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Studies of liver phosphorylase in hepatic injuries II. Alteration in isozyme pattern

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Michio Kobayashi

Abstract

Enzyme deviations in injured livers were studied by analyzing isozyme patterns of phosphorylase using a newly developed electrophoretic method, which separates six molecular species of this enzyme, i.e. M,FM,F,L,L', and FL'. In hepatic injuries caused by CCl4 and galactosamine intoxications of rats, F appeared in early stages and L' (and FL') in later stages of the injuries with a concurrent decrease or loss of L, which is a sole isozyme component of adult liver. In injured livers of patients with hepatitis and cirrhosis of the liver, increases in FL' activity were also found. Appearance of F was found only in hepatocellular carcinoma. The results obtained with phosphorylase isozyme analysis support the idea that an undifferentiated gene expression takes place in the injured livers of non-malignant hepatic disorders.

KEYWORDS: phosphorylase isozyme pattern, carbon tetrachloride liver injury, galatosamine liver injury, partial hepatectomy, AH 130 asctites hepatoma

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STUDIES OF LIVER PHOSPHORYLASE IN HEPATIC INJURIES
II. ALTERATION IN ISOZYME PATTERN

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Abstract. Enzyme deviations in injured livers were studied by analyzing isozyme patterns of phosphorylase using a newly developed electrophoretic method, which separates six molecular species of this enzyme, *i.e.* M, FM, F, L, L′, and FL′. In hepatic injuries caused by CCl_4 and galactosamine intoxications of rats, F appeared in early stages and L′ (and FL′) in later stages of the injuries with a concurrent decrease or loss of L, which is a sole isozyme component of adult liver. In injured livers of patients with hepatitis and cirrhosis of the liver, increases in FL′ activity were also found. Appearance of F was found only in hepatocellular carcinoma. The results obtained with phosphorylase isozyme analysis support the idea that an undifferentiated gene expression takes place in the injured livers of non-malignant hepatic disorders.

Key words: phosphorylase isozyme pattern, carbon tetrachloride liver injury, galatosamine liver injury, partial hepatectomy, AH 130 asctites hepatoma

Since the discovery by Wosilait and Sutherland (1) that the glycogen phosphorylase (PLase; 1, 4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2. 4. 1. 1) in liver differs from that of skeletal muscle, at least three isozymes of PLase, namely fetal, muscle and liver types (2) or I, III and L, respectively, and two hybrids, II and LI (3), have been demonstrated in mammalian organs. Polyacrylamide gel disc electrophoresis with or without added glycogen in the separating gel is commonly used for separation of those PLase isozymes. Gels with glycogen separate muscle type from liver type without yielding the hybrid bands (2, 4). Gels without glycogen, which separate hybrid molecules from others, fail to resolve muscle and liver PLase's (3, 5). The author established a new method of distinguishing all of those molecular species in one run of electrophoresis on a gel form of cellulose acetate membrane with a discontinuous buffer system (6). The present study was undertaken with the newly developed technique of PLase isozyme separation in an attempt to demonstrate isozyme alteration in livers of hepatotoxin-intoxicated rats and of patients with liver diseases.

Sato and others (2) reported that fetal type PLase appeared commonly in

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various rat hepatomas, together with variable activities of liver type, depending on the degree of differentiation. The appearance of fetal or prototype isozymes of other carbohydrate metabolizing enzymes in injured livers has been well documented by Taketa and others (7-9). These results led us to postulate that the fetal PLase may resurge in injured livers. The present communication deals with the results which substantiate the above assumption. In addition, the disappearance of adult type liver PLase concomitant with the appearance of another fetal liver or adult spleen type of PLase is described.

MATERIALS AND METHODS

Experimental conditions of rats and clinical data of patients with various liver diseases were given in a previous report (10), unless otherwise stated. Fetal livers obtained from a 21-day pregnant rat were pooled and processed identically. Enzyme extracts for elecrophoretic separation of PLase isozymes were prepared according to the method of Sato and others (2) with the following slight modifications*: when tissues contained no appreciable amounts of glycogen, supernatants ($105{,}000 \times g$, 45 min) were directly subjected to electrophoresis; and when tissues contained considerable amounts of glycogen, precipitates ($105{,}000 \times g$, 45 min) were suspended in the homogenizing medium described previously (10), treated with human salivary α-amylase and centrifuged for 45 min at $105{,}000 \times g$, the resulting supernatants being used for electrophoresis. Either procedure gave identical PLase isozyme patterns. For tissues with low PLase activities and negligible glycogen contents, such as those of rat and human spleen and AH 130 ascites tumor cells, PLase was partially purified by passing the supernatant through a column of 5′-AMP Sepharose (11) and concentrated with a Collodion bag (Sartorius, Goettingen).

The enzyme extracts having PLase activities of about 0.01-0.03 units (one unit was defined as one μmole of substrate reacted per min at 37°C) were applied per 1 cm onto a Cellogel (Chemetron, Mirano) membrane (10 cm bridge distance) at 1 cm distance from the cathodic end. The anodic and membrane buffers consisted of 0.38 M glycine-Tris (0.38 M with respect to glycine), pH 8.3, and the cathodic buffer 0.035 M asparagine-Tris (0.035 M with respect to asparagine), pH 7.3. Electrophoresis was performed in a cold room (2-4°C) at a constant voltage of 48 V/cm for 2 h. After electrophoresis, the membrane was incubated for 30 min at 37°C in a mixture of 40 mM Tris-maleate buffer, pH 6.1, 2% glycogen, 40 mM G-1-P (disodium salt) and 5 mM EDTA (disodium salt*), and with 3 mM AMP and 0.75 M sodium sulfate (Na_2SO_4) unless otherwise indicated. For human liver PLase with limited total activities available, Cellogel membranes after electrophoresis were placed by inverting the surface down onto a 1% agar plate containing the above constituents except that Na_2SO_4 concentration was reduced to 0.38 M with identical results, and incubated for two to three h. Newly synthesized glycogen was developed by the method of Takeo *et al.* (4).

* Personal communication from K. Sato.

RESULTS

Isozyme pattern of rat tissues. Heart muscle PLase, employed as a reference isozyme source, was separated upon electrophoresis into three major bands corresponding to the PLase isozymes of I, II and III (I<II<III) reported by Yonezawa *et al.* (3). Adult liver PLase gave a widely spread zone located between the positions of II to III and composed of more than seven distinct subbands with lower intensity at each end. In contrast with the adult liver isozyme, fetal liver had a major zone of PLase with a mobility corresponding to III and with four to five subbands, a zone with two to four less intense subbands at position II and one faint but discernible subbands at the position of I (not clearly seen in the photograph). The isozyme pattern of spleen PLase was similar to that of fetal liver except for the appearance of one extra band anodic to the position of I, corresponding to the band of monomeric PLase I (12). The cathodic band of heart muscle isozyme never resolved into subbands. Thus, six species of isozyme bands or groups of subbands could be distinguished by the present electrophoretic method. They were tentatively designated, taking the results of other reports (2, 3) into account, as F, FM and M for heart muscle, L for adult liver, F′, F, FL′ and L′ for spleen and fetal liver (Fig 1).

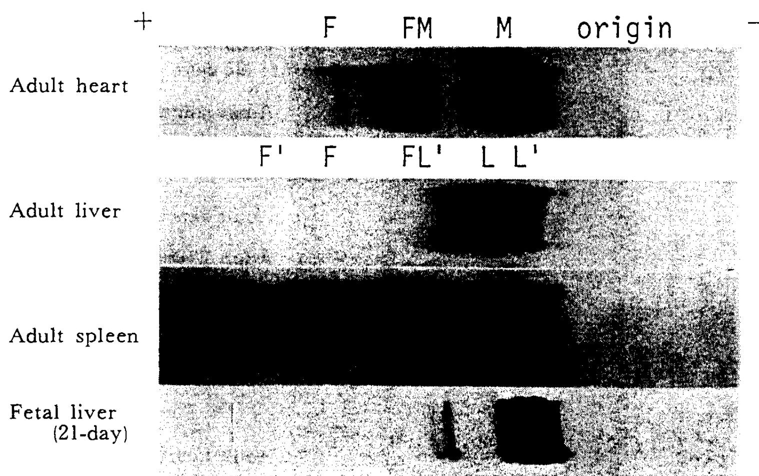

Fig. 1. Isozyme patterns of PLase in rat tissues. F band in fetal liver is present but not shown clearly in the photograph.

Developmental change of isozyme pattern. In accordance with the postnatal development, more anodic subbands of L′ became intense and the opposite ones of L′ became faint, finally giving the adult type liver isozyme of L. Concomitantly, FL′ and F bands became less marked to an undetectable level in the adult liver (Fig. . 2).

+ F FL' L L' origin −

Adult liver (51 day)

Young liver (19 day)

New born liver

Fetal liver (21 day)

Fig. 2. Developmental change of isozyme pattern in liver Plase.

+ −

Staining

Adult heart

Adult liver

+AMP+Na_2SO_4

Fetal liver (21 day)

Adult spleen

Adult heart

Adult liver

+AMP−Na_2SO_4

Fetal liver (21 day)

Adult spleen

Adult heart

Adult liver

−AMP−Na_2SO_4

Fetal liver (21 day)

Adult spleen

Fig. 3. Effect of AMP and Na_2SO_4 on PLase isozymes. Enzyme extacts were prepared from well-fed rats. F and FL′ bands are not visible in this photograph because of the small activities of PLase applied.

Characterization of isozymes. All the isozyme bands were stained most intensely in the presence of both Na_2SO_4 and AMP. When Na_2SO_4 was omitted from the incubation medium, F, FL′, FM and M still retained their activities, but L and L′ bands were not detected. By removing both of the activators from the staining system, none of the isozymes were visualized, confirming that the PLase isozymes were all in *b* form (Fig. 3). These data also gave validity to the present tentative designation of the isozyme bands.

Isozyme patterns in injured rat livers. In CCl_4-treated rat livers, subbands of L became blurred in 24 h and 48 h, and F and FL′ resurged in some rats in 48 and 72 h with a concomitant reduction in the L mobility, which became identical with that of L′ in 1 week (Fig. 4). Gal-NH_2 intoxication gave similar alterations

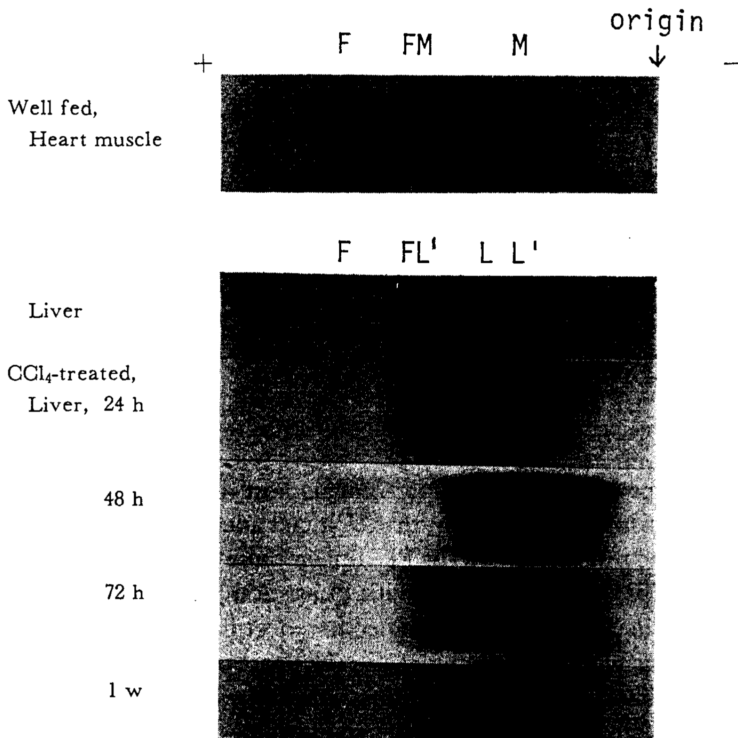

Fig. 4. Isozyme patterns of PLase in CCl_4-treated rat livers.

in the isozyme pattern, although the degree of the change was slightly less than in CCl_4 injury (Fig. 5). Partial hepatectomy also caused similar but smaller isozyme changes with a retarded appearance of F in 1 week (Fig. 6). Ascites hepatoma AH 130 had an isozyme pattern of most intense F and faint L′ with a

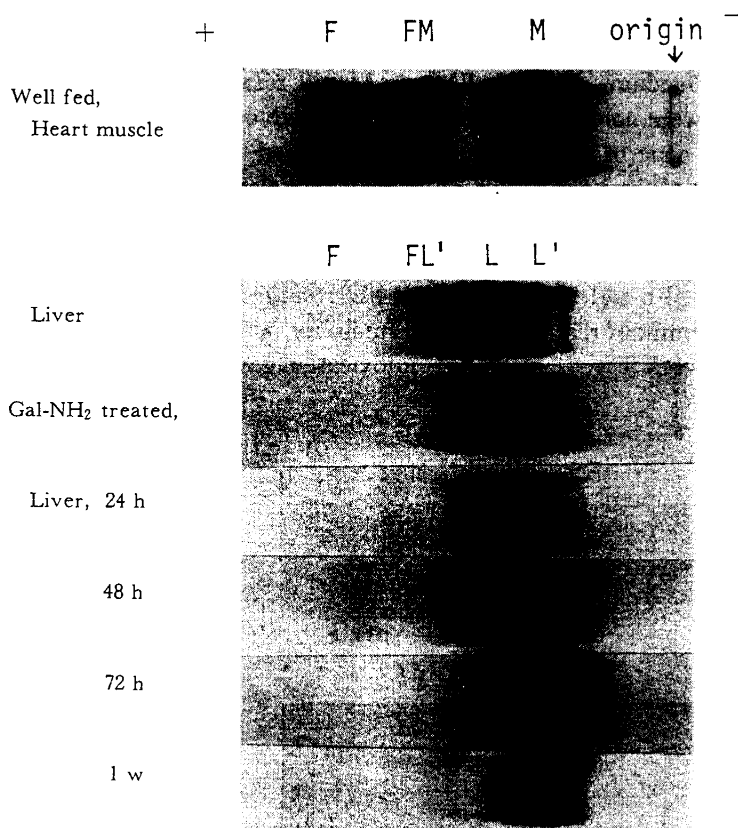

Fig. 5. Isozyme patterns of PLase in Gal-NH_2-treated rat livers.

total absence of adult type isozyme L. Tumor-bearing host liver still retained L isozyme, which was indistinguishable from that of intact adult liver (Fig. 7).

The incidence of the resurgence of F or FL′ under these experimental conditions is listed in Tables 1 and 2. together with PLase activities. The low incidence of F appearance in Gal-NH_2 injury seemed to reflect the variability in the extent of hepatic injury as well as the difference in the phase of liver damage (*cf.* Table 1. in Ref. 10.). Thus, the Gal-NH_2 injury was less severe than the CCl_4 intoxication and it was slightly retarded. Although Form *a* isozymes were found to migrate slightly faster than Form *b* isozymes (the results not shown here), the blurred subbands of L in injured livers appeared not to be due to the presence of relatively large amounts of *a* form, because the partially hepatectomized or sham-operated livers had considerable activities of PLase *a* with discrete subbands. Furthermore, when a PLase extract mostly of *a* form was subjected to elecrophoresis, discrete subbands similar to those of *b* form were yielded. The blurred

band may be due to some modifications of PLase molecules with high activity as a result of hepatic injury.

Isozyme pattern in human livers. Isozyme patterns of liver PLase in human liver diseases are shown in Fig. 8. Although F band was not demonstrated in

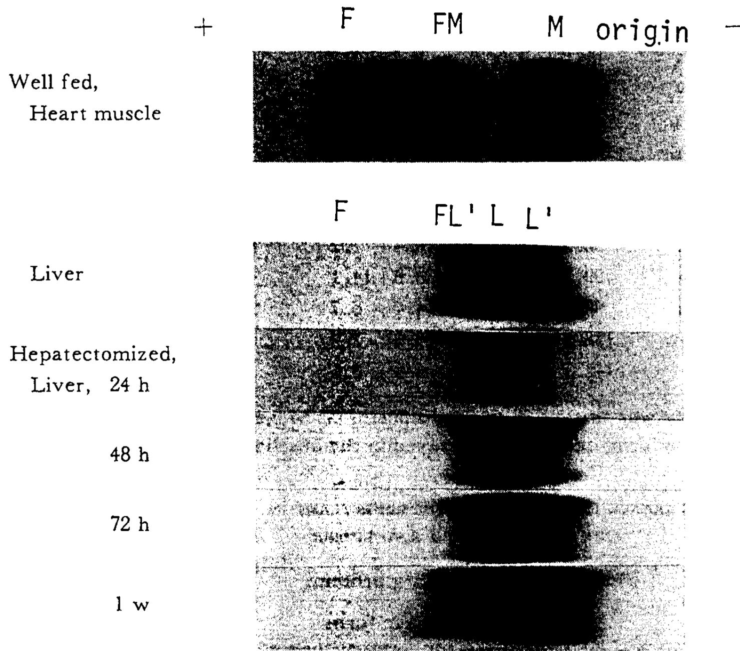

Fig. 6. Isozyme patterns of PLase in partially hepatectomized rat livers.

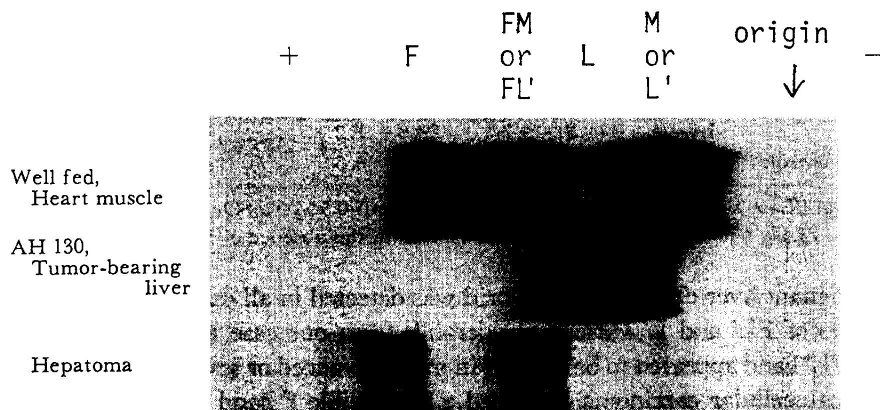

Fig. 7. Isozyme pattern of PLase in ascites hepatoma AH 130.

TABLE 1. APPEARANCE OF FETAL TYPE PHOSPHORYLASE IN CCl_4 AND GALACTOSAMINE-TREATED, AND CONTROL RAT LIVERS

Experimental conditions		PLase activity[a] (Units/g protein)		Appearance of F type
		Total	Active form	
CCl_4,	24 h	107.7± 6.6	77.6±16.6	0/5
	48 h	76.2±11.4	70.4± 2.4	6/6
	72 h	60.6± 4.6	9.7± 3.2	5/6
	1 w	145.9±18.5	17.1± 1.9	1/3
Galactosamine,	24 h	99.6±18.4	124.5±24.8	0/5
	48 h	108.6± 2.1	127.7± 2.1	0/4
	72 h	72.7±11.1	13.8± 4.2	3/5
	1 w	117.0± 0.2	76.5± 6.8	0/5
Control,	24 h	90.3± 4.8	10.4± 1.2	0/4
	48 h	99.2± 5.0	28.8±14.1	0/4
	72 h	98.0± 3.7	14.4± 6.7	0/5
	1 w	138.9± 8.4	16.6± 2.9	0/2
Well-fed control,		141.1± 5.0	6.3± 0.5	0/4

a Total PLase activity was assayed in the presence of 0.5 M Na_2SO_4 together with 1 mM AMP. PLase *a* (active form) activity was assayed in the absence of Na_2SO_4 and AMP.

TABLE 2. APPEARANCE OF FETAL TYPE PHOSPHORYLASE IN HEPATECTOMIZED AND SHAM-OPERATED RAT LIVERS

Experimental conditions		PLase activity[a] (Units/g protein)		Appearance of F type
		Total	Active form	
Hepatectomized,	24 h	116.5±10.2	111.1±11.9	0/5
	48 h	106.9± 7.0	71.1±11.6	0/5
	72 h	94.7± 2.9	63.0± 5.8	0/4
	1 w	96.6± 1.7	24.0± 6.3	2/3
Sham-operated,	24 h	120.9± 2.2	101.0±12.8	0/3
	48 h	181.3± 5.3	43.4±11.0	0/4
	72 h	134.5±14.9	75.9± 0.3	0/2
	1 w	114.8± 6.7	29.8± 1.2	0/2
Well-fed control,		141.1± 5.0	6.3± 0.5	0/4

a Total PLase activity was assayed in the presence of 0.5 M Na_2SO_4 together with 1 mM AMP. PLase *a* (active form) activity was assayed in the absence of Na_2SO_4 and AMP.

non-malignant liver diseases, FL′ hybrid was detected in all cases. The relative intensities of FL′ and L′ varied considerably from one case to another: more intense FL′ band appeared to be present in more advanced or severe liver diseases.

Hepatocellular carcinoma possessed a discernible F band as was found for a rat ascites hepatoma. Spleen PLase provided a reference isozyme pattern to

show the identity of the L′ in spleen with the L′ in liver. Thus no apparent L isozyme was observed in those human cases.

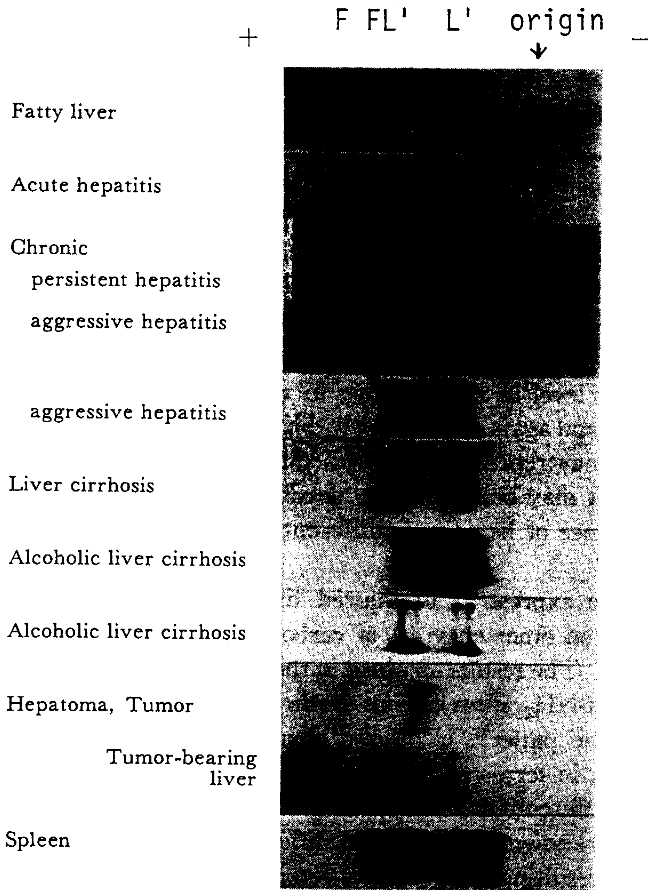

Fig. 8. Isozyme patterns of PLase in human livers, hepatoma and spleen.

DISCUSSION

By the development of a technique for electrophoretic separation of PLase isozymes, the presence of six molecular species including two hybrid forms was demonstrated in rat tissues. Furthermore, the isozymes other than M were shown to be composed of several distinct subbands, which had been separated hitherto as broad single bands. The small differences in the mobility of broad bands, which were frequently observed between L and L′ (2), can be now understood as increases in slow moving subcomponents and decreases in fast migrating ones and *vice versa*.

Thus, the adult liver possesses a specific isozyme L (or a group of subbands) distinct from the slowest component L′ (or a group of subbands) of fetal liver or spleen type isozymes. Obviously, the rat hepatoma is now found to have a fetal or spleen type isozyme designated as L′, which is considered as an undifferentiated molecular species of PLase. Since the hybrid band with an intermediate mobility of F and L′ appeard as the subcomponents of L decreased and those of L′ increased, formerly described LI hybrid (3) should be more properly termed as FL′, a hybrid of F and L′. It is, therefore, evident that there are two types of undifferentiated deviation of Plase; one is the shift of L subbands toward L′ and another is the resurgence of F and FL′. These changes appear to be mutually correlated in most cases of liver cell dedifferentiation, although the linking is not a strong one. Furthermore, L′ activity appears to decrease as the F activity is further intensified, as may be seen in a most undifferentiated hepatoma AH 130. Similar changes in onco-fetal protein are found in α-fetoprotein appearance in hepatoma patients. It's level in serum is low in well-differentiated, high in moderately differentiated, and again low in poorly differentiated hepatomas (13). Thus, the presence of L′ may represent an intermediate differentiation and of F the least differentiation. It may be, therefore, possible to determine the degree of PLase deviation and hence of hepatocyte differentiation by analysis of PLase isozyme pattern.

Undifferentiated gene expression in injured livers has been extensively studied in our laboratory on other enzymes of carbohydrate metabolism (7-9) and α-fetoprotein (14-17). The results obtained in the present study with PLase isozyme in CCl_4 and Gal-NH_2 intoxications confirmed the previous results, namely the altered isozyme patterns in injured livers resembled those of fetal liver and ascites hepatoma in terms of the decrease or loss of adult liver PLase and the appearance of undifferentiated molecular species, PLase's L′ and F.

Partial hepatectomy is known to produce a dedifferentiated enzyme pattern, although the extent of the resulting enzyme deviation is relatively small (7). It is thus of some interest to see whether the appearance of PLase F in injured liver is due to the regeneration of hepatocyte following liver cell necrosis or to the hepatic injury *per se*. The regenerative process in partially hepatectomized rat liver starts several hours earlier than that in hepatotoxin-injured rat liver (14), and the extent of enhancement in DNA synthesis is nearly equal in both regenerative manipulations (14). Accordingly, the appearance of F band would be expected to be detected earlier in the partial hepatectomy than in the liver injury. In contrary to this assumption, PLase F was not detected for three days after partial hepatectomy, when an intense F band was already demonstrated in the injured livers. Furthermore, F band was observed in the livers one week after hepatectomy, when F band was no longer detectable in the injured livers. These results

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indicate that the F component of PLase resurges in hepatic injury itself as well as in a later stage of hepatocyte regeneration invoked by partial hepatectomy. In other words, the appearance of PLase F may be independent of increased DNA synthesis, as is the case for elevation of α-fetoprotein (16, 17).

Another change in electrophoretic pattern of PLase related to hepatic damage is the blurring of L subbands in its early stages. This is probably due to a subtle change in PLase L molecule resulting from liver injury and not to the presence of *a* form in the enzyme extract as mentioned under Results.

With human livers obtained from patients with parenchymal liver diseases, similar undifferentiated PLase patterns were obtained, although the F band appeared only in a hepatocellular carcinoma. There was, however, increased intensity of FL′ band in the livers of non-malignant hepatic lesion, indicating the increase in F component in injured livers. The appearance of FL′ hybrid is pretty strong evidence that the increases in F and L′ components occur within the same damaged hepatocytes. Thus, the increased FL′ activity is considered not to be derived from the infiltration of other cells with a high activity of F component.

All these results obtained with PLase isozyme support the idea that the undifferentiated gene expression can be brought about by hepatic injury, which has been put foreward by Taketa *et al.* (7-9) based on the data of other key enzymes of carbohydrate metabolism in injured livers.

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