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HETEROGENEITY OF ESTERASES AND CELL TYPES IN RAT LIVER

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SUMMARY

In rat liver, six bands of esterase activity with β-naphthyl acetate were detected by electrophoresis on the cellulose acetate membrane.

By comparing the esterase zymograms from adult liver, lymph nodes, spleen, intestinal mucosa, and hepatocyte fraction, it is apparent that Bands 3 and 6 are derived from hepatocytes, while Bands 2, 4, and 5 are mainly localized in hepatic reticuloendothelial cells. Band 1 was obscure in adult liver.

During the development of rat liver, Band 1 was detectable from the prenatal upto the 2 weeks postnatal liver and then it disappeared after an increase in the intensity of Band 3. Band 6 appeared 2 weeks after birth showing a higher activity than in adult.

INTRODUCTION

Multiple molecular forms of esterases have been demonstrated in liver by electrophoresis on starch[1–4] and acrylamide gels[5,6]. However, there is very little information about the significance of individual isozymes in the liver tissue.

On the other hand, it has been proposed from the study on acid phosphatase isozymes that the heterogeneous cells in liver tissue, such as parenchymal hepatocytes and hepatic reticuloendothelial cells, possess different isozyme patterns[7].

The present study deals with the relationship between the heterogeneity of esterases and cell types which constitute the liver tissue, and also with the alteration of isozyme patterns during the development of liver to adult stage.

MATERIALS AND METHODS

Male Wistar rats weighing approx. 200 g were used. Fetal and newborn rats were also used. Rats were killed by decapitation and the organs were removed and homogenized in 0.25 M sucrose solution, to make 20% (w/v) of the homogenate. The organs used in this study were liver, lymph nodes, spleen, and intestinal mucosa

Abbreviation: PCMB, *p*-chloromercuribenzoate.

which was obtained by scraping a portion of the intestine. The hepatocytes isolated from the liver tissue[8] were also homogenized.

The soluble fractions for electrophoresis were prepared by centrifugation of the individual homogenates treated by the addition of 0.25% Triton X-100 (at 25 000 × *g* for 20 min).

Assay of esterase activity

The esterase activity was determined with β-naphthyl acetate as substrate, by the slightly modified method of Nachlas and Seligman[9]. In this study, instead of using a stabilized tetrazonium salt for the colorimetric measurement at 540 nm, the amount of β-naphthol released was determined directly at 328 nm at room temperature (approx. 24 °C) at intervals of 30 s for 10 min. 1 unit of esterase activity is defined as the amount of enzyme which hydrolyzes 1 μmole of substrate in 1 min.

Determination of protein

The protein content was determined according to the method of Lowry *et al.*[10], using bovine serum albumin (Armour) as a standard.

Electrophoresis

Electrophoresis on the cellulose acetate membrane (Sepraphore III, Gelman, 2.5 cm × 17 cm) was carried out using Veronal buffer (pH 8.6, $I = 0.07$) at 250 V for 2 h at 10 °C.

After electrophoresis, the strips were stained on the filtered Noble agar (Difco) solution (1%) containing 0.025 M Veronal buffer (pH 7.2), 0.4 mg/ml β-naphthyl acetate dissolved in 0.04 ml of acetone and 0.5 mg/ml Fast Blue RR. Incubation time was about 10 min at 37 °C.

The stained membrane was transparent after immersion in decahydronaphthalene and was scanned by a densitometer at 540 nm.

In inhibition studies, the inhibitor was incorporated into the reaction medium. For the spectrophotometric determination, diisopropylphosphorofluoridate (DFP) was used at a concentration of $1 \cdot 10^{-5}$ M. For the staining of enzyme activity after electrophoresis, DFP ($1 \cdot 10^{-4}$ M), eserine sulfate ($1 \cdot 10^{-4}$ M), and *p*-chloromercuribenzoate (PCMB, $1 \cdot 10^{-3}$ M) were used.

RESULTS

Esterase patterns of rat liver on cellulose acetate membranes

Six bands of esterase activity were detected by electrophoresis on cellulose acetate membranes as shown in Fig. 1. Among these bands, the far anodic band designated as Band 1 was a very faint band in adult liver. Bands 2 and 3 existing near the origin overlapped under our conditions, but these bands were distinguished by DFP inhibition, as shown in Fig. 3. Bands 4 and 5 occurred on the cathodic side. Band 6, moving farthest toward the cathode, corresponded to the albumin position.

Effect of DFP, eserine, and PCMB on the esterase activity

When the esterase activity on cellulose acetate membrane was scanned by a densitometer at 540 nm, three zones were obtained. The first zone at the origin con-

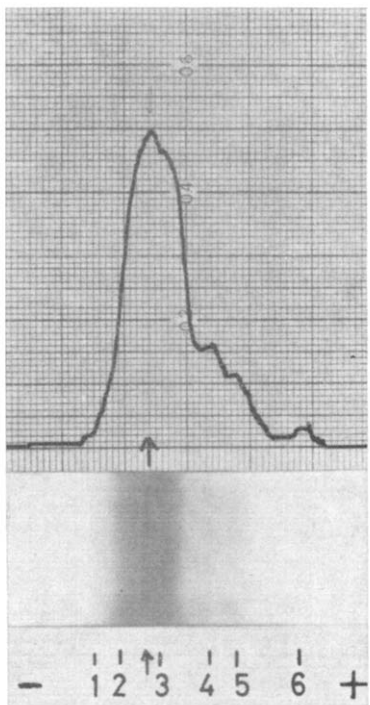

Fig. 1. Electrophoretic pattern of rat liver esterase on the cellulose acetate membrane and the corresponding densitometric recording. The arrows indicate the origin.

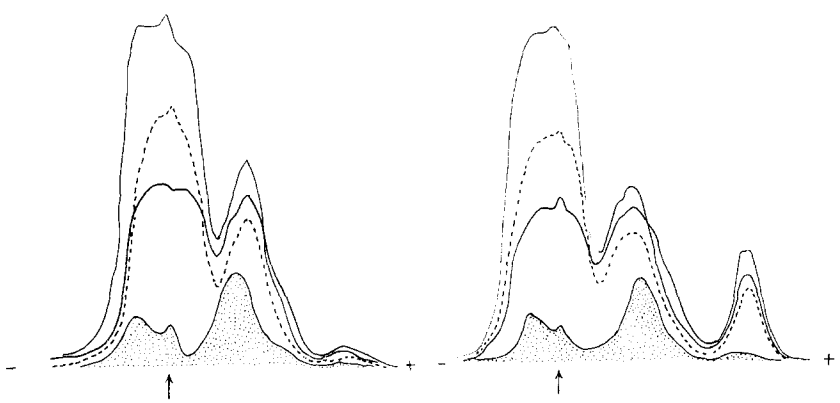

Fig. 2. Effect of inhibitors on the electrophoretic pattern of esterase activity in normal liver (left graph) and in the liver of a rat fed 3′-methyl-4-dimethylaminoazobenzene for 6 weeks (right graph). ———, control; dotted area, DFP ($1 \cdot 10^{-4}$ M); ---------, eserine ($1 \cdot 10^{-4}$ M); ———, PCMB ($1 \cdot 10^{-3}$ M).

tained Bands 1, 2, and 3. The second zone consisted of Bands 4 and 5, and the third small zone at the cathodic side corresponded to Band 6. When the same volume of liver extract was applied on the cellulose acetate membrane and stained by the medium containing one of the inhibitors, electrophoretic patterns changed with each inhibitor used (Fig. 2).

As shown in Fig. 2 (left graph) DFP strongly inhibited the first and third zones, but inhibited the second zone slightly. In the first zone, DFP inhibition of Band 2 was less than that of Band 3. This zymogram is shown in Fig. 3. Eserine inhibited the second and third zones, while it did not inhibit the first zone. PCMB activated the first and/or second zone, and inhibited the third slightly. Although Band 1 is hardly detectable in adult liver, so far as we determined in the intestinal mucosa, it was very sensitive to DFP and resistant to eserine and PCMB.

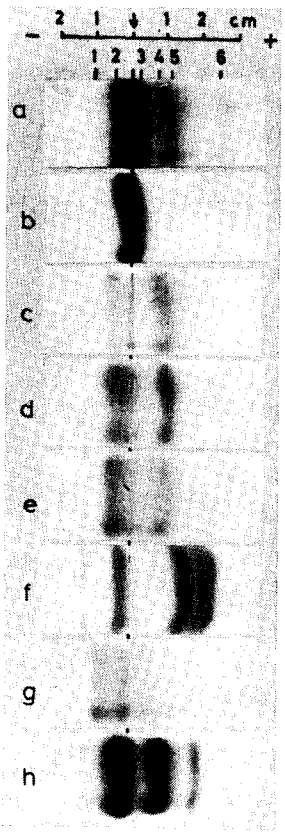

Fig. 3. Zymograms of esterases in various rat tissue extracts on cellulose acetate membranes. (a) Adult liver, (b) hepatocyte fraction, (c) adult liver with addition of DFP ($1 \cdot 10^{-4}$ M), (d) lymph node, (e) spleen, (f) intestinal mucosa, (g) fetal liver, (h) hepatoma, induced by 3'-methyl-4-dimethylaminoazobenzene. The arrow indicates the origin.

Fig. 2 (right graph) shows the pattern of the liver of a rat fed 3'-methyl-4-(dimethylamino)azobenzene for 6 weeks. Since the third zone appeared with high intensity in this liver, it was possible to elucidate the effect of inhibitors on Band 6. The third zone was strongly inhibited by DFP, and slightly by eserine and PCMB.

Isozymatic patterns of esterases in various tissues and hepatocytes

As shown in Fig. 3, the esterase zymogram in the hepatocyte Fraction (b) consisted mostly of Band 3 and partly of Band 6. When the membrane strip applied with

adult liver extract was stained by a medium containing DFP (c), Bands 3 and 6 disappeared, and this pattern resembled those of lymph nodes (d) and spleen (e). From these results, Band 3 and also Band 6 appear to be derived from hepatocytes and these isozymes were inhibited strongly by DFP. On the other hand, Bands 2, 4 and 5 seem to originate from nonhepatocytes, mainly from hepatic reticuloendothelial cells.

The pattern of intestinal mucosa (f) was different from those of lymph nodes and spleen. It showed Band 6 with strong intensity and apparent presence of Band 1, and these two bands seem to be related to fetal (g), neonatal liver, and hepatic cancer (h). Another strong band near Band 6 in intestinal mucosa was not detected in liver (a), but in serum. This band was sensitive to DFP and resistant to eserine.

Table I shows the enzyme activities and the effect of DFP on various tissue and hepatocytes. The specific activities of esterase in hepatocytes and intestinal mucosa were higher than that of adult liver tissue, whereas the activities of lymph nodes, spleen, and fetal liver were lower. The esterase activities in adult liver, especially hepatocytes, and intestinal mucosa were sensitive to DFP, while those in lymph nodes, spleen, and fetal liver were resistant to this inhibitor.

TABLE I

ESTERASE ACTIVITIES IN VARIOUS TISSUES AND HEPATOCYTE FRACTION

Mean values ± S.D.

Tissue	*No. of rats*	*munits/mg protein*	*Activity with DFP ($1 \cdot 10^{-5}$ M) (%)*
Adult			
Liver	5	444.2 ± 24.72	42.9 ± 4.19
Intestinal mucosa	5	1262.6 ± 233.29	24.5 ± 3.56
Lymph nodes	2*	66.2 ± 9.04	97.6 ± 2.40
Spleen	3	58.4 ± 7.52	87.6 ± 10.03
Hepatocyte fraction	3	732.0 ± 38.11	25.0 ± 0.64
Fetal liver	3 (litters)	94.7 ± 22.52	81.6 ± 9.10

* In each experiment, 4 lymph nodes were pooled.

Changes of esterase isozymatic pattern in developing liver

Fig. 4 shows the total esterase activity in developing liver. The activity per g of liver weight increased gradually upto 4 weeks after birth and then it leveled off. The liver weight in these stages increased gradually. The average weight of neonatal liver after 1, 2, 3, 4 and 5 weeks, and of adult liver were about 0.3, 0.5, 1.2, 2.0, 2.4 and 6.3 g, respectively. Upto 3 weeks of postnatal age, the esterase activity was hardly inhibited by DFP, thereafter DFP inhibition increased to adult stage. This fact indicates that, in developing liver, esterase isozymes altered qualitatively and quantitatively.

Fig. 5 shows the scanning pattern of esterase. Band 1 of esterase isozyme was observed in the liver from fetus to 1 week after birth, but Band 6 was not detected in them (a–d). After 2 weeks, diminishing of Band 1 and an increase in the volume of the first zone, band 6 was observed. Thereafter, Band 6 occurred more distinctly in the liver of 4 and 5 weeks postnatal age (e, f). This can be seen also in Fig. 6, which indicates the esterase activity in isozymes separated by electrophoresis. Though these

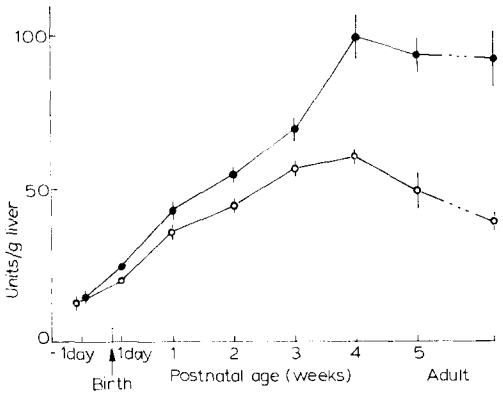

Fig. 4. Esterase activity in the developing rat liver. ●—●, total esterase activity; ○—○, DFP ($1 \cdot 10^{-5}$ M) resistant esterase activity. The vertical bars represent the range of the standard deviation. The value for fetal liver is the mean of 3 litters. The value of 1 day after birth represents the mean of duplicate experiments with the pooled livers of five rats. The values after 1 week represent the means of 3–4 animals.

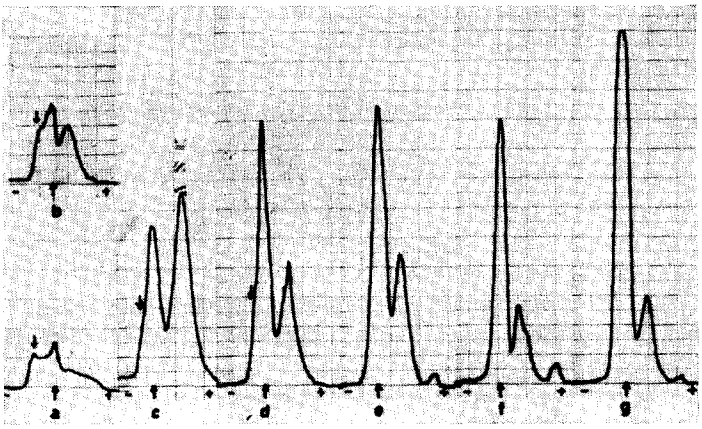

Fig. 5. Electrophoretic pattern of esterase in the developing rat liver. (a) Fetal liver (1 day before birth), (b) newborn, 1 day; (c) 5 days, (d) 1 week, (e) 3 weeks, (f) 5 weeks, (g) adult liver. Total esterase activity applied on the membranes (munits): (a) 8, (b) 21, (c, d, e, f, g) 40–45. ↑ indicates the origin; ↓ indicates the position of Band 1.

values were roughly calculated by a densitometer, it appears that the activity (per ml of liver extract) in the second zone (Bands 4 and 5) was not changed through the developing stage, and an increase in the activity of Band 3 is correlated with the development and differentiation of hepatocytes.

DISCUSSION

Though evidence exists that many enzymes occur in multiple molecular forms, the significance of this heterogeneity is not, as yet, apparent. As for esterases of rat liver, the existence of multiple forms has been demonstrated under various condi-

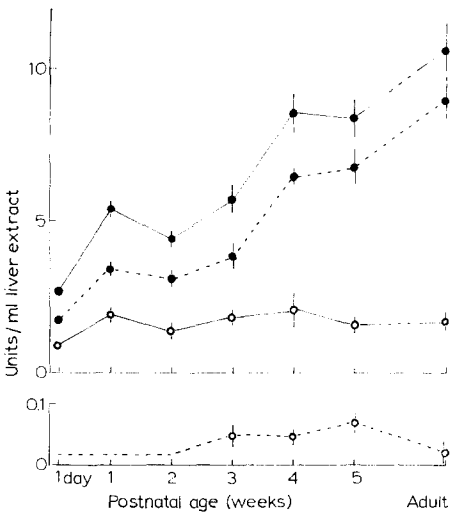

Fig. 6. Densitometric determination of esterase activities in developing liver separated by electrophoresis. ●—●, total esterase activity; ●---●, activity in the first zone; ○—○, activity in the second zone; ○---○, activity in the third zone. Each point, after 1 week of postnatal age, represents the mean and standard deviation of 3–4 animals. The value of 1 day after birth was determined with the pooled livers of five rats.

tions[1–6], but scarcely anyone paid attention to the heterogeneity in the cellular population of the hepatic tissue.

The present study has been performed with a different method from the others in that a cellulose acetate membrane for the electrophoresis and extraction by Triton X-100 to solubilize the esterases bound with the endoplasmic reticulum[11] were used. The zymogram of liver on the cellulose acetate membrane merely presented six bands of esterase activity with β-naphthyl acetate and this smaller number of bands seemed to be rather convenient to study the interrelationship between individual esterase forms and cell types.

From the effect of DFP and eserine[5,6,12], it appears that the esterases represented by Bands 1, 3 and 6 are in the category of carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1), while the other forms (Bands 2, 4, and 5) belong to the arylesterase (aryl-ester hydrolase, EC 3.1.1.2). The effect of PCMB suggests that Bands 3 and 6 are not essentially of the same type, since only Band 6 was slightly inhibited by PCMB. The definite explanations about number and properties of these esterases await the isolation of individual esterases.

From the DFP sensitivity and esterase zymograms, it may be concluded that Bands 3 and 6 belong to hepatocytes and Bands 2, 4 and 5 are related to the reticuloendothelial cells. Further, it is considered that the DFP-sensitive carboxylesterases are mainly located in the hepatocytes while the arylesterases are preferentially located in the reticuloendothelial cells in the liver. In this connection, Band 1 appears to be rather related to hepatocytes. These considerations seem to be supported by the autoradiographic study of Barnard *et al.*[13] on mouse liver. They observed that the caroboxylesterase sensitive to [^{3}H]DFP was concentrated in the hepatocytes, especially in the endoplasmic reticulum, and further, few grains of [^{3}H]DFP were in the reticuloendothelial cells, erythrocytes or leucocytes.

During liver development, the reticuloendothelial cell type of esterase which is represented by Bands 4 and 5 was hardly changed. This appears to agree with the result by Holmes and Masters[6] who showed a relatively little change in the arylesterase during the development stage, in spite of an increase in the carboxylesterase.

On the other hand, Bands 1, 3 and 6 showed a significant alteration during the development. Band 1 was demonstrated on the zymograms of livers from prenatal to 2 weeks postnatal and then it disappeared, while Band 6 was detected 2 weeks after birth and this activity in infant liver was higher than that in adult liver. The activity of Band 3 increased upto the adult stage. Therefore, it may be concluded that among these bands, Band 1 of esterase is the most immature or fetal type, Band 6 is infant type and Band 3 is adult type.

Ontogenetically, it is interesting that the intestine and the fetal and neonatal livers, both of which originate from the foregut, possess Band 1 and 6. In addition, both bands were detected in hepatic cancer and also Band 6 was increased in the precancerous liver.

The results showed that the different types of cell in the liver display the different forms of esterase as well as acid phosphatase (EC 3.1.3.2)[7] and also a possibility to elucidate the grade of cell differentiation by these patterns.

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