Inhibitory Effect of Ethanol and Colchicine on the Intracellular Processing of β -Glucuronidase Which Occurs in the Golgi Complex

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To investigate the effect of ethanol or colchicine on the intracellular proteolytic processing of lysosomal β -glucuronidase, which is considered to occur in the Golgi complex in the intracellular sorting pathway, three rat liver Golgi subfractions, GF-1, GF-2, and GF-3, were isolated from ethanol- or colchicine-treated rats, and the electrophoretic patterns of the extracted Golgi β -glucuronidase on polyacrylamide gel were examined. The isolated Golgi subfractions from the drug-treated rats gave a better yield of fraction than that from the control rats. The enzymatic characterization of these three subfractions showed no significant contamination by other subcellular structures such as plasma membranes, microsomes, or lysosomes, and no inhibitory effect of the drugs was observed. On the other hand, suppressed galactosyltransferase activity, a marker enzyme of the Golgi complex, was detected in the colchicine-treated rats. The electrophoretic pattern of Golgi β -glucuronidase on polyacrylamide gel revealed one major band which moved to the same position as the lysosomal enzyme type in the control rats. In contrast, in the ethanol- and colchicine-treated rats, Golgi β -glucuronidase was found to have two major bands stained for enzyme activity resulting from a mixture of microsomal- and lysosomal-type enzymes. These results suggested that the post-translational modification step, during conversion from a microsomal-type enzyme to a lysosomal-type enzyme, was apparently inhibited. Taken together, these findings indicated that ethanol or colchicine administration to rats caused an inhibitory effect on the intracellular post-translational modification of Golgi β -glucuronidase destined for targeting to the lysosomes.

Key words colchicine; ethanol; Golgi complex; β -glucuronidase; intracellular processing; lysosome

It is well established that newly synthesized lysosomal enzymes are translocated into the rough endoplasmic reticulum, where the signal peptide cleavage and core glycosylation of the selected asparagine residues of the nascent protein takes place.^{1–4)} The lysosomal hydrolases are then transported to the Golgi complex where the mannose 6-phosphate residues on their oligosaccharide moieties are formed, and these phosphomannosyl residues are recognized by specific receptors (mannose 6-phosphate receptors).^{5,6)} This intracellular recognition system allows for the segregation of lysosomal soluble hydrolases from proteins destined for secretion.

β -Glucuronidase is a well-known lysosomal hydrolase which is considered to follow the same intracellular sorting pathway as other lysosomal enzymes. On the other hand, β -glucuronidase is also an unusual protein in that considerable amounts of this enzyme are localized in the endoplasmic reticulum as well as in the lysosomes.^{7–17)} This unusual localization of β -glucuronidase in the endoplasmic reticulum was explained by the presence of egasyn, which is the microsomal resident protein which stabilizes β -glucuronidase in the endoplasmic reticulum.^{18–22)} However, the physiological importance of this microsomal β -glucuronidase-egasyn complex remains to be determined.

Although the microsomal and lysosomal β -glucuronidases are catalytically and immunologically identical, they differ somewhat in isoelectric points and in the size of the subunit component.^{12–14)} A previous report¹³⁾ from our laboratory demonstrated that these post-translational processing events for β -glucuronidase possibly proceed in the Golgi complex in the intracellular sorting pathway, since the biochemical properties and the elec-

trophoretic behavior of Golgi β -glucuronidase were indistinguishable from those of the lysosomal enzyme. It was also demonstrated that Golgi β -glucuronidase has a heterogenous acidic isoelectric point of pI 5.7–6.6 which was quite similar to that of lysosomal β -glucuronidase.¹³⁾ In contrast, microsomal β -glucuronidase showed a neutral isoelectric focusing pattern. Furthermore, as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Golgi β -glucuronidase was found to be comprised of heterogenous subunit components of 79 and 74 kDa which resembled those of lysosomal enzymes (unpublished data), while microsomal β -glucuronidase had a single polypeptide component of 79 kDa. Therefore, these results imply that the polypeptide portion of β -glucuronidase is proteolytically processed at the NH₂-terminus or COOH-terminus to generate a heterogenous component of the enzyme in the Golgi complex by processing proteinase during the intracellular sorting pathway; this results therefore indicate that the subunit component of a microsomal-type enzyme is converted to that of a lysosomal-type enzyme post-translationally in the Golgi complex. However, a detailed biochemical analysis of the maturation mechanism for β -glucuronidase has not been elucidated until now.

In order to investigate the post-translational modification mechanism of β -glucuronidase in the Golgi complex, we tried to isolate a sufficient number of Golgi subfractions for biochemical analysis; however, the recovery of prepared Golgi subfractions was limited. To increase the recovery of Golgi subfractions, a simple and convenient procedure has been reported for the isolation of Golgi subfractions from rat liver, in good purity and sufficient quantity, by employing rats pretreated with ethanol²³⁾ or

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anti-secretory agents such as colchicine and vinblastine.²⁴⁾ Ethanol intoxication in rats causes the enhanced synthesis of very low density lipoprotein (VLDL) and results in an increase in secretory vesicles in a hepatocytes. Therefore, sufficient amounts of secretory vesicle-rich fractions are obtained from rat liver, and biochemical analysis of this has been carried out previously.²³⁾ However, a recent paper demonstrated that chronic ethanol treatment apparently altered the normal microheterogeneity pattern of transferrin as a consequence of decreased activity of Golgi sialyltransferase and increased activity of sialidase at the plasma membrane, therefore implying that one of the targets that may be sensitive to chronic ethanol treatment may be the Golgi complex.²⁵⁾ On the other hand, microtubule depolymerizing agents such as colchicine and vinblastine are known to inhibit the transport vesicles from fusing with prelysosomal compartments²⁶⁾ or are known to cause the accumulation of secretory vesicles.²⁴⁾ Previous data showed that the administration of colchicine to rats caused an increase in the protein recovery of Golgi subfractions.²⁴⁾ Meanwhile, a significant inhibitory effect on the proteolytic conversion from the proalbumin to the serum-type albumin was also reported.²⁷⁾

In this paper, to investigate the effect of the administration of ethanol or colchicine on the microheterogeneity of β -glucuronidase in the Golgi complex, Golgi subfractions were prepared from ethanol- or colchicine-treated rat livers and the electrophoretic properties of β -glucuronidase were examined in the Golgi subfractions. We present evidence showing that the post-translational modification step in the Golgi complex converting the microsomal β -glucuronidase to the lysosomal β -glucuronidase is indeed changed by the administration of ethanol as well as colchicine.

MATERIALS AND METHODS

Animals Male Wistar rats (250 g) were used.

Materials Materials were obtained from the following sources: uridine 5'-diphosphate (UDP)-[¹⁴C]galactose from New England Nuclear Corp. (Boston, MA), UDP-galactose, glucose-6-phosphate disodium salt, *p*-nitrophenyl β -*N*-acetylglucosamine, naphthol-AS-BI- β -D-glucuronide, Fast Red-Violet LB salt, and bovine serum albumin were from Sigma, St. Louis, MO. Adenosine 5'-monophosphate (AMP) was obtained from Kojin Co., Ltd. (Tokyo, Japan). *p*-Nitrophenyl β -D-glucuronide was prepared by the method of Kato *et al.*²⁸⁾ Pharmalyte (pH 5–8), proteins used as molecular weight standards, were obtained from Pharmacia LKB Biotechnology, Inc. (UK). Colchicine was purchased from Merck. All other chemicals used were of reagent grade materials from various commercial sources.

Enzyme Assays UDP-galactose: *N*-acetylglucosamine galactosyltransferase activity was determined by a slight modification of the method of Fleischer *et al.*²⁹⁾ The reaction mixture contained 10 μ mol of sodium cacodylate (pH 6.3), 2 μ mol of MgCl₂, 8 μ mol of MnCl₂, 1 mg of Triton X-100, 3 μ mol of *N*-acetylglucosamine, and 76 nmol of UDP-[¹⁴C]galactose (3.2 $\times$ 10⁵ dpm/mol). To this was added 10–75 μ l of a suspension of cell fraction (Golgi

fraction containing about 20–100 μ g of protein) and the final volume was adjusted to 200 μ l with distilled water. After a 10-min incubation at 37 °C, the reaction was stopped by passing the mixture through a column (0.5 $\times$ 5 cm) packed with about 1 ml of Dowex 1 $\times$ 8 (200–400 mesh) in the Cl[−] form, and the column was eluted with 3 $\times$ 0.4 ml samples of distilled water directly into scintillation vials containing 15 ml of Bray's solution.³⁰⁾ Radioactivity was counted on a Packard scintillation spectrometer (model 3375). The blank contained all the ingredients of the assay except *N*-acetylglucosamine. The difference in radioactivity between the experimental samples and the blank is considered to represent the amounts of galactose transferred to *N*-acetylglucosamine during the reaction. The unit of specific activity is nmol/h/mg protein. β -Glucuronidase activity was determined as described,¹²⁾ with *p*-nitrophenyl β -D-glucuronide under the standard assay conditions. One unit of β -glucuronidase was taken as the activity that catalyzed the release of 1 μ mol of *p*-nitrophenol per min from the substrate. Specific activity was expressed as units per mg of protein. Acid phosphatase was assayed with *p*-nitrophenylphosphate as a substrate.³¹⁾ β -*N*-Acetylglucosaminidase³²⁾ and glucose-6-phosphatase³³⁾ were assayed as described previously. All acid hydrolases were assayed in the presence of 0.1% Triton X-100. 5'-Nucleotidase (AMPase) was assayed by a slight modification of the method of Emmelot *et al.*³⁴⁾ The reaction mixture contained 5 mM AMP, 10 mM MgCl₂, 0.1% Triton X-100, and 0.2 M Tris-HCl buffer (pH 7.5). To this was added 50 μ l of a suspension of cell fraction (the Golgi fraction containing about 50–100 μ g of protein), and the final volume was adjusted to 200 μ l with distilled water. Incubation was carried out at 37 °C for 20–40 min, and the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The mixture was centrifuged to remove any insoluble material and was analyzed for inorganic phosphate by the procedure of Fiske and Subbarow.³⁵⁾

Protein Determination Protein was determined by the method of Lowry *et al.*³⁶⁾

Preparation of Antisera Antisera against rat liver lysosomal β -glucuronidase was prepared by injecting the purified enzyme into rabbits.¹²⁾ The enzyme was emulsified with an equal volume of Freund's complete adjuvant and injected into rabbits intracutaneously three times at two-week intervals at a dose of 0.3 mg/rabbit. The rabbits were bled 10 d after the last injection. Antibodies were purified by immunoaffinity chromatography using antigen-Sepharose 4B as described.¹²⁾ Ouchterlony gel diffusion analysis was performed at room temperature in 1% agarose gel in 50 mM phosphate buffer (pH 7.5) containing 0.9% NaCl and 0.02% NaN₃.

Nondenaturing PAGE Nondenaturing PAGE was performed by the method of Davis³⁷⁾ in Tris-Cl (pH 8.9). 7.5% Gels were prepared in 0.8 $\times$ 10 cm glass tubes and the current flow was 4 mA/tube. Staining of β -glucuronidase activity in the polyacrylamide gel was done by a slight modification of the method of Ohishi and Shioya³⁸⁾ using 0.01% naphthol-AS-BI- β -D-glucuronide as the substrate and 0.04% Fast Red-Violet LB salt as the

coupling agent.

Isoelectric Focusing Isoelectric focusing on the polyacrylamide gels was performed by a slight modification of the method as described by Owens *et al.*³⁹⁾ The gels (in 0.6×15 cm glass tubes) contained 4% acrylamide, 6 M urea, and 1.25% Pharmalyte (pH 5–8). The lower electrode solution was 6 M urea containing 0.1 M H_2SO_4 , and the upper electrode solution was 1% NaOH. Electric focusing was initiated using a current of 1.5 mA per gel and the voltage was increased to 350 V. After focusing for 18–20 h at 4°C, the gels were removed from the tubes and stained for enzyme activity according to the slight modification of Ohishi and Shioya.³⁸⁾ Sucrose gradient isoelectric focusing was carried out in an LKB 8101 column (110 ml capacity) according to the manufacturer's instructions. The pH gradient was stabilized with a stepwise sucrose gradient of 0–40% (w/w). The samples of β -glucuronidase were layered in the middle of the gradient and electrofocused at 600 V for 60 h. After isoelectric focusing, 2 ml fractions were collected, and the enzyme activity or pH of each fraction was measured. Analytical isoelectric focusing on the polyacrylamide gels with Pharmalyte 3–10 was done according to the manuals provided by Pharmacia LKB Biotechnology, Inc. Gels were stained for enzyme activity as described.¹²⁾

SDS-PAGE SDS-PAGE was performed by the method of Laemmli.⁴⁰⁾ The gels were stained for protein with Coomassie brilliant blue and destained in 7% acetic acid.

Preparation of the Subcellular Fractions of Rat Liver In the case of ethanol treatment, 1.5 ml of 50% ethanol solution per 100 g body weight was orally administered at 90 min before sacrifice. In the case of colchicine treatment, 0.5 ml of the prepared colchicine solution (0.1 mg/ml) per 100 g body weight was injected intraperitoneally at 3 h before killing, and then rat liver subcellular fractions were prepared. The livers of 6 rats (40 g) were minced by passing them through the stainless-steel sieve (100 mesh),⁴¹⁾ mixed with 4 volumes of ice-cold 0.25 M sucrose, and then homogenized for 3 strokes with a Potter-Elvehjem homogenizer with a Teflon Pestle. The whole homogenate (20% homogenate) was centrifuged at $650 \times g$ for 5 min to remove cell debris and unbroken cells. The resultant supernatant was centrifuged at $10000 \times g$ for 10 min and then at $105000 \times g$ for 90 min to isolate the mitochondrial-lysosomal fraction and the total microsomal fraction, respectively. The pellets of total microsomal fraction were suspended in a small volume (about 18 ml) of 0.25 M sucrose and sufficient amounts of 2.1 M sucrose (about 22 ml) were added to obtain a 1.17 M sucrose concentration. Three Golgi subfractions were isolated from this suspension by the method of Ehrenreich *et al.*²³⁾ and Redman *et al.*²⁴⁾ The concentration of the total sucrose solution was determined at 25°C using an Abbe refractometer (Atago Kogakukikai Co., Tokyo) and all operations were carried out at 4°C.

Solubilization of β -Glucuronidase from Rat Liver Subcellular Fractions The rat liver microsomal and Golgi fractions were treated with 1% Triton X-100 or 0.26% sodium deoxycholate [DOC (final concentration)] according to the method of Dallner *et al.*⁴²⁾ after

hypotonic-shock treatment, and then centrifuged at $105000 \times g$ for 1 h to separate the supernatants and the tightly packed membranes. The supernatants thus obtained were used for PAGE analysis as the microsomal or Golgi content fractions. Since the amounts of β -glucuronidase obtained from the Golgi subfractions were limited, 3–5 preparations of the Golgi subfractions were combined and then solubilized with the buffer containing 1% Triton X-100. To the extracted Golgi fraction was added a specific IgG against β -glucuronidase and the resultant immunoprecipitate was analyzed by SDS-PAGE without 2-mercaptoethanol. On the other hand, the mitochondrial-lysosomal fraction was submitted to osmotic-shock treatment by resuspending the samples in the hypotonic solution (0.025 M sucrose), followed by centrifugation at $105000 \times g$ for 60 min. The obtained pellets were washed with 0.2 M KCl solution and then centrifuged again at $105000 \times g$ for 60 min. These supernatants were combined and used as the lysosomal content fraction. The microsomal and lysosomal content fractions were subjected to ammonium sulfate fractionation and organic solvent fractionation as described previously.¹³⁾

RESULTS AND DISCUSSION

Yields and Enzymatic Characterization of Golgi Subfractions Firstly, three Golgi subfractions were prepared from the control, the ethanol-, and the colchicine-treated rats, respectively, by the method of Ehrenreich *et al.*,²³⁾ and the morphology of the Golgi fraction was investigated by electron microscopy. A thin-section electron micrograph of the Golgi subfractions showed that the light Golgi fraction (GF-1) of control rat liver consisted of numerous secretory vesicles containing many VLDL particles, while the heavy Golgi fraction (GF-3) was largely composed of flattened saccular elements with VLDL contents at the bulbous ends. The intermediate Golgi fraction (GF-2) consisted of a mixture of flattened Golgi cisternae and small vesicles containing VLDL marker (data not shown). These morphological studies correlated well with those reported previously,²³⁾ and no significant alterations were found among the Golgi fractions from the control and drug-treated rat livers.

Secondly, the effects of ethanol or colchicine on the protein recovery and enzymatic characterization of the Golgi subfractions were investigated. Colchicine is known to depolymerize microtubules due to its binding with tubulin. As a result, colchicine causes an increase in the secretory vesicles in rat liver, although the detailed mechanisms of this action are not well understood. Also, previous study demonstrated that ethanol administration enhanced the synthesis of VLDL and resulted in an increase in secretory vesicles of the hepatocytes. Therefore, the effect of ethanol on the function of the Golgi complex was also investigated in rat liver. As shown in Table 1, colchicine pretreatment enhanced the accumulation of protein recovery in the Golgi subfractions, and a better yield of GF-1 was observed. These results suggested that secretory vesicles were accumulated in the Golgi subfraction of GF-1 in response to each drug.

The specific activities of galactosyltransferase and other

Table 1. Recovery of Proteins and Enzymes of the Golgi Subfractions Isolated from Ethanol- and Colchicine-Treated Rat Livers

Proteins and enzymes	Recovery (%)					
	Ethanol			Colchicine		
	GF-1	GF-2	GF-3	GF-1	GF-2	GF-3
Proteins	188.2	150.4	107.4	256.3	155.8	111.6
Galactosyltransferase	224.7	91.8	88.9	80.3	67.8	80.7
Glucose-6-phosphatase	44.3	60.0	112.0	56.5	75.4	79.3
5'-Nucleotidase	135.4	112.5	102.1	124.2	95.7	101.4
Acid phosphatase	81.3	93.8	91.2	66.9	73.7	88.1
β -Glucuronidase	61.8	100.2	110.5	50.0	103.3	100.0
β -N-Acetylglucosaminidase	65.1	65.1	66.7	68.6	54.7	52.0

Percent values represent the recovery of proteins and enzymes in the Golgi subfractions relative to the control Golgi subfractions. Ethanol was administered orally 90 min before sacrifice in a dose of 0.6 g/body weight and colchicine was injected intraperitoneally 3 h before killing in a dose of 0.05 mg/100 g body weight.

enzyme activities in the isolated Golgi subfractions are also shown in Table 1. A slightly depressed enzyme activity of galactosyltransferase in the colchicine-treated rats was found in the Golgi subfractions, whereas the highest specific activity of the enzyme in the GF-1 fraction was observed as well as a lower enzyme activity in the GF-2 or GF-3 fractions of the ethanol-treated rats. These results were compatible with those reported previously.⁴³⁾ The distinct results obtained from the different treatments cannot be explained at present. It is not clear whether colchicine has an inhibitory effect on the galactosyltransferase activity in the Golgi complex or, alternatively, the depressed activity is caused by the secondary effect of colchicine within the cell. To characterize the purity of isolated Golgi subfractions, the enzyme levels known as markers specific for other subcellular organelles were determined. Since the Golgi subfractions were prepared from the total microsomal fraction by virtue of different densities, microsomal contamination in the Golgi fraction was concerned. The enzyme level of the microsomal marker enzyme, glucose-6-phosphatase, was very low, suggesting that the microsomal fraction contamination in the Golgi fraction was small. Although 5'-nucleotidase was considered to be a marker enzyme of plasma membranes, a previous study demonstrated *via* morphological study that the 5'-nucleotidase was localized to the Golgi cisternae or secretory vesicles.⁴⁴⁾ In the present study, a considerably high specific activity of 5'-nucleotidase was detected in the GF-3 fraction, suggesting that plasma membrane contamination in the GF-3 fraction occurred. The level of enzyme activity of lysosomal acid hydrolases, including acid phosphatase, β -glucuronidase, and β -N-acetylglucosaminidase, was shown to be relatively low. Therefore, the Golgi subfractions thus obtained were proved to be fairly homogeneous with respect to their biochemical properties and morphological analysis. In the ethanol or colchicine-treated rats, the level of enzyme activities of the subcellular fractions remained unchanged in the three Golgi subfractions.

Effect of Ethanol or Colchicine on the Electrophoretic Properties of Golgi β -Glucuronidase Electrophoretic patterns of rat liver microsomal, Golgi, and lysosomal β -glucuronidases in the ethanol or colchicine-treated rats were analyzed and compared with those of the control liver enzyme by nondenaturing PAGE. The microsomal

and Golgi subfractions were subjected to hypo-osmotic shock treatment before solubilization with 0.26% DOC. On the other hand, the lysosomal fraction was prepared only by hypo-osmotic treatment. The solubilized enzymes from each fractions were loaded on nondenaturing PAGE, and the enzyme activities were stained as described in Materials and Methods.

As shown in Fig. 1, ethanol or colchicine pretreatment of the rats caused the Golgi β -glucuronidase to have a two major bands; the faster mobility band corresponded approximately with that of lysosomal enzymes, and the slower mobility band corresponded with that of the microsomal enzymes. Since the Golgi fraction was subjected to extensive osmotic shock treatment before solubilization, the possibility of lysosomal enzyme contamination in the prepared Golgi fraction was excluded. Therefore, these results implied that the post-translational modification step which converted microsomal β -glucuronidase to lysosomal β -glucuronidase in the Golgi complex was altered due to the ethanol- or colchicine-treated rats livers.

Next, to investigate the electrophoretic pattern of Golgi β -glucuronidase by isoelectric focusing on polyacrylamide gels, the Golgi subfractions isolated from the ethanol- or colchicine-administered rats were treated with a solubilization buffer containing 1% Triton X-100, and the activity staining pattern of Golgi β -glucuronidase was analyzed and compared with that of the control enzyme after electrophoresis. As shown in Fig. 2, the Golgi enzyme in the control rat livers revealed heterogenous activity bands with a more acidic pattern than those of microsomal enzymes, and those activity bands of the Golgi enzymes seemed to have a similar pattern to the lysosomal enzymes. These results were consistent with the data published previously by Tsuji *et al.*¹³⁾ In contrast, a significant change in isoelectric focusing patterns on the polyacrylamide gels in the ethanol- or colchicine-administered rats was observed. These results demonstrated that Golgi β -glucuronidase has a mixture of a neutral part and an acidic part in the heterogenous bands, suggesting that the intracellular conversion from the microsomal enzyme type to the lysosomal enzyme type was significantly inhibited due to the administration of ethanol or colchicine.

Next, the effect of ethanol and colchicine on the subunit structure of Golgi β -glucuronidase was characterized by

Fig. 1. Nondenaturing PAGE Analysis for the Golgi β -Glucuronidases from the Control Rats (I), the Ethanol-Treated Rats (II), and the Colchicine-Treated Rats (III)

The rat liver β -glucuronidases extracted from the microsomal fraction, the Golgi fraction, and the lysosomal fraction were electrophoresed on 7.5% polyacrylamide gel and the gels were stained for enzyme activity (0.04 units of each activity) as described in Materials and Methods. The microsomal and lysosomal fractions were prepared from the control rats, while the Golgi subfractions were prepared from the ethanol-treated rats (II) or from the colchicine-treated rats (III). The three prepared Golgi subfractions were solubilized and combined for the electrophoretic analysis. The position of the microsomal or lysosomal type enzymes is shown by arrows, respectively, at left in the colchicine-treated rats (III). (I) A, microsomal β -glucuronidase; B, Golgi β -glucuronidase; C, lysosomal β -glucuronidase. (II) A, microsomal β -glucuronidase; B, Golgi β -glucuronidase; C, lysosomal β -glucuronidase. (III) A, microsomal β -glucuronidase; B, Golgi β -glucuronidase; C, lysosomal β -glucuronidase.

Fig. 2. Electrophoretic Comparison of the Isoelectric Focusing Patterns of Golgi β -Glucuronidases Extracted from the Control Rat Livers (I), the Ethanol-Treated Rat Livers (II), and the Colchicine-Treated Rat Livers (III)

The rat liver β -glucuronidases extracted from the microsomal fraction (A), the Golgi fraction (B), and the lysosomal fraction (C) were applied to the isoelectric focusing on polyacrylamide gels with Pharmalyte (pH 5–8), and the gels were stained for enzyme activity after electrophoresis, as described in Materials and Methods. The microsomal and lysosomal fractions were prepared from the control rats, while the Golgi subfractions were prepared from the ethanol-treated rats (II) or from the colchicine-treated rats (III).

SDS-PAGE. Since the amounts of β -glucuronidase obtained from the Golgi subfractions were limited, 3–5 preparations of the Golgi subfractions were combined and then solubilized with the buffer containing 1% Triton X-100. To the extracted Golgi fraction was added a specific IgG against β -glucuronidase, and the resultant immunoprecipitate was analyzed by SDS-PAGE without 2-mercaptoethanol. The result is shown in Fig. 3. The native Golgi β -glucuronidase was demonstrated to have three distinct bands, of which subunit a with 79000 Da was most intensely stained. This result suggests that the proteolytic processing step to generate the processed form of the

74000 Da polypeptide of β -glucuronidase proceeds within the Golgi complex. The electrophoretic pattern of Golgi enzyme seemed to be similar to that of the lysosomal type enzyme, and the heterogeneous subunit structure of Golgi β -glucuronidase was not changed by the administration of ethanol or colchicine. However, we cannot determine by SDS-PAGE analysis whether a minor modification in processing the polypeptide of β -glucuronidase from the NH₂-terminus or COOH-terminus occurs in the Golgi complex, or whether this processing mechanism is changed by the administration of ethanol or colchicine. Therefore, the amino acid sequence of the NH₂- and COOH-terminus

Fig. 3. SDS-PAGE of the Golgi β -Glucuronidase Prepared from the Control Rat Livers (I) and the Colchicine-Treated Rat Livers (II)

The rat liver β -glucuronidases extracted from the microsomal fraction (A), the Golgi fraction (B), and the lysosomal fraction (C) were immunoprecipitated with specific IgG, and the resultant immunoprecipitates were subjected to SDS-PAGE in the absence of 2-mercaptoethanol. The microsomal and lysosomal fractions were prepared from the control rats, while the Golgi subfractions were prepared from the control rats (I) or the colchicine-treated rats (II). The detailed experimental procedure is described in Materials and Methods. The gel was stained for protein with Coomassie brilliant blue. The bands marked by a, b, and c show the subunit component of lysosomal β -glucuronidase, respectively (II). The subunit band c is not discernible on the SDS-gel. In the control rat livers (I), the position of each microsomal and lysosomal β -glucuronidase is indicated by arrows at right. The positions of immunoglobulin G and its heavy chains are marked by IgG and H, respectively.

of processed β -glucuronidase remains to be elucidated.

As to Golgi β -glucuronidase, it was previously demonstrated by Tsuji *et al.*¹³⁾ that the electrophoretic pattern on polyacrylamide gel of the Golgi β -glucuronidase, with a relatively acidic pI of 5.7–6.6, resembled that of a lysosomal enzyme, but apparently differed from that of a microsomal enzyme. These results indicate that the post-translational modification of β -glucuronidase in generating a lysosomal-type enzyme starts to take place in the Golgi complex. The Golgi fraction isolated by Tsuji *et al.*¹³⁾ is a modification of the procedure described by Fleischer and Fleischer⁴⁵⁾ and by Morr *et al.*,⁴⁶⁾ and is different from the preparation of Golgi subfraction presented here. However, it should be noted that the electrophoretic behavior of Golgi β -glucuronidase with a single major band is approximately similar to that of the lysosomal enzyme (Fig. 1); therefore, the data presented here are in good accord with those of the previous data.¹³⁾ To further determine the inhibitory effect of ethanol or colchicine on other subcellular fractions, the electrophoretic properties of β -glucuronidase extracted from the microsomal fraction was examined by applying it to the column isoelectric focusing, and each enzyme activity peak of microsomal enzyme was compared with that of the control microsomal enzyme. The result is shown in Fig. 4. The result showed that the isoelectric point of control microsomal β -glucuronidase, of pI 6.98, had moved to a more alkaline position, pI 7.20, in the ethanol-treated rats, and to a pI of 7.12 in the colchicine-treated rats, suggesting that the electrophoretic nature of the microsomal enzyme was somehow changed due to the administration of ethanol or colchicine. Therefore, we supposed that the electrophoretically altered

Fig. 4. Isoelectric Focusing of the Microsomal β -Glucuronidase Isolated from the Control Rat Livers, the Ethanol-Treated Rat Livers, and the Colchicine-Treated Rat Livers

Each of the extracted microsomal fractions was subjected to isoelectric focusing on the column at 4°C with pH 5–8 Pharylyte. Fractions of 2 ml were collected after the isoelectric focusing for 60 h at a constant voltage of 600 V, and the pH of each fraction was determined. The enzyme activities extracted from the control rats (○), the ethanol-treated rats (■), and the colchicine-treated rats (▲) were determined.

microsomal-type enzyme was transported to the Golgi complex and then accumulated without undergoing a further modification step in the ethanol- or colchicine-treated rat livers. These data suggested that ethanol or colchicine alone had an inhibitory effect on the intracellular post-translational modification step for the conversion of a microsomal-type enzyme to a lysosomal-type enzyme. A recent paper described the fact that the administration of chronic ethanol to rats had an apparent inhibitory effect on the sialylation of transferrin in the Golgi complex, suggesting that chronic ethanol intoxication to rats has a significant influence on the Golgi enzymes regulating sialylation.²⁵⁾ According to our previous investigation, both the microsomal and lysosomal β -glucuronidases were found to have high-mannose type oligosaccharides, suggesting that sialylation did not occur in β -glucuronidase in the Golgi complex.¹⁴⁾ Therefore, the maturation step for the oligosaccharide moiety of Golgi β -glucuronidase may not be changed due to the administration of ethanol or colchicine. Since it was demonstrated previously²⁷⁾ that colchicine treatment caused a significant inhibitory effect on the proteolytic conversion of secretory protein, albumin, in the Golgi complex, it is possible to speculate that colchicine itself may be inhibitory on the expression of processing proteinase in the Golgi complex. Alternatively, if the modification step in generating the lysosomal β -glucuronidase operates in the acidic endosomes, but not

in the Golgi complex, it is reasonable to assess that the efficient transport of β -glucuronidase from the Golgi complex to the endosomes is considerably delayed by the action of colchicine; therefore, we observed an inhibitory effect on the processing of β -glucuronidase in the Golgi subfractions prepared from the colchicine-treated rat livers.

Taken together, the data thus obtained led us to assume that microsomal β -glucuronidase was transported through the Golgi complex to the lysosomes, and that the microsomal enzyme was processed and converted to the lysosomal enzyme in the Golgi complex. Due to the administration of ethanol or colchicine to rats, the modification process of microsomal-type enzymes in generating the lysosomal-type enzymes was significantly inhibited. It was interesting to note that the observed inhibitory effect of ethanol on Golgi β -glucuronidase resembled that of colchicine, which was in contrast to the case of rat albumin reported previously,²⁷⁾ because only colchicine was found to be inhibitory for the maturation of rat serum albumin. At present, detailed mechanisms of the post-translational modification of β -glucuronidase in the Golgi complex has not yet been clarified. Further analysis is now in progress to demonstrate the intracellular processing activity of β -glucuronidase.

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