CYTOMORPHOMETRY OF DEVELOPING RAT LIVER AND ITS APPLICATION TO ENZYMIC DIFFERENTIATION

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ABSTRACT

Quantitative stereological methods have been adapted for the measurement of the volume of liver attributable to parenchymal, hematopoietic, and Kupffer cells and for the measurement of the relative and absolute number (per unit volume) of these cell types and the mean volume of the parenchymal cell. These morphological parameters are the main ones for interpreting the biochemical differentiation of liver. Quantitative changes in these parameters, in rat liver between the 15th day of gestation and adult life, are presented. Despite the large number of hematopoietic cells, the parenchymal cells fill more than half of the liver volume between the 15th and 18th days of gestation and 0.85 of the liver volume at term. The fraction of liver volume occupied by Kupffer cells is never more than 0.02; the number of Kupffer cells per cubic centimeter increases less than twofold between fetal and adult life. The mean volume of individual parenchymal cells undergoes a threefold rise during late fetal life, declines in the neonatal period, and doubles between the 12th and 28th postnatal days. With the morphometric data obtained, it is impossible to convert enzyme concentrations (units per gram, determined in homogenates of whole liver) to enzyme amounts per unit volume of parenchymal or hematopoietic tissue or per individual cell of either type. In late fetal liver, only rises in enzyme concentration less than twofold may be attributed to the enrichment of parenchymal tissue at the expense of hematopoietic elements. The sudden upsurge, by more than twofold, of hepatic enzymes of the late fetal cluster (and also of the neonatal and late suckling cluster) reflects rises per parenchymal mass and per parenchymal cell. Thyroxine and glucagon, the administration of which to fetal rats promotes enzyme differentiation in liver, are without appreciable effect on the cytological parameters studied. Hydrocortisone accelerates the involution of hematopoietic tissue in fetal liver. Enzymes that are diminished by prenatal injection of hydrocortisone may be concentrated in hematopoietic cells.

INTRODUCTION

Much information has accumulated in the last decade about enzymic differentiation in mammalian liver during late gestation and early postnatal life. Recently, hormones have been identified which promote the developmental formation of distinct groups of enzymes in fetal and neonatal rat

liver (1, 2). The cytology of the liver is a much older subject but the microscopic descriptions are largely qualitative in nature, whereas the biochemical information is quantitative. Study of the relationships between enzymic and cellular compositions is thus precluded by the lack of quantitative morphological data. Homogenates of whole liver are usually employed in biochemical studies, in lieu of physical separation of the various cell types. Cytomorphometry, utilizing thin sections obtained from tissue with well-preserved three-dimensional structure, is a method of choice for determining the cellular composition of the tissue from which such homogenates are prepared. The present study applied the recently described methods of quantitative stereology (3, 4) to determine the cellular composition of rat liver during development and to test the extent to which it may change upon the administration of hormones which are known to promote enzymic differentiation.

Loss of hematopoietic tissue is a major cellular event during prenatal differentiation of liver (5). The rate of this loss, in terms of tissue mass and cell number, can now be quantitatively correlated with the coincident increases and decreases in the concentration of hepatic enzymes. Determination of the mean volume of a parenchymal cell as a function of fetal and postnatal age provides an additional way to analyze the nature of changing enzyme patterns.

METHODS AND MATERIALS

Animals

Rats of the inbred NEDH strain were used. Fetal age was read off a curve of body weight against day of gestation, established by Gonzalez (6). When indicated, fetal rats were injected with hormones intraperitoneally through the uterine wall of the laparotomized, anesthetized dam. The amounts of thyroxine, glucagon, and hydrocortisone acetate, respectively, were 30 μg, 0.25 mg, and 0.125 mg per fetus, given 24, 5, and 36 hr before assay, respectively.

Counting of Nuclei in Homogenates

Portions of freshly excised liver were homogenized in 4 vol of 0.15 N KCl, diluted (1:20) with 0.9% NaCl, then further diluted (1:20) in a white blood cell pipette with 45% acetic acid saturated with orcein, and counted in two 0.9 mm^3 chambers of a Spencer Bright Line Hematocytometer (American Optical Corporation, Buffalo, N. Y.) with Neubauer ruling as previously described (7). The results ($N_v$homog) are expressed as nuclei per cubic centimeter of liver.

The Method of Morphometry

Morphometry utilizes measurements in two-dimensional samples for obtaining quantitative information about three-dimensional structures. It requires well-preserved tissue sections with a reproducible thickness of not more than 1 μ. Therefore, the tissues were handled and embedded as usually done for electron microscopy. Immediately after the rats were killed, pieces of liver were placed in Karnovsky's fixative (8), diced, transferred to fresh fixative for 4 hr, and thoroughly washed with buffer. The tissue was postfixed in Palade's ice-cold 1% OsO_4 (9) for 1–2 hr, dehydrated in alcohol, and finally embedded in Luft's Epon 812 (10). The plastic-embedded liver blocks were sectioned with a Porter-Blum ultramicrotome and stained with toluidine blue. Six blocks from each liver were sectioned and examined under oil immersion in a Zeiss microscope (Carl Zeiss, Inc., New York) at a magnification of ×1250. Parenchymal, hematopoietic, and Kupffer cells were easily identifiable from the shape and staining properties of their nuclei.

The principles involved in the quantitative, stereological, or morphometric analysis of tissues have been described recently (3, 4). The fraction of tissue volume occupied by component i ($V_v{}^i$) is proportional to the fraction of the area (of a thin section) occupied by this component. This area in turn is proportional to the frequency with which fixed points (probes) of a superimposed lattice fall on it. $V_v{}^i$ is thus given by the number of points falling on component i over the total number of probe points on the field. We used an eight point lattice for the differential scoring of parenchymal, hematopoietic, and Kupffer cells and of unidentifiable space. $V_v{}^P$, $V_v{}^H$, $V_v{}^K$, and $V_v{}^X$ (their sum = 1) are the corresponding volume fractions obtained. The selection of fields from any section was random; occasional fields that were empty or imperfectly preserved in more than one-third of the area were avoided.

The sections (six per liver = one per block) and fields (six per section) used for determining volume fractions with the above point-scoring method were also used for the differential counting of cell nuclei in a viewing area of 13,273 × 10^{-8} cm^2. The ratios of the numbers of parenchymal (nonmitotic plus mitotic), hematopoietic, and Kupffer cell nuclei to the total number of nuclei per field provide the relative numbers of each of these nuclei ($N_n{}^P + N_n{}^H + N_n{}^K = 1$). N^{MP} refers to that fraction of parenchymal nuclei which were in the process of mitosis.

The absolute number of all nuclei per cubic centimeter of liver (N_v) was calculated according to

Loud (4)[1]: $N_v = N_A/\bar{D}$, where N_A is the number of nuclei per square centimeter and $\bar{D}$ is 8.1 × 10^{-4} cm, the average diameter of parenchymal cell nuclei. The error arising from the somewhat smaller size of hematopoietic and Kupffer cell nuclei (6–7 × 10^{-4} cm) was small, and no correction was applied. By multiplying the total (N_v) with the fractions $N_n{}^P$, $N_n{}^H$, and $N_n{}^K$, we obtain the number of parenchymal ($N_v{}^P$), hematopoietic ($N_v{}^H$), and Kupffer ($N_v{}^K$) cell nuclei per cubic centimeter of liver. For liver up to the age of 12 days, binuclear cells were rare and we assumed all cells to be mononuclear. Using the reported frequency of multinuclear parenchymal cells at later ages (11), N_v, N_{homog}, $N_v{}^P$, and V^P were corrected to refer to cell number for 28- and 100-day old rats; Table I shows the uncorrected as well as the corrected (in italics) values. Actual counts in homogenates are given for comparison.

The volume fraction divided by the number of cells of type i per unit volume ($V_v{}^i/N_v{}^i$) provides the mean absolute volume of such cells. V^P, V^H, and V^K denote the mean volumes of parenchymal, hematopoietic, and Kupffer cells thus calculated.

Statistical Significance of the Results

Each value in Table I refers to the mean of results with a single liver. Volume fractions (V_v) were obtained by scoring 8 points in each of six fields in each of six sections, that is, 288 points per liver. The relative number of nuclei (N^P, etc.) were obtained by counting in six fields (each containing 25–140 nuclei, depending on age) in each of six sections. The least significant difference (LSD) at the 1% confidence level was calculated by analysis of variance performed on the data which had been subjected to arcsine (for V_v) or square root (for N_n) transformation for proportions. Differences between groups of livers in excess of this LSD are referred to as "significant" or are labeled "$P > 0.01$."

RESULTS

In rat liver, biochemical differentiation proceeds extensively during the last few days of gestation and during early postnatal life. For our morphometric analysis, therefore, we chose livers at several late fetal ages and at 2, 12, and 28 days of postnatal age. 100-day old rats were considered "adults". Table I summarizes the cytomorphometric data obtained in this study. The first two groups of columns (volume fractions and relative number of nuclei) and N_v (total number of nuclei per cubic centimeter) provided the basis for calculating the concentration of the different types of nuclei ($N_v{}^P$, $N_v{}^H$, and $N_v{}^K$) and the mean cell volumes. The number of nuclei determined in homogenates, N_{homog} (not used in the calculations), agree fairly well with their morphometric determination (N_v) and provide an independent check of this method.

It should be noted that the volume fractions are based on a constant (i.e., unit volume of liver) so that they can be thought of as "concentration." The first number in Table I means, for example, that the parenchymal cell volume ($V_v{}^P$) occupied 0.57 cm³ of 1.0 cm³ liver. On the other hand, the values under relative number of nuclei involve fractions with a variable denominator (namely, the age-dependent total number of nuclei per field), and only the next set of columns give the actual concentration of nuclei per unit volume. This distinction between relative and absolute concentration (denoted by subscripts n and v, respectively) is important: for example, $N_n{}^P$ (parenchymal nuclei relative to total) increases between fetal and adult life but the actual concentration of parenchymal cells per gram, $N_v{}^P$, decreases because the mean volume of these cells, V^P, increases with age.

Volume fractions were determined by scoring 288 points for each liver. More than 30 of these points would fall on major components (with volume fractions over 0.1), and for these there is good agreement between triplicate livers. In the case of minor components (with volume fractions under 0.1), there is a much greater per cent error so that many more samples would have to be scored to establish significant changes with age.

To determine the relative number of nuclei a total of 900–5040 were counted per liver so that, even for those representing a tenth of the total, the values are based on at least 90 counts. There is thus usually good agreement for $N_n{}^P$, $N_n{}^H$, and $N_n{}^K$ between triplicate livers. The derived values of cells per cubic centimeter ($N_v{}^P$, etc.) are, of course, correspondingly accurate. However, the accuracy of cell volumes is limited by the accuracy of the volume fraction involved in the calculation.

During the gestational age of 15–18 days over half the liver *volume* is occupied by parenchymal tissue (see $V_v{}^P$), but since the parenchymal cells are relatively large (see cell volumes) their *number* is only about one-third of the total (see $N_n{}^P$). The reverse is true for hematopoietic cells: they occupy one-third of the liver volume (see $V_v{}^H$) but they account for more than half of the cells (see $N_n{}^H$).

[1] Based on DeHoff, R. T., and F. N. Rhines. 1961. *Trans. AIME.* **221**:975.

TABLE I

Morphometric Observations of Normal and Hormone-Treated Rat Livers at Different Ages

Age in days* (g body, liver wt)	Volume fractions				Relative number of nuclei				Nuclei per cm³ × 10^{-8}					Cell volume ($\mu^3 \times 10^{-3}$)		
	$V_v{}^P$	$V_v{}^H$ ($\Sigma V_v = 1$)	$V_v{}^K$	$V_v{}^X$	$N_n{}^P$	$N_n{}^H$ ($\Sigma N_n = 1$)	$N_n{}^K$	$N_n{}^{MP}$ ($N_n{}^P = 1$)	N_v homog	N_v	$N_v{}^P$	$N_v{}^H$	$N_v{}^K$	V^P	V^H	V^K
F 15.2	0.57	0.40	0.011	0.021	0.32	0.64	0.044	0.115	10.9	13.0	4.16	8.32	0.572	1.370	0.48	0.19
(0.2, 0.03)	0.59	0.39	0.014	0.014	0.37	0.59	0.049	0.110		11.9	4.40	7.02	0.582	1.34	0.56	0.24
	0.64	0.32	0.010	0.031	0.36	0.61	0.038	0.088		10.7	3.85	6.53	0.407	1.66	0.49	0.24
F 18.2	0.57	0.36	0.024	0.04	0.33	0.63	0.047	0.092	9.9	11.9	3.93	7.50	0.559	1.45	0.48	0.43
(1.0, 0.08)	0.52	0.43	0.010	0.04	0.32	0.63	0.047	0.094		11.9	3.81	7.50	0.559	1.36	0.57	0.18
	0.60	0.32	0.014	0.07	0.35	0.61	0.043	0.072		12.5	4.38	7.63	0.538	1.41	0.42	0.26
F 20.4	0.63	0.23	0.024	0.118	0.35	0.59	0.058	0.083		8.5	2.98	5.01	0.493	2.12	0.46	0.49
(2.9, 0.22)	0.69	0.24	0.018	0.049	0.33	0.62	0.049	0.073		8.6	2.84	5.33	0.421	2.43	0.45	0.43
	0.73	0.19	0.017	0.073	0.36	0.59	0.059	0.084		7.9	2.84	4.66	0.466	2.57	0.41	0.36
F 20.6	0.63	0.31	0.013	0.042	0.30	0.62	0.074	0.039	10.0	9.1	2.73	5.64	0.673	2.31	0.55	0.19
(3.2, 0.24)	0.68	0.26	0.018	0.042	0.35	0.57	0.076	0.048		7.8	2.73	4.44	0.593	2.49	0.58	0.30
	0.66	0.26	0.031	0.045	0.38	0.54	0.079	0.037		7.6	2.89	4.10	0.600	2.28	0.63	0.52
F 21.6	0.88	0.07	0	0.072	0.38	0.54	0.079	0.039	4.1	3.3	1.25	1.78	0.261	7.01	0.39	
(4.45, 0.33)	0.84	0.11	0	0.05	0.49	0.38	0.130	0.033		4.3	2.11	1.63	0.559	3.99	0.67	
	0.83	0.10	0.009	0.065	0.43	0.47	0.110	0.038		3.7	1.59	1.74	0.407	5.20	0.57	0.22
P 2	0.78	0.086	0.048	0.08	0.53	0.30	0.170	0.014	5.3	6.1	3.23	1.83	1.04	2.41	0.47	0.46
(8.67, 0.24)	0.85	0.019	0.086	0.078	0.50	0.31	0.184	0.029		6.2	3.10	1.92	1.14	2.74	0.099	0.75
	0.87	0.039	0.029	0.06	0.55	0.28	0.214	0.027		6.1	3.36	1.71	1.30	2.59	0.23	0.22
P 12	0.88	0.011	0.030	0.083	0.72	0.044	0.24	0.0008	5.0	4.8	3.16	0.211	1.15	2.54	0.52	0.26
(21, 0.69)	0.87	0.024	0.038	0.069	0.71	0.054	0.23	0.0000	4.8	4.7	3.34	0.254	1.09	2.61	0.95	0.35
	0.87	0.004	0.021	0.104	0.69	0.048	0.26	0.0009		4.4	3.04	0.211	1.14	2.86	0.19	0.18
P 28	0.90	0.011	0.011	0.076	0.73	0.068	0.20	0.0063	2.9	2.2	5.61	0.750	0.439	5.6	0.73	0.25
(49.8, 1.84)									*2.4*	*1.8*	*1.24*			*6.74*		
	0.91	0.010	0.035	0.073	0.74	0.077	0.18	0.0026	2.3	2.6	1.92	0.199	0.475	4.72	0.50	0.73
									1.91	*2.2*	*1.47*			*6.20*		
	0.90	0.011	0.011	0.076	0.76	0.079	0.16	0.0061		2.8	2.13	0.219	0.439	4.23	0.50	0.25
										2.3	*1.64*			*5.49*		

P 100	0.87	0	0.021	0.111	0.63	0.014	0.35	0.0000	2.4	2.1	1.32	0.030	0.748	6.58	0	0.28
(287, 9.8)									*2.14*	*1.9*	*1.08*			*8.02*		
	0.87	0.007	0.021	0.104	0.60	0.030	0.37	0.0000		1.8	1.08	0.055	0.660	8.05	1.28	0.32
										1.6	*0.89*			*9.82*		
	0.87	0	0.020	0.110	0.61	0.016	0.37	0.0000		2.0	1.22	0.032	0.738	7.13	0	0.27
										1.8	*1.00*			*8.63*		
F 20.3 (HC)	0.82	0.104	0.0071	0.066	0.45	0.47	0.083	0.046	5.6	5.5	2.48	2.58	0.456	3.31	0.40	0.16
(2.7, 0.21)	0.84	0.076	0.0010	0.069	0.47	0.46	0.068	0.031	6.1	5.4	2.54	2.48	0.367	3.31	0.31	0.03
	0.71	0.130	0.045	0.111	0.46	0.47	0.068	0.043		5.8	2.67	2.73	0.393	2.66	0.48	1.14
F20.4 (T_4)	0.70	0.27	0.011	0.021	0.29	0.63	0.080	0.068	10.6	9.1	2.64	5.73	0.701	2.65	0.47	0.15
(2.8, 0.23)	0.77	0.16	0.018	0.056	0.36	0.57	0.070	0.069		6.7	2.41	3.82	0.516	3.19	0.42	0.35
	0.79	0.19	0.011	0.010	0.38	0.55	0.069	0.085		6.8	2.58	3.74	0.469	3.06	0.51	0.23
F 20.6 (G)	0.62	0.29	0.010	0.083	0.37	0.57	0.064	0.039		10.2	3.77	5.81	0.653	1.64	0.50	0.15
(2.9, 0.22)	0.67	0.24	0.024	0.066	0.38	0.56	0.062	0.056		8.9	3.38	4.98	0.552	1.98	0.48	0.43
	0.62	0.29	0.024	0.066	0.37	0.57	0.060	0.058		8.1	3.00	4.62	0.486	2.07	0.63	0.49

Rats were untreated (above dashed line) or injected (below dashed line) with hydrocortisone (HC), thyroxine (T_4), or glucagon (G), as described in Materials and Methods. Three rat livers were examined in each age or treatment group so that each row across refers to an individual liver. For 28-day old and adult (100-day old) liver, the N_v values corrected for multinucleated cells (in italics under the uncorrected values) report the number of cells; cell volume data calculated with these values are also in italics. V, volume; N, number of nuclei. Superscripts P, H, or K refer to parenchymal, hematopoietic, or Kupffer cells (respectively), and X to unidentifiable space, presumably extracellular. Subscript v indicates that the quantities are expressed per unit volume; subscript n indicates fraction of a variable total number, normalized as 1.0. N_n^{MP} refers to the fraction of parenchymal nuclei in mitosis. N_v homog refers to total number of nuclei per cubic centimeter of liver determined in homogenates.

* F, fetal; P, postnatal.

By the end of gestation the difference between the mean volumes of the two types of cells is even greater so that despite the fact that the *number* of hematopoietic cells per gram is similar to that of parenchymal cells, most of the liver mass (V_v^P = 0.88) is attributable to parenchymal tissue. Neonatal liver still contains a small but significant number of hematopoietic cells, but after the 12th postnatal day the number is too small for accurate determination by the present method. The number of Kupffer cells per cubic centimeter (N_v^K) of liver is significantly greater in adult liver than in fetal liver.

The mitotic rate for parenchymal cells decreases from 11% to about 4% during the last 7 days of gestation (see N^{MP}) and then decreases even more.

The lower part of Table I refers to fetal rats given an injection of hydrocortisone, thyroxine, or glucagon. The two latter hormones had no effect on any of the parameters studied whereas hydrocortisone affected several parameters. Mainly, it decreased the number of hematopoietic cells and increased the volume of parenchymal cells. For a clearer overview, the most important data from Table I are shown graphically in Figs. 1–3.

The increase of parenchymal volume fraction and the decrease of hematopoietic volume fraction with age are depicted in Fig. 1; an injection of hydrocortisone to fetal rats accelerates both processes. Fig. 2 illustrates the biphasic change in the volume of parenchymal cell with age. The sudden rise in volume before term followed by a decrease in volume in the neonatal period is associated, in time at least, with the rapid deposition and depletion of liver glycogen. After a stable period of 10 days, a doubling of cell volume occurs between the 12th and 28th postnatal day. This morphometric determination of mean cell volume does not depend on any assumptions about the shape of parenchymal cells. Calculation from measured cell diameters, that would necessitate such assumptions, was avoided. However, the mean cell diameters were measured independently and are shown in Fig. 2 to illustrate that their change with age is qualitatively similar to that of cell volume and quantitatively about as much as expected for spheroids.

Fig. 3 illustrates the changes in cell number with age. The large decrease in total number of cells per cubic centimeter during late fetal life is largely due to the diminished number of hematopoietic cells. The coincident *decrease* in the number of parenchymal cells results from an increase in the mean volume of these cells (see Fig. 2) and is not in contradiction to the rise in the parenchymal volume fraction which occurs during this time (see Fig. 1). After birth the increased number and, later, the diminished number of cells per unit volume are inversely related to the changes in the volume of parenchymal cells (cf. Figs. 2, 3). Hydrocortisone treatment decreased the number of hematopoietic cells.

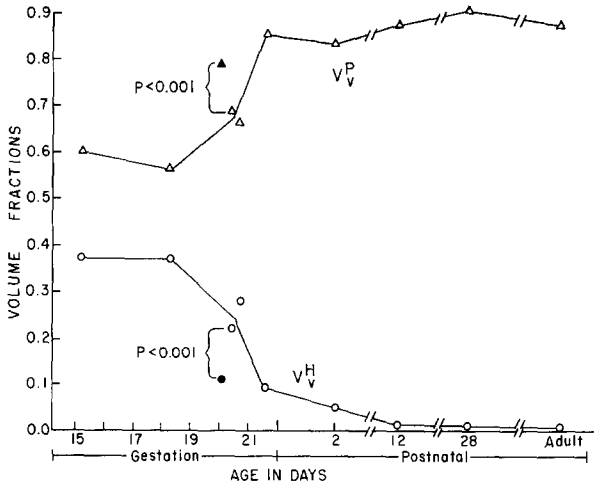

FIGURE 1 The parenchymal (V_v^P, △) and hematopoietic (V_v^H, ○) volume fractions of liver as a function of age. Dark symbols (▲, ●) refer to rats injected with hydrocortisone. Each point is a mean of results with three livers, shown in Table I.

Some of the morphometric parameters for adult liver now studied have been previously determined. The volume fraction for parenchymal cells was reported to be 0.83 (12), which is similar to our value of 0.87. The mean parenchymal cell volume, assuming one nucleus per cell, was reported to be 4940 (12) and 5400 (4) as opposed to our value of 7250, and the number of nuclei ×

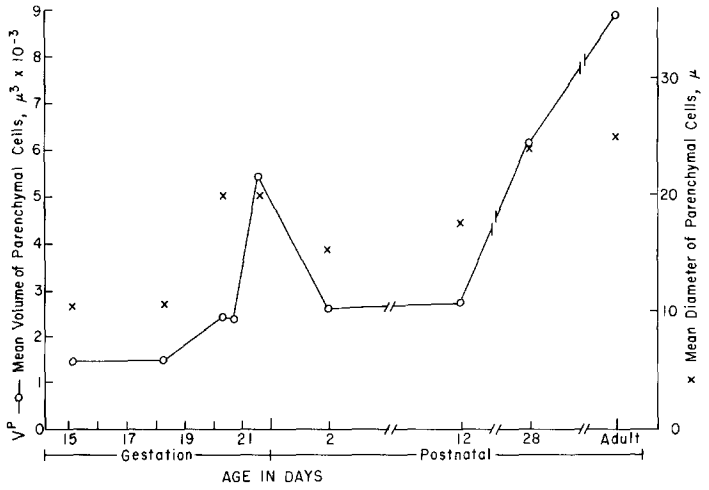

FIGURE 2 The volume of parenchymal cells (—○—) and their mean diameter as a function of age (×). Each point for cell volume (left ordinate) is a mean of results with three livers shown in Table I. Each point for cell diameter (right ordinate) is a mean of 180 measurements (10 cells in six sections from each of three livers of each age).

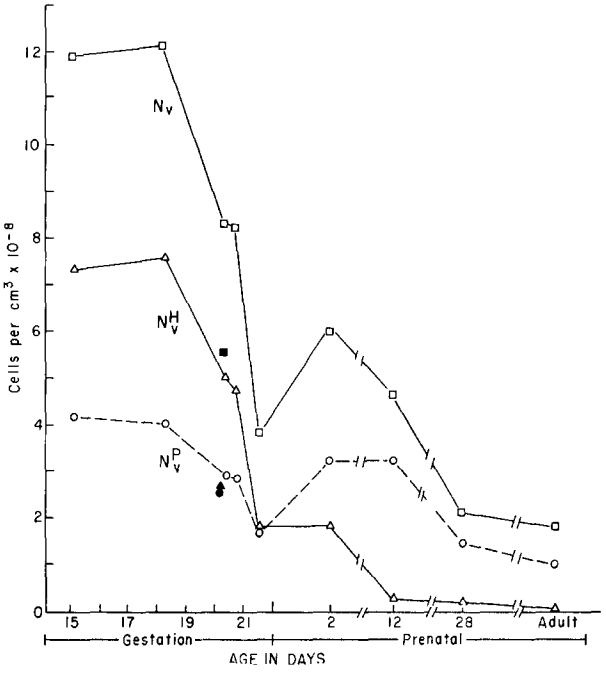

FIGURE 3 The number of total (N_v, □), parenchymal ($N_v{}^P$, ○) and hematopoietic ($N_v{}^H$, △) cells per cubic centimeter of liver during development. Dark symbols (■, ●, ▲) refer to fetal rats injected with hydrocortisone. Each point is a mean of results with three livers shown in Table I. In this and subsequent figures, the italicized (corrected) values of Table I were used for 28-day old and adult livers.

10^{-8} per cubic centimeter of liver was reported to be 1.69 (12) as opposed to our value of 1.2. These discrepancies indicate a larger cell volume in our material and may be due to preparative techniques but are more likely to be due to genuine strain, age, or dietary differences. The larger cell volume is borne out by the appropriately larger average cell diameters found by us, 24 μ (in Fig. 2) as opposed to 17.7 μ according to Weibel et al. (12), i.e., by a measurement independent of the morphometric determination of volume fractions used to calculate the actual cell volumes.

Figs. 4–8 illustrate applications of the new morphological information to the analysis of enzyme concentrations. Glycogen synthetase (EC 2.4.1.11) exemplifies one of the several enzymes that rise in concentration during fetal life. Let us assume that this enzyme is restricted to hepatocytes. During the fetal period covered in Table I the significant involution of hematopoietic mass (V_v^H = 0.37 to 0.09) is associated with a less than twofold rise in parenchymal mass (V_v^P = 0.60 to 0.85), so that the rise of glycogen synthetase per gram of hepatocyte is very similar to that per gram of total liver (Fig. 4). The enzyme also increases, severalfold, per parenchymal cell. Its rise in the liver, therefore, cannot be attributed simply to more parenchymal cells. The prenatal upsurge of such enzymes clearly reflects the changing enzymic composition of parenchymal tissue and not the changing cellular composition of liver.

After the second postnatal day the concentration

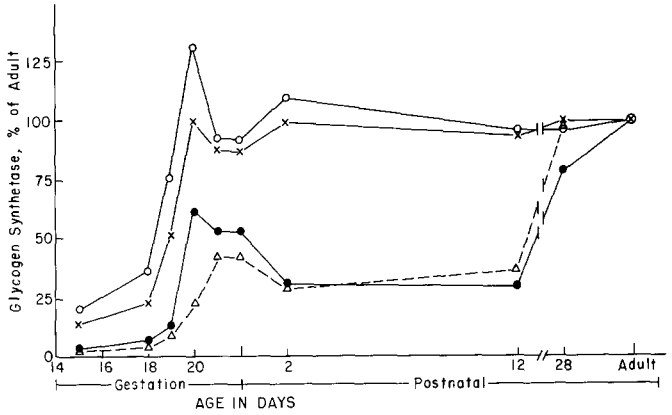

FIGURE 4 Developmental formation of glycogen synthetase in rat liver. Units of glycogen synthetase per gram of liver (13) (×) were divided by V_v^P to obtain units per cubic centimeter parenchymal tissue (○), by N_v to obtain units per cell (△) and by N_v^P to obtain units per parenchymal cell (●). They are all expressed as per cent of the corresponding adult value.

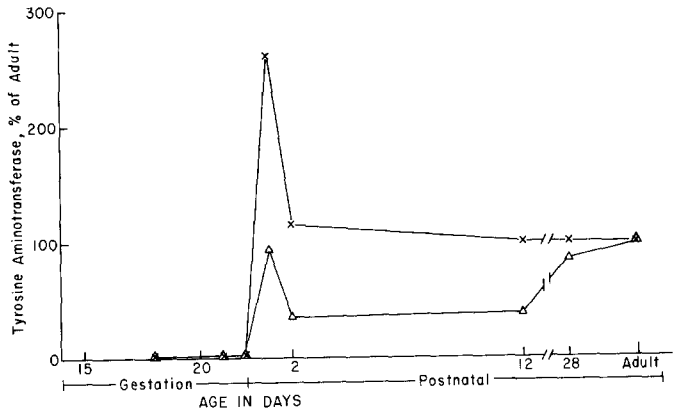

FIGURE 5 The neonatal emergence of liver tyrosine aminotransferase. Enzyme units are expressed per gram of liver (14) (×) and per cell (△).

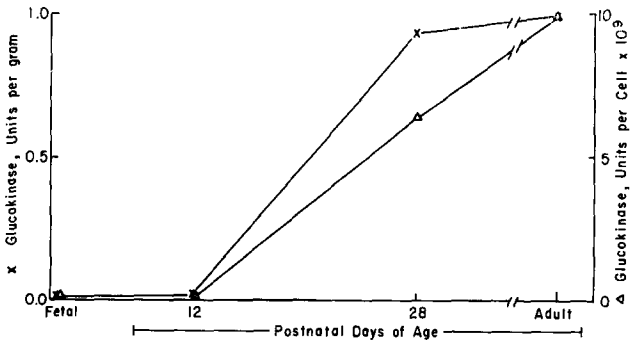

FIGURE 6 The emergence of glucokinase during the late suckling period. Enzyme units are expressed per gram (15) (×) and per cell (△).

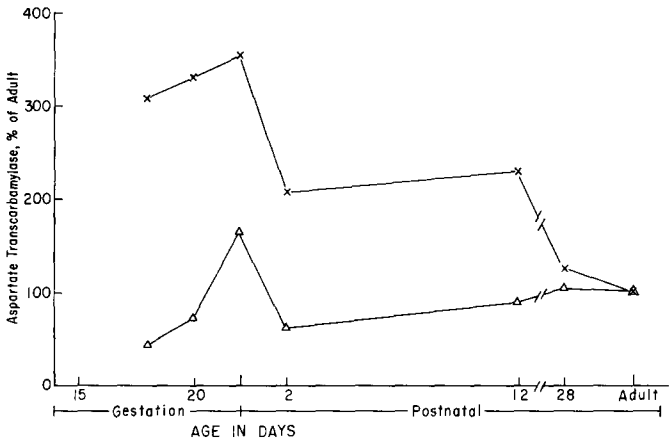

FIGURE 7 Changes in aspartate transcarbamylase with age. Enzyme units are expressed per gram of liver[2] (×) and per cell (△).

of glycogen synthetase remains constant per gram but between the 12th and 28th day the enzyme increases per cell in parallel with the increase in mean cell volume.

Enzymes of the neonatal cluster rise precipitously (per gram of liver or per cell) immediately after birth. The enzyme used to illustrate this phenomenon (tyrosine aminotransferase, Fig. 5) also undergoes a second rise per cell (and remains constant per gram of liver) between the 12th and 28th postnatal day.

For enzymes of the late suckling cluster, which are undetectable before the 12th postnatal day, the rise per gram of liver is as striking as the rise per cell. In Fig. 6, for example, glucokinase (EC 2.7.1.12) reaches its adult concentration before the 28th day, but the amount of it per cell continues to rise.

Enzymes with stable or falling concentrations per gram of liver in the prenatal period may appear to increase per cell, due to the decrease in cell number during fetal life. One such enzyme is aspartate transcarbamylase (EC 2.1.3.2) (Fig. 7). There is no reason to assume that this enzyme is concentrated in hematopoietic tissue (since its concentration does not diminish with the normal or hydrocortisone-stimulated involution of this tissue), so that its rise per cell in the prenatal period must reflect an enrichment of the parenchymal cells. The enzyme clearly decreases (per gram or per cell) in the neonatal period, and, unlike other enzymes (Figs. 4, 5, 8), it shows no rise per cell when the cell volume doubles between the 12th and 28th postnatal day.

Phosphoserine phosphohydrolase (EC 3.1.3.3) may in part be associated with hematopoietic cells: the same hydrocortisone treatment which reduced the hematopoietic volume fractions (from 0.22 to

[2] Herzfeld, A., and W. E. Knox. Unpublished.

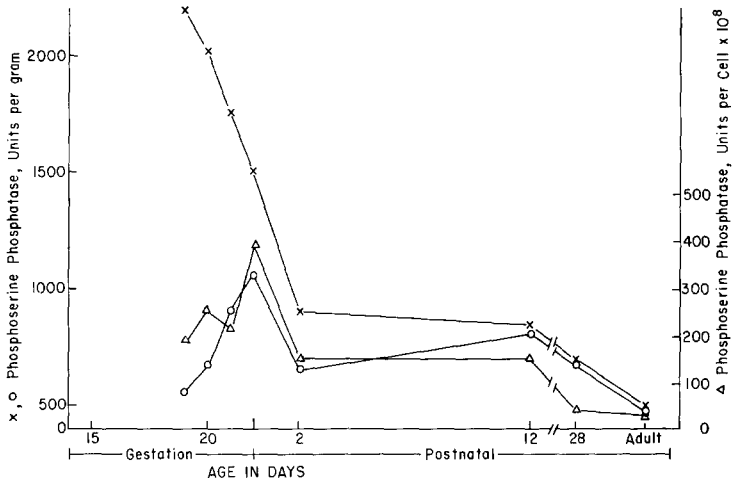

FIGURE 8 Developmental changes in the amount of phosphoserine phosphatase in rat liver. Crosses (×) refer to concentration (units per gram of liver) (16). These values minus the enzyme units in the hematopoietic tissue (calculated with the assumptions explained in the text) give units per cubic centimeter of parenchymal tissue (—○—). Triangles (△) refer to total enzyme units per cell.

0.103, see Fig. 1) also reduced the concentration of phosphoserine phosphohydrolase from 1870 to 1210 units (15). If we assume that this reduction of enzyme concentration in liver was entirely due to the involution of hematopoietic tissue (i.e., that each 0.1 cm^3 of this tissue contains 600 enzyme units), we can calculate, by difference, the amount of enzyme in the parenchymal portion of each gram (or cubic centimeter) of liver throughout development. The resultant curve (open circles in Fig. 8) shows that the amount of the enzyme in the parenchymal tissue in the prenatal period may increase while its concentration in the liver as a whole decreases.

DISCUSSION

Cytological descriptions of tissues often fail to clarify whether "more" of a certain type of cell, at one developmental stage compared to another, refers to absolute or relative cell numbers or to the contribution of this component to tissue mass. To take an extreme example, Kupffer cells represent less than 3% of the total liver volume (see V_v^K) in both adult and fetal livers. They also represent 4% of the total number of cells in fetal liver (see N_n^K), but in adult liver (due to the large volume and consequent low frequency of parenchymal cells) they represent 37% of the number of cells. While this rise with age in relative number is almost 10-fold, the rise in absolute number (i.e., per unit volume) is only 1.3-fold (see N_v^K). This example merely illustrates the necessity for specifying the basis on which "more" or "less" cells are to be judged.

The present studies represent an effort to translate known cytological features of developing liver into quantitative terms like "concentration" so that they can be correlated with the parameters of biochemical differentiation in a meaningful manner. Since enzymic differentiation in liver has been recognized to proceed most actively during the late fetal, the neonatal, and the late suckling periods (2), cytological changes which occur at these times are of prime importance.

The Late Fetal Period

It has often been suggested that observed increases in enzyme concentrations in mammalian liver around the time of birth reflect "merely" an increase in the proportion of hepatocytes at the expense of hematopoietic tissue. With the data now obtained, this question is no longer a matter of debate but one of simple calculation. During the last 4 days of gestation, the fraction of liver volume occupied by parenchymal hepatocytes nearly doubles. Thus the concentration of an enzyme (assumed to be located in parenchymal cells) that only doubles per gram of liver during this period remains unchanged per gram of parenchymal mass. However, the dozen or so

enzymes (see a typical example in Fig. 4) that constitute the late fetal cluster (2) rise much more than twofold during this period. This clearly represents the differentially increased synthesis of a group of enzymes, resulting in significant changes in the biochemical nature of the hepatocyte. Since the number of hepatocytes per gram of liver at most doubles during late gestation, any enzyme that more than doubles per gram of hepatic tissue also increases per parenchymal cell.

The hormones that have so far been found to alter the course of enzymic differentiation in fetal liver were the ones that were tested for their possible effects on the morphological features of this tissue.

Glucagon and thyroxine, which induce prematurely the formation of several enzymes in fetal liver, did not alter the cytological parameters now quantified. Their effects on enzymes of the same liver are, however, considerable. For example, an injection of glucagon to fetal rats raises the level of tyrosine aminotransferase from insignificant to near adult values, and an injection of thyroxine more than doubles the level of glucose-6-phosphatase (1). One can, therefore, conclude that the enzymic changes did not result from alterations in the proportions of cell types. Instead, the proportions of components within the cells changed. There may be structural counterparts within the cell of some of these biochemical changes: the electron microscope morphometry of subcellular organelles during the normal and induced enzymic differentiation of hepatocytes is currently under study.

It has been suggested by Nagel (17) that an increase in glucocorticoid secretion accelerates the maturation and consequent involution of hematopoietic tissue of fetal liver. Our results are in accord with this suggestion and show that livers of fetal rats given an injection of hydrocortisone lose half of their hematopoietic cells; there is a corresponding rise in the parenchymal volume fraction. An approach is thus available for testing, indirectly, the cellular localization of biochemical constituents: enzymes which decrease in liver during late gestation, and decrease more extensively upon an injection of hydrocortisone, may well be concentrated in the hematopoietic tissue. Phosphoserine phosphatase provides an example of this type of enzyme.

The Neonatal Period

At birth there is already very little hematopoietic tissue in rat liver and its further diminution during the neonatal period is insignificant in relation to changes in enzymic concentrations. The mean volume of parenchymal cells is the only cytological parameter that varies strikingly: its increase just before birth, concurrently with extensive glycogen deposition, is followed by a 50% decrease during the neonatal depletion of liver glycogen. Thus, during the first 2 postnatal days, a less than twofold increase in the concentration of some preexisting enzymes may not reflect any increase per cell. However, the precipitous rise of enzymes of the neonatal cluster, i.e., those that are undetectable in the prenatal period (see example in Fig. 5), reflects, of course, major increases per parenchymal cell as well as per parenchymal mass.

The Late Suckling Period

The period between the 2nd and 12th postnatal day is relatively uneventful from the point of view of enzymic differentiation (2), and the present studies show that cytological changes are also minimal during this time. The subsequent period (age 12–28 days) of rapid enzymic differentiation is accompanied by a sudden increase in the volume of parenchymal cells. However, enzymes of the late suckling cluster, which are undetectable before the 12th day, rise just as strikingly per gram as per cell (see typical example in Fig. 6). Enzymes of earlier clusters have stable concentrations per gram of liver during this period, but they also increase per cell (see Figs. 4, 5). It seems to be a frequent occurrence that enzymes increase in concentration in cells at least in proportion to the increase in cell size.

The Bases of Expressing Enzyme Concentrations

The vast majority of publications express enzyme amounts per unit weight of tissue (i.e., as concentration). Cells or milligrams DNA are frequently advocated as more "physiologically meaningful" bases for comparison, and these are unsound only if their own changing concentrations in the tissues are not recorded. However, even the interpretive value of such tissue bases is uncertain. In the case of liver enzymes located in

parenchymal cells, the large *number* of hematopoietic or Kupffer cells at certain stages of development can introduce major, physiologically irrelevant alterations in the denominator of such a ratio. For example, the sudden diminution of hematopoietic cells shifts the curve of glycogen synthetase per cell (see Fig. 4) so that it peaks two days later than the curves calculated on the parenchymal cell (or mass) basis. The only reasonable procedure is to record primary concentrations (i.e., per gram) for both constituents (enzymes, total protein, dry weight, etc.) and cytological parameters (number of cells, volume attributable to cell types, etc.); any derived concentrations (i.e., ratios of enzyme to cell number, DNA, or dry weight, etc.) can then be used for interpretive purposes (18) with clear knowledge of the shifting tissue composition.

Consideration of enzyme amount per cell of the type which actually contains the enzyme is highly relevant to our understanding of the cell as an economic unit. Since the size of the nucleus does not change much during an increase of cell volume one could assume that the metabolic requirements of the nucleus remain constant while those of the cytoplasm increase. To maintain unchanged function, the enzymic potential for the synthesis or catabolism of cytoplasmic constituents may increase per enlarging cell (i.e., remain constant per unit volume of cytoplasm) while enzymes concerned with the nucleus should remain constant per cell (i.e., decrease per gram of tissue when cell volume increases). Several enzymes which emerge during late fetal or neonatal life remain constant per gram (i.e., increase per cell) during the postnatal doubling of the parenchymal cell volume (e.g., glycogen synthetase, tyrosine aminotransferase, and glucokinase in Figs. 4–6), suggesting that they are concerned with the maintenance of cytoplasmic functions. On the other hand, enzymes which are high in fetal liver and may be necessary for proliferation might be expected to remain low per cell after the establishment of minimal rates of mitosis. Aspartate transcarbamylase, an obligatory enzyme in pyrimidine synthesis, remains constant per cell between the 12th and 28th postnatal day, suggesting that more cytoplasm may not necessitate more of the functions subserved by this enzyme. With many more quantitative enzyme studies in different instances of cell growth, one will begin to be able to distinguish metabolic requirements determined by cytoplasmic mass from those determined by the cell as a functional unit.

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