fetal life, reached a maximum during the 2nd postnatal week, and declined thereafter. To indicate the relative contribution of nuclear to total enzyme activity, Fig. 1 also depicts the activity of whole homogenates. It may be seen that the decrease in total activity after the 5th postnatal week could be accounted for by the loss of activity from the nuclear fraction. The difference between the total and nuclear activities was essentially due to the activity of the mitochondrial fraction (see Fig. 2) though because of some loss of activity during the fractionation procedure the sum of nuclear and mitochondrial activity was 15–25% less than that measured in whole homogenates.

The amount of mitochondrial protein as a function of age is shown in Fig. 2. It may be seen that fetal liver contained about one-third as much as did adult liver. The mitochondrial protein content remained constant during the last 4 days of gesta-

tion and rose most rapidly during the first 5 post-natal days. The levels of glutamate dehydrogenase are depicted for comparison; those of aspartate aminotransferase and cytochrome c oxidase (determined in the same preparation) will be seen in Fig. 3. For purposes of contrasting the developmental accumulation of five mitochondrial enzymes with each other and with the total mitochondrial protein, Fig. 3 depicts all values as per cent of the corresponding value in adult liver. It may be seen that aspartate aminotransferase, cytochrome c oxidase, and glutamate dehydrogenase were formed rapidly during the last few days of gestation. Values for the two other enzymes in Fig. 3 are from previously published work (17–18); the rise in L-glutaminase was most swift during neonatal life and significant ornithine aminotransferase formation was delayed until the 3rd postnatal week. The total rise in the amount of mitochondrial protein per gram of tissue was only threefold. The severalfold rises in enzyme levels occurred without any (prenatal period) or with relatively little (postnatal period) rise in total
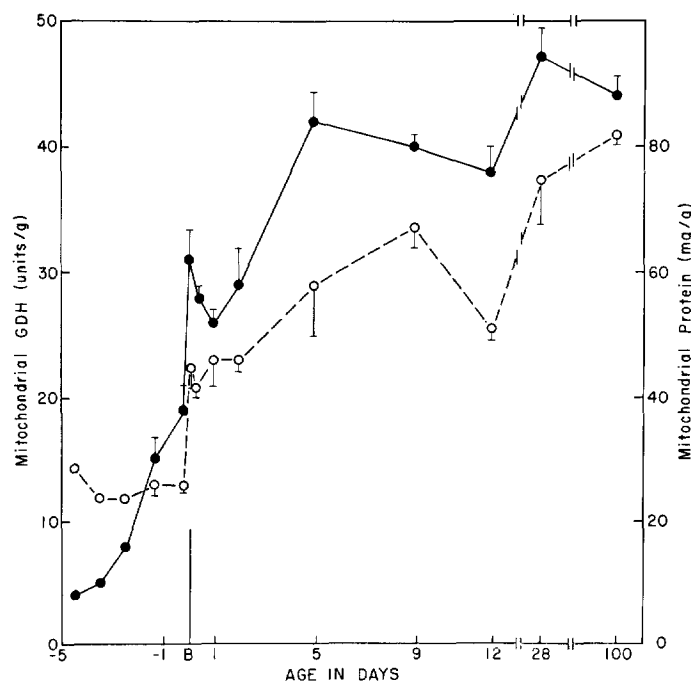

Figure 2 Changes with age of mitochondrial glutamate dehydrogenase (GDH) and protein concentrations in rat liver. Mitochondrial preparations were analyzed for glutamate dehydrogenase activity (●——●) (left ordinate) and protein content (○--○) (right ordinate). Prenatal points refer to a pool of livers from 1 litter or to means of three to four such pools. Bars = 1 SD. Points after birth are averages of three to six livers from different litters.
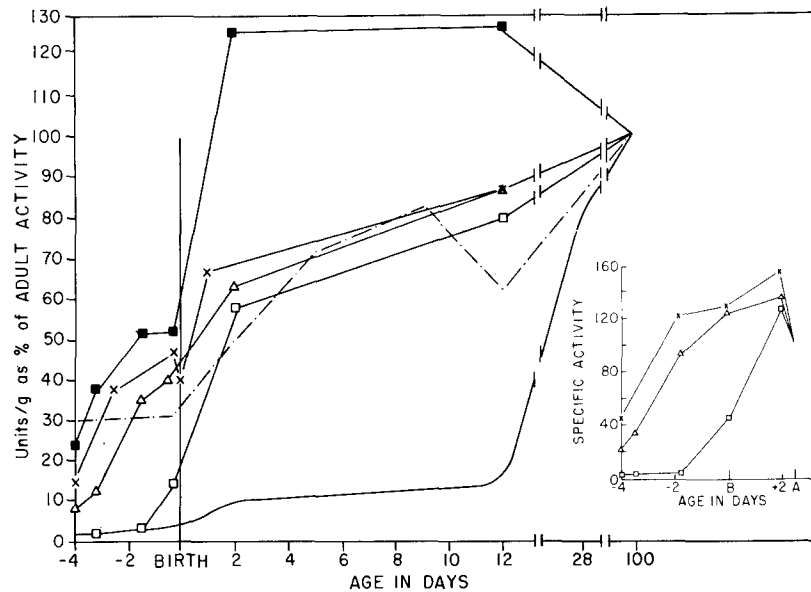
FIGURE 3 The developmental formation of mitochondrial enzymes in rat liver. The activities (units per gram) of glutamate dehydrogenase (△———△), cytochrome c oxidase (×———×), and aspartate amino-transferase (■———■) in mitochondria are expressed as per cent of adult activities (for absolute values see Table I). Mitochondrial protein content per gram of liver (1 U = 1 mg) as per cent of that in adult liver (•———•) is calculated from Fig. 2. L-Glutaminase (□———□) and ornithine aminotransferase (———) were calculated from previously published data (17, 18); the absolute values (micromoles per minute per gram) in adult liver are 3.1 ± 0.5 for ornithine aminotransferase and 10.0 ± 2.7 for glutaminase. The *insert* depicts enzyme activities per milligram of mitochondrial protein at the time of most rapid change. A = adult.

mitochondrial protein. Only during early post-natal life was there parallelism between increases in some enzyme levels and in mitochondrial protein.

The insert in Fig. 3 serves to emphasize the different purpose served by expressing enzyme levels in an alternate way. The fact that the *specific activities* of glutamate dehydrogenase and cytochrome c oxidase have reached their final adult levels by the time of birth is relevant to the differentiation of mitochondria per se. Their activity per gram, on the other hand, (which at this time is only 50% of the adult value), is an indicator of the extent to which the *cell* or *tissue* has approached its mature composition.

Electron micrographs typified in Fig. 4 were used for the morphometric analyses of liver during development. The results are shown in Table II. It may be seen that, per unit volume of hepato-cyte cytoplasm, both the volume occupied by mitochondria $(V_v{}^{MT})$ and the number of mito-chondria $(N_v{}^{MT})$ remained essentially constant during the last 6 days of gestation. Both have

risen by the 2nd postnatal day. Since the number of mitochondria increased more than the total volume occupied by them, the mean volume of mitochondria (third column) must decrease with age. The surface area of rough ER per unit volume of cytoplasm $(S_v{}^{RER})$ did not change significantly during development and that of the smooth ER $(S_v{}^{SER})$ rose between the 2nd and the 12th post-natal day (Table II).

Developmental changes in the biochemical and structural constituents of liver are summarized in Table III. In order to compare the rates at which the concentrations of these different types of constituents approach maturity, they have been uniformly expressed as per cent of the appropriate adult value. We are assuming that values per unit volume of hepatocyte cytoplasm (sub-script v) are comparable to those per unit weight of whole liver (subscript g), since on the 16th day of gestation already 60% of the liver volume is parenchymal tissue (the adult value is 88% so that in terms of Table III the hepatocyte concen-
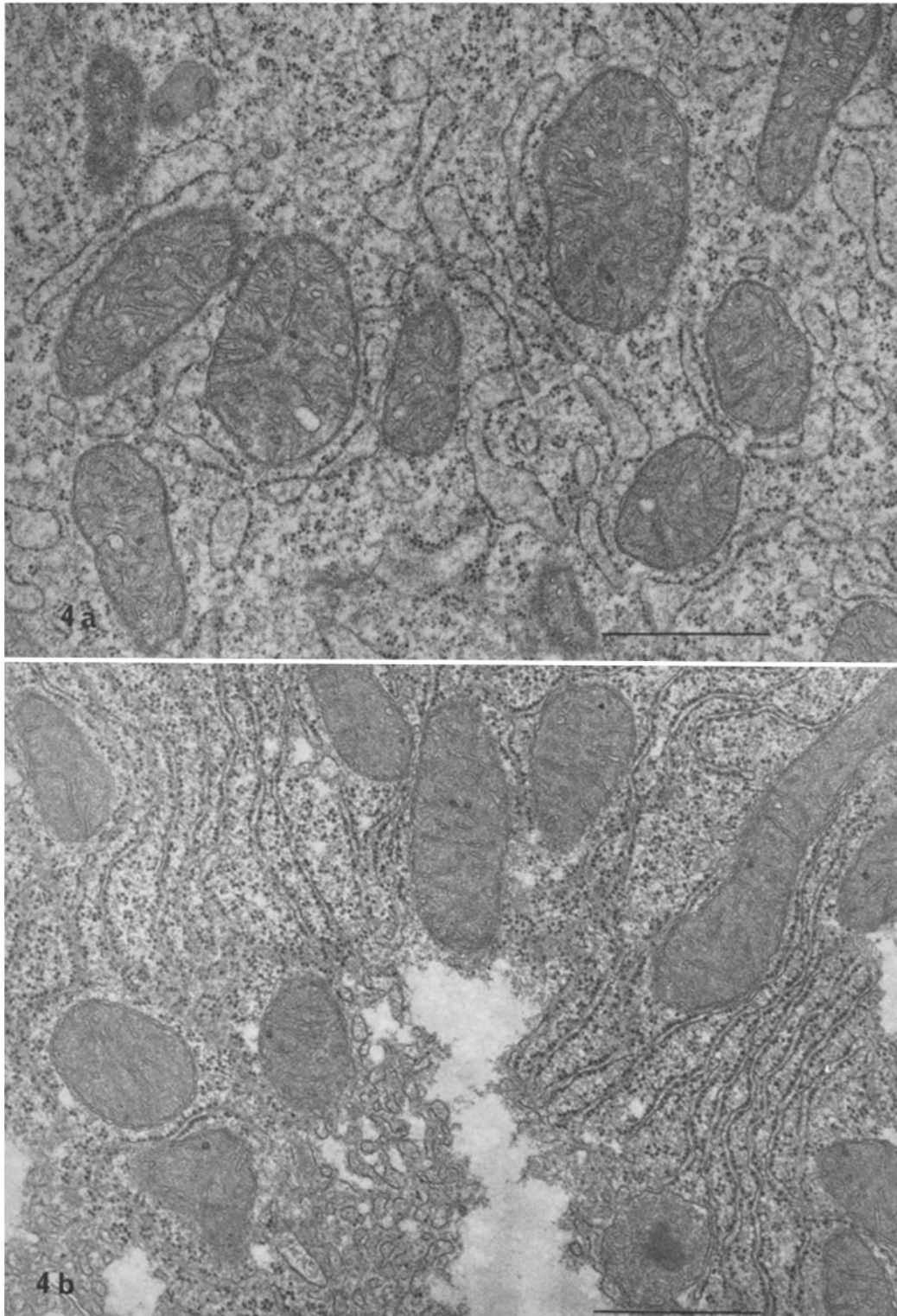
FIGURE 4 (a) Fetal liver, 17th day of gestation; (b) adult liver. Bar = 1 μm. × 25,000.

TABLE II

The Quantification of Subcellular Structures by Electron Microscope Stereology

The volume (cm^3) occupied by mitochondria ($V_v{}^{MT}$), their number ($N_v{}^{MT}$), and the surface areas (m^2) of rough and smooth ER ($S_v{}^{RER}$ and $S_v{}^{SER}$) are expressed per cubic centimeter of hepatocyte cytoplasm. The values are means (±standard deviation) of results with 5–10 fetal (from two to three different litters) and 3–4 postnatal livers. Mean volume of mitochondria = $V_v{}^{MT}/N_v{}^{MT}$.

Age	Mitochondria			Endoplasmic reticulum	
	$V_v{}^{MT}$	$N_v{}^{MT} \times 10^{-9}$	Mean vol.	$S_v{}^{RER}$	$S_v{}^{SER}$
(days)			*μm^3*		
Gestation					
15.2–17.6	0.135 ± 0.02	96.4 ± 25.4	1.40	4.04 ± 0.35	0.59 ± 0.22
18.25–18.6	0.143 ± 0.05	94.4 ± 31.6	1.50	4.15 ± 0.52	0.51 ± 0.26
20.3–20.6	0.134 ± 0.03	70.0 ± 18.7	1.90	4.33 ± 0.64	0.77 ± 0.33
21.6–21.9	0.132 ± 0.02	90.5 ± 15.1	1.46	5.05 ± 0.34	0.74 ± 0.37
Postnatal					
2	0.23 ± 0.04	221 ± 30	0.96	4.70 ± 0.9	0.59 ± 0.50
12	0.19 ± 0.03	180 ± 26	1.06	4.43 ± 0.6	2.42 ± 0.71
28	0.214 ± 0.04	258 ± 16	0.83	3.31 ± 1.3	3.83 ± 1.61
100	0.200 ± 0.05	199 ± 49	1.00	3.44 ± 0.5	2.29 ± 1.07

tration of fetal liver is no less than 70% of adult liver).

It may be seen that the different subcellular compartments approached their final adult levels at distinctly different times: rough ER during early fetal life, mitochondria ($V_v{}^{MT}$) and soluble protein during neonatal life and smooth ER by the end of the 2nd week. The relatively late increase in the density of mitochondria (protein per unit volume) is an obvious consequence of the greater (but slower) per cent increase in total mitochondrial protein than in total volume occupied by mitochondria.

The disparity between the rates of accumulation of mitochondrial enzymes and of total mitochondrial protein has already been illustrated in Fig. 3. The developmental formation of a microsomal enzyme, glucose-6-phosphatase (illustrated in Table III), clearly does not coincide with quantitative changes in the surface area of RER or SER. The many soluble enzymes which emerge in liver around the 3rd postnatal week (4), i.e. after there is no further change in the concentration of subcellular organelles or soluble protein, are exemplified here by tryptophan pyrrolase.

The last line of Table III does not refer to concentration but gives the absolute mean volume of parenchymal cells (V^P). The transient peak of V^P at term (1, 21) which can in part be ex-

plained by the extensive deposition and subsequent depletion of glycogen is not relevant to the present discussion, but the major increase after the 12th postnatal day is of interest. Assuming that the volume occupied by nuclei is relatively small and constant, changes in V^P reflect changes in cytoplasmic volume. Thus, on the 12th postnatal day the amount of cytoplasm per cell is still small (about 30% of adult) but the concentration of organelles (and of soluble protein) within it is already maximal (see 12 day column, lines 1–7). It is the same as that in the cytoplasm that the cell is to acquire during subsequent weeks. Only the concentration of enzymes (two of the many are illustrated in Table III) appears to change after the 12th postnatal day.

DISCUSSION

There are probably several enzymes which change their subcellular distribution during development (22); the present study includes one example, glutamate dehydrogenase, which is concentrated in the mitochondria of adult liver but which, during fetal life, appears to accumulate at similar rates in both the nuclear and mitochondrial fractions. The other enzymes studied in the same preparations remained mitochondrial throughout development. Their increases with age have been observed before; we merely quantified and timed

TABLE III

Various Aspects of Intracellular Differentiation of Rat Liver Hepatocytes

All values are expressed as percent of those in adult liver. Data for the first six lines are from Table II and Fig. 2. Glucose-6-phosphatase (G6Pase) and tryptophan oxygenase (TP) activity units are from previous reports (20, 19). The number of parenchymal cells per gram of liver and their mean volume (V^P, $100\% = 9.02 \times 10^3 \ \mu m^3$) are from previous morphometric studies (1). Capital letters V, N, S and E refer to the type of measurement, i.e., volume, number, surface area, and enzyme activity, respectively. Superscripts indicate the particular entities subjected to such measurements (Sol = soluble fraction obtained by centrifuging at 100,000 g for 60 min) and subscripts indicate the tissue base, i.e., unit volume of hepatocyte cytoplasm (v) or gram of whole liver (g). $\text{Density}^{MT} = \text{protein}_g^{MT}/V_v^{MT}$.

	Amount of constituents (% of adult)				
Age in days:	16 (Gestation)	22	2 (Postnatal)	12	28
V_v^{MT}	67	66	115	195	107
N_v^{MT}	48	46	110	91	129
Protein_g^{MT}	29	32	56	62	90
Density^{MT}	15	40	46	80	87
S_v^{SER}	26	33	36	106	169*
S_v^{RER}	117	146	136	128	96
Protein_g^{Sol}	63	86	90	92	100
E_g^{G6Pase}	<5	50	200	100	—
E_g^{TP}	<5	<5	<5	<5	100
V^P	16	58	29	30	70

* Not significantly different from 12 day value, see Table II.

these increases more precisely. Our study of subcellular organelles requires closer scrutiny since the method of quantitative stereology is too new to have received adequate tests of its reproducibility.

The only morphometric study of developing liver, that of Rohr et al. (21), includes one fetal age (3 days before birth) and does not go beyond the 8th postnatal day. However, it reports, in agreement with our observation, a neonatal rise in the volume (V_v^{MT}) and a greater rise in the number of mitochondria (N_v^{MT}). In absolute terms, too, the changes observed are in reasonable

agreement. V_v^{MT} rises from 0.10 to 0.20 (our values: 0.13–0.23) and N_v^{MT} from 42 to 124 $\times 10^9$ (our values: 94–221 $\times 10^9$). Rohr et al. (21) did not determine the surface area of the ER but found no major change in the volume of rough ER and an increase in that of smooth ER between days 1 and 8; these results are in harmony with our conclusions based on surface area measurement. Our values for adult liver ($V_v^{MT} = 0.2$, $N_v^{MT} = 199$) can be compared with those of Weibel et al. (13) ($V_v^{MT} = 0.18$, $N_v^{MT} = 280$) and Loud (23) ($N_v^{MT} = 208$). Our values for S^{RER} (3.4) and S^{SER} (2.3) are similar to those of Loud (23) (3.25 and 2.06); the values of Weibel et al. (6.2 and 4.7) were calculated for two membrane surfaces (14). Thus, the agreement between morphometric data from different laboratories, and with different rat strains, is probably as good as those for biochemical determinations with well-established methods.

From the point of view of enzymic composition the late fetal rat liver is very far from having reached its fully differentiated state. Some three-dozen enzymes characteristic of adult liver are still essentially absent (24). In contrast, some of the morphological features of fetal hepatocytes appear to be well developed. On the 16th day of gestation the mitochondrial mass and the number of mitochondria have already reached 67 and 48% of their adult levels, respectively, and the area of RER is about the same as in adult liver. The rise in the concentration of mitochondria is restricted to the neonatal period, whereas the upsurge of mitochondrial as well as of other enzymes occurs in three main clusters: during the late fetal, the neonatal, and the late suckling period (3rd postnatal week) (4). Enzymes of the ER, as discussed by Siekevitz and co-workers (25, 26), also do not rise in parallel with the compartment as a whole; the area of smooth ER increases during the period (2nd and 12th postnatal day), which is relatively uneventful from the point of view of enzymic differentiation (4). Thus, judged by the enzymes usually studied, the amount and composition of subcellular compartments appear to change independently. The structural proteins which must rise in parallel with any enlargement of the compartment as a whole are largely unknown.

The mature state of an organ is attained by separate "differentiations" (i.e., changes in structure or composition) of its elements. The specific

criterion of differentiation varies according to the complexity of the element under consideration. *Mitochondria* differentiate when the relative amounts of enzymes change within them, but from the point of view of *cytoplasm* a mere quantitative change in the concentration of mitochondria represents differentiation. Considering the *cell* as a unit, the acquisition of more of the same kind of cytoplasm, to surround the single nucleus, can be considered as differentiation. On a higher level of complexity, that of the whole organ, changes in the relative number of cell types with or without modification of intracellular enzyme patterns represents differentiation. The realization of this hierarchical nature of differentiation should solve a practical problem, that of the proper choice of bases (now ranging arbitrarily from a particular protein fraction or DNA to whole body weight) for expressing enzyme activities. Observations on cytochrome *c* oxidase and glutamate dehydrogenase (see Fig. 3) activities provide simple examples. After birth, but not before birth, these enzymes remain constant relative to the amount of mitochondrial protein but rise per gram of liver (or per total protein) and, therefore, relative to other constituents of the tissue. This type of change can alter the functional balance of the tissue because a metabolic pathway can encompass enzymes of several compartments. Thus, gram liver is the proper base of expression when using enzymes as indicators of the differentiation of the *tissue*, whereas milligram mitochondrial protein is the proper base when judging their contribution to the differentiation of *mitochondria*. The relationship between the part and the larger unit is, of course, such that differentiation of the latter does not necessarily involve the former but differentiation of the part contributes to that of the whole.

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