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Changes in Lysosomal Enzymes in Experimental Hepatic Damage[1)]

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An investigation has been in order to elucidate the mechanism of the elevation of serum enzyme activities and about the changes in subcellular distribution during development of experimentally produced hepatic damage in the rats. Two enzymes have been studied: acid phosphatase (Acid Pase) and N-acetyl-β-glucosaminidase (NAG). Three different methods of inducing hepatic damage have been used: administration of carbon tetrachloride (CCl_4), dimethylnitrosamine (DMNA), and thioacetamide (TAA).

In acute hepatic damage, an increase in soluble activity was found to occur for two enzymes studied. The extent of this increase was slightly much in the activity of NAG as compared with that of Acid Pase.

The changes in serum NAG activity was remarkably much in the case of any drugs. During the development stages of chronic administration of CCl_4, the subceliular distribution pattern of lysosomal enzymes after 4 weeks were similar to that of lysosomal enzymes after 12 weeks.

At 4 weeks after the administration of CCl_4, the changes of lysosomal enzymes activities in the plasma was similar to that of acute hepatic damage by the treatment with DMNA and TAA.

Furthermore, in the plasma at 12 weeks after the administration of CCl_4, the variation of lysosomal enzymes activities was clearly differed from that of acute hepatic damage.

It is assumed that the changes in NAG activity is minor due to fibroblasts other than hepatic parenchymal cells for the origin of the increased serum NAG activity during the CCl_4-chronic hepatic damage.

Keywords——N-acetyly-β-glucosaminidase; acid phosphatase; subcellular distribution; acute hepatic damage; chronic hepatic damage; hydroxyproline; histological changes

N-Acetyl-β-glucosaminidase (E. C. 3.2.1.30) is widely distributed in mammalian tissues and is associated with degradation of glycoproteins and mucopolysaccharides.[3)] This enzyme is mainly located to lysosomal membranes. It has been reported that NAG activity in human serum was elevated above the normal range in patients with acute hepatitis and chronic hepatitis.[4–6)] Liver was the tissue used almost exclusively in the studies of the intracellular distribution of the enzyme and of its changes under various conditions.[7–9)] Little has been known, however, about the intracellular distribution pattern of the enzyme occurring in experimental hepatic damage.

In the present experiments, in order to elucidate the mechanism of the elevation of the serum enzyme activity in hepatic damage, the interrelation between the serum enzyme activity and the intracellular distribution pattern of the enzyme in the liver was investigated under various conditions.

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Methods

Animals and Drugs——Male albino rats of the Wistar strain, weighing approximately 200 g at the beginning of the experiment, were used.

They were fed on a standard semisynthetic diet. For acute hepatic damage, animals were single given carbon tetrachloride (CCl_4, 1 ml/kg), dimethylnitrosamine (DMNA, 100 mg/kg), thioacetamide (TAA, 200 mg/kg), respectively, through an intraperitoneal injection and sacrificed after 24 hr. Chronic hepatic damage was induced by the subcutaneous injection of CCl_4 (50%, 2 ml/kg) twice a week for periods of 4 weeks and 12 weeks, and sacrificed at three days after final administration.

Cell Fractionation of Rat Liver——Rats were killed by decapitation and the liver was removed, and weighed. The liver homogenates (10%, w/v) were prepared in an ice-cold 0.25 M sucrose solution at five strokes (1000 rev./min) in a Potter-Elevehjem type homogenizer with a teflon pestle. Fractions of subcellular particles were prepared from the sucrose homogenates of rat liver according to the method described by De Duve, *et al.*[10]

Histology and Assay Methods——A small piece of the liver was taken from the right lobe for histological study and the determination of hydroxyproline. Estimation of hydroxyproline contents in the livers was performed by the method of H. Stegeman, *et al.*[11]

Enzyme Assays——NAG was determined according to the method of Walker, *et al.*[12] with a slight modification by using *p*-nitrophenyl-N-acetyl-β-glucosaminide as substrate. Acid Pase was determined according to the method of Appelmans, *et al.*[13] by using sodium β-glycerophosphate as substrate and the liberated inorganic phosphate was measured according to the method of Lindberg and Ernster.[14] Acid Pase was used as lysosomal markers. The total activity was obtained by the addition of a nonionic detergent Triton X-100. The results presented throughout this report are expressed as total activity (μ mol of *p*-nitrophenol/g liver per min for the liver, and μ mol of *p*-nitrophenol/1 ml of plasma per min for the plasma).

Protein was determined colorimetrically by the method of Lowry, *et al.*[15] with crystalline bovine serum albumin as a standard.

The obtained results were analyzed for stastical significance by determining student'st test.[16]

Results

Histological Changes in the Liver of Rats with Chronic Hepatic Damage

The histological changes was examined in order to judge liver fiber formation. Samples were taken from each liver, fixed in 10% (v/v) formalin, embedded in paraffin and stained with hematoxylin and eosin.

Fig. 1 shows the histological figures of the control rat liver. The hepatic lobular structures were maintained and the radially oriented lobules were found at regular interval around the central veins.

In the liver of rats at 4 weeks after repeated administration of CCl_4, as shown in Fig. 2, the vacuoles, *i.e.* a large number of fat droplets were found along with a slight necrosis and degeneration.

In the liver of rats at 12 weeks after repeated administration of CCl_4, as shown in Fig. 3, the degree of fat droplets contents was similar to that at 4 weeks, but the formation of pseudolobules was found.

The results of histological examination was represented the fiber formation in the liver of rats repeatedly received with CCl_4 for 12 weeks.

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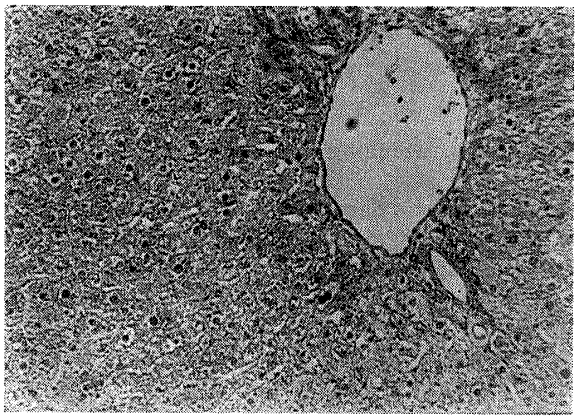

Fig. 1. Light Micrograph of Liver from Control Animals

The hepatic lobular structures were maintained and the radially oriented lobules was found at regular interval around the central veins (H and E stain. ×100).

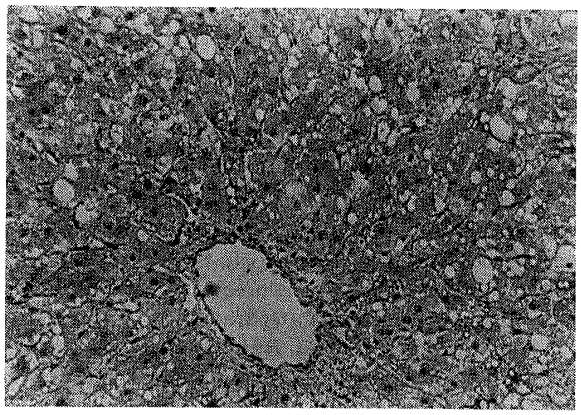

Fig. 2. Section from the Liver of Rats at 4 Weeks after the Administration of CCl_4

The light micrograph exhibits with only a few centrilobular parenchymal necrosis and and inflammatory infiltration. Adjacent to the necrotic zone can be observed many hydropic hepatocytes (H and E stain. ×100).

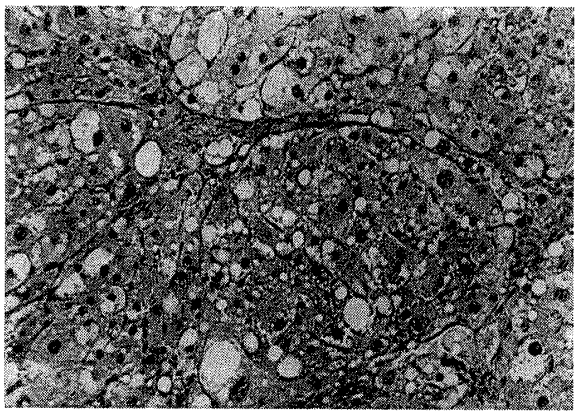

Fig. 3. Section from the Liver of Rats at 12 Weeks after the Administration of CCl_4

At this magnification, the vacuolization and the amount of fat droplets can be seen same degree as in Fig. 2. Also the advanced fiber formation can be observed (H and E stain. ×100).

Biochemical Changes in the Liver of Rats after the Treatment with Hepatotoxins

Rats were once treated with CCl_4 (1 ml/kg), DMNA (100 mg/kg), TAA (200 mg/kg). Table I shows the biochemical changes in the liver of rats at 24 hr after the treatment with different hepatotoxins.

The increase in the body weight was inhibited by the treatment of these drugs and the liver weight was inclined to decrease by the treatment of DMNA. Changes in the lysosomal protein was seen to increase slightly in the liver of rats treated with DMNA and TAA. Hydroxyproline contents were slightly increased in the liver of rats treated with any drugs.

Table II shows the biochemical changes in the liver of rats at 4 weeks or 12 weeks after the administration of CCl_4. The increase in the body weight was inhibited by the CCl_4-chronic treatments.

The liver weight was increased in each case and was found to be hypertropic. The changes in the lysosomal protein was seen to incline to increase slightly.

In order to examine chronic hepatic damage biochemically, hydroxyproline contents in the liver were determined as the index of fiber formation.

As shown in Table II, hydroxyproline contents in the liver were increased 2-fold in the liver of rats at 4 weeks and 12 weeks after the administration of CCl_4, respectively, as compared with controls. It may be difficult to judge liver fiber formation biochemically from the determination of hydroxyproline contents in the liver.

Changes in Subcellular Distribution of Enzymes in Acute Hepatic Damage

The results presented in Table III show the changes in subcellular distribution pattern of lysosomal enzymes activities in acute hepatic damage with CCl_4, DMNA, or TAA. With respect to the intracellular distribution pattern, the total activities were significantly decreased in the lysosomal fractions and significantly enhanced in the supernatant fractions.

TABLE I. Biochemical Changes in the Livers of Rats after the Administration of Three Hepatotoxins

Hepatotoxins	Body weights, final (g)	Liver weight (g/100 g b.w.)	Lysosomal protein (mg/g liver)	Hydroxyproline (μg/g liver)
Control	224±9	4.3±0.1	13.1±1.2	178.0± 7.0
CCl_4[a]	214±8[b]	4.3±0.1[b]	14.7±1.0[b]	198.9±12.0[b]
DMNA[a]	205±6[b]	3.1±0.1[e]	17.8±1.0[d]	298.8±23.6[e]
TAA[a]	196±3[c]	4.5±0.1[b]	16.1±0.7[d]	227.0± 7.4[e]

Values are given as means±standard errors of five rats.
a) Acute hepatic damage was caused by given these drugs through a single intraperitoneal injection. Rats were sacrificed after 24 hr.
b) Not significantly different from value in controls, N.S.
c) Significantly different from value in controls, $p<0.05$.
d) Significantly different from value in controls, $p<0.02$.
e) Significantly different from value in controls, $p<0.01$.

TABLE II. Biochemical Changes in the Liver of Rats after the Repeated Administration of CCl_4

Group	Body weights, final (g)	Liver weight (g/100 g b.w.)	Lysosomal protein (mg/g liver)	Hydroxyproline contents (μg/g liver)
Control	358±16	4.3±0.2	18.7±0.4	178.7±12.7
4 weeks[a]	275± 6[e]	6.4±0.3[e]	21.0±0.3[d]	314.3±21.4[e]
Control	480± 9	3.5±0.1	14.5±0.7	241.8±14.7
12 weeks[a]	361± 6[e]	6.5±0.6[e]	15.9±0.7[b]	515.6±83.6[d]

Values are given as means±standard errors of five rats.
a) Chronic hepatic damage was caused by given CCl_4through subcutaneous injection twice a week for above periods.
b) Not significantly different from value in controls, N.S.
c) Significantly different from value in controls, $p<0.05$.
d) Significantly different from value in controls, $p<0.02$.
e) Significantly different from value in controls, $p<0.01$.

From the view of the behavior of lysosomes, the S/L ratio was expressed as percent of the activity in the supernatant fractions to that in the lysosomal fractions.

In the case of NAG, the values by CCl_4, DMNA, TAA treatments were reduced as compared with controls in the lysosomal fractions.

TABLE III. Intracellular Distribution of Lysosomal Enzymes Activities in the Liver of Rats after Acute Hepatic Damage

Group	Enzyme activities (μmol/g liver/min)		S/L ratio[a]
	Lysosomes	Supernatants	
NAG			
Control	1.30±0.08	0.11±0.01	0.08
CCl_4	0.83±0.11[b]	0.18±0.02[b]	0.22
DMNA	0.56±0.02[d]	0.49±0.03[c]	0.88
TAA	1.09±0.05[b]	0.17±0.04[b]	0.16
Acid Pase			
Control	1.35±0.07	0.42±0.05	0.31
CCl_4	1.06±0.06[c]	0.88±0.06[c]	0.83
DMNA	0.47±0.04[d]	0.63±0.05[c]	1.34
TAA	0.45±0.03[d]	0.50±0.03[b]	1.11

Values are given as means±standard errors of five rats.
a) Activity of supernatant fraction/activity lysosomal fraction ×100.
b) $p<0.05$. *c*) $p<0.02$. *d*) $p<0.01$.

On the other hand, those values were significantly increased in the supernatant fractions. In particular, the subcellular distribution pattern in NAG activity was pronounced changed by the treatment with DMNA.

These results may be only the quantitative changes. Similar variation can be seen for the activity of Acid Pase on the intracellular distribution pattern. The release to the supernatant fraction of Acid Pase, however, was tempted to slightly higher than that of NAG.

It is considered that this evidence is due to different localization mode of both enzymes within lysosomes.

Plasma Enzymes in Acute Hepatic Damage

The changes in lysosomal enzymes activities in the plasma in acute hepatic damage is shown in Table IV.

TABLE IV. Plasma Enzymes Activities in the Rats Treated with Hepatotoxins

Hepatotoxins	Enzyme activity[a] (μmol/ml plasma/min)	
	NAG[b]	Acid Pase[c]
Control	0.014±0.001	0.026±0.002
CCl_4	0.026±0.002	0.038±0.003
DMNA	0.131±0.02	0.067±0.003
TAA	0.167±0.03	0.066±0.004

Values are given as means±standard errors of five rats.
a) Differences of the values betweem treated and control groups, were statistically significant ($p<0.01$).
b) N-Acetyl-β-glucosaminidase.
c) Acid phosphatase.

The enzyme activity of the plasma by the treatment with CCl_4 was found to be increase 2-fold as compared with controls. In the case of the treatment with DMNA and TAA, their activities were increased 9.4-fold, 11.9-fold, respectively, as compared with controls, while Acid Pase activity in the plasma was increased 1.5—2.6 fold as compared with controls by the treatment with these drugs. Thus, the changes in NAG in the plasma was seen to be markedly elevated as compared with that of Acid Pase.

It is considered that this difference is due to the stability of both enzymes in the blood and to organ origin for the elevation of blood enzymes by the hepatotoxins. Furthermore, the changes in the enzymes activities in the plasma were found to respond well relatively to the changes in intracellular distribution of the liver.

Changes in Subcellular Distribution of Enzymes after the CCl_4-Chronic Treatment

Next changes in the behavior of enzymes of intracellular particles were examined in the liver at 4 weeks and 12 weeks after the administration of CCl_4 as a model of chronic hepatic damage.

Its results are shown in Table V. For the activity of NAG in the liver after 4 weeks, the values in the lysosomal fractions were not changed and those in the supernatant fractions were tempted to increase slightly.

On the other hand, this change was not significant in lysosomal fractions in the liver after 12 weeks as well as after 4 weeks.

In the supernatant fractions, their values were elevated above 2-fold as compared with controls. In the case of Acid Pase in the liver after 4 weeks, activity in the lysosomal fractions was decreased and that of supernatant fractions were increased about 2-fold.

On the other hand, after 12 weeks, the activity was significant unchanged in lysosomal fractions and increased about 1.5-fold in supernatant fractions. The extent of leakage of

TABLE V. Intracellular Distribution of Lysosomal Enzymes Activities in the Liver of Rats after the Repeated Administration of CCl_4

Group	Enzme activity (μmol/g liver/min)		S/L ratio[a]
	Lysosomes	Supernatants	
NAG			
Control	0.971±0.05	0.292±0.02	0.30
4 weeks[b]	0.820±0.02[d]	0.380±0.01[e]	0.46
Control	0.558±0.04	0.067±0.01	0.12
12 weeks[c]	0.581±0.05[d]	0.150±0.02[e]	0.26
Acid Pase			
Control	0.976±0.01	0.317±0.01	0.32
4 weeks[b]	0.553±0.05[f]	0.661±0.04[f]	1.20
Control	0.550±0.07	0.238±0.03	0.43
12 weeks[c]	0.600±0.01[d]	0.367±0.03[e]	0.61

Values are expressed as means±standard errors of five rats.
a) Activity of supernatant fraction/activity of lysosomal fraction.
b, *c*) CCl_4 was repeatedly dosed to the rats for 4 or 12 weeeks.
d) N.S. *e*) $p<0.05$. *f*) $p<0.01$.

both enzymes from lysosomes to supernatants was not much than the changes found during acute hepatic damage.

Plasma Enzymes in CCl_4-Chronic Treatment

Table VI gives the results obtained for the elevation of lysosomal enzymes activities in the plasma after the administration of CCl_4.

TABLE VI. Plasma Enzymes Activities in the Rats after the Administration of CCl_4

Group	Enzyme activity (μmol/ml plasma/min)	
	NAG[a]	Acid Pase[b]
Control	0.016±0.003	0.042±0.005
4 weeks[c]	0.052±0.015[f]	0.162±0.029[f]
Control	0.018±0.003	0.039±0.002
12 weeks[d]	0.030±0.004[e]	0.049±0.004[e]

Values are expressed as means±standard errors of five rats.
a) N-Acetyl-β-glucosaminidase.
b) Acid phosphatase.
c), *d*) Chronic hepatic damage was caused by given CCl_4 through subcutaneous injection twice a week for above periods.
e) Not significantly different from value in controls, N.S.
f) Significantly different from value in controls, $p<0.01$.

After 4 weeks, the activity was increased 3.3-fold for NAG, 3.9-fold for Acid Pase, respectively, as compared with controls. After 12 weeks, NAG and Acid Pase activity was not significantly changed.

Similarity in the elevation pattern was found between acute hepatic damage and 4 weeks after the administration of CCl_4. As regard to the activity between NAG and Acid Pase, marked difference was not observed as well as the change in intracellular distribution.

The changes at 12 weeks after the administration of CCl_4 were a smaller tendency in both the activity of NAG and the activity of Acid Pase as compared with changes in 4 weeks. The variations of enzymes activities in the plasma at 12 weeks after the administration of CCl_4 were clearly differed the changes of these enzymes activities in the plasma in acute hepatic

damage.

Discussion

There are numerous reports showing that CCl_4, DMNA and TAA cause extensive central necrosis in the liver 24 hr after their administration to rats. Furthermore, both CCl_4 and DMNA also cause a fatty liver, the early signs of which are easily detectable 5—8 hr after the administration of the poison. In acute hepatic damage by these poisons, there is little report about the behaivor of NAG up to data.[17]

Present study was carried out in purpose to know whether the difference about the changes in the subcellular distribution pattern of lysosomal enzymes are observed or not. In acute hepatic damage by the treatment with these drugs, as is shown in Table III, both the total activity of NAG and Acid Pase show the decreases in the lysosomal fraction and show the increases in the supernatant fractions.

The fact that Acid Pase is released to supernatants in acute hepatic damage agreeded with the report described by Ugazio *et al.*[18,19] This phenomenon represents the fact that the lysosomal membranes are destroyed. But this evidence may be caused by labilization of lysosomal membranes in these liver injuries, and there was no unequivocal in present study. Similar behavior was found also for NAG as well as for Acid Pase. The extent of this release from the particles, as well as the report described by Ugazio, *et al.*[19] is, however, different from one enzyme to another. As for NAG, there are new data on this phenomenon.

Also, the difference of S/L ratios between NAG and Acid Pase is presumed to be different for localization mode of these enzymes in lysosomes. In acute hepatic damage, there are substantial increases in the activities of NAG than Acid Pase in the plasma (Table IV). For example, NAG activity elevates above 5-fold by the treatment with DMNA and TAA.

Since administration of DMNA and TAA to rats are known to affect primarily the liver and leakage of enzymes from this organ into the blood is well documented, it may be thought that the increases of NAG activity in the plasma is not originated from kidney, leukocyte in these liver injuries.

On the other hand, as Acid Pase is due to be mainly hepatic and prostatic in origin, the changes of Acid Pase in the plasma may not be larger than that of NAG. It is thought that the stability of the enzymes in the plasma affects elevation pattern. In addition, there may be difference in the mode of each liver injury.

Histological examination by light microscopy indicated that the livers at 4 weeks after the administration of CCl_4 to rats are regarded as acute hepatic damage (Fig. 2). NAG is considered to be involved in the catabolism of acid mucopolysaccharides.

Koizumi *et al.*[17] reported abnormality of acid mucopolysaccharides in the liver and the elevation of NAG activity in the serum in CCl_4-chronic hepatic damage (Fig. 3). The hydroxyproline contents in the liver were estimated as the index of fiber formation.

Previous reports[20] showed that hydroxyproline contents increased 4-fold as compared with controls in 12 weeks after the administration of CCl_4 to rats. In present experiments, hydroxyproline contents in the liver by the biochemical method are not shown prominent rise in 12 weeks after the administration of CCl_4 to rats as compared with controls (Table II). Thus, both methods were not necessarily parallel relationship.

The changes in the activities of lysosomal enzymes both liver and plasma after the chronic administration of CCl_4 to rats are shown in Table V. The changes in intracellular distribution

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of lysosomal enzymes at 4 weeks after the administration of CCl_4 was found to be similar to those of acute hepatic damage (Table III, V).

In CCl_4-chronic hepatic damage, no significant change was observed in the intracellular distribution pattern of these enzymes (Table V).

Furthermore, in the plasma at 12 weeks after the administration of CCl_4, the variation of lysosomal enzymes activities were clearly differed from that of acute hepatic damage. In addition, the variation of lysosomal enzymes activities in the plasma during the proceed of CCl_4-chronic hepatic damage was less than those in acute hepatic damage (Table VI).

From such considerations it is concluded that the variation of NAG activity in the plasma of chronic hepatic damage may not be attributed to the other tissues such as fibroblast than liver parenchymal cells.

Furthermore, it is considered that the another particles except for lysosomes affect intracellular distribution pattern of both NAG and Acid Pase.

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