EXOCYTOTIC EXCRETION OF DEXTRAN SULFATES FROM LIVER TO BILE

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Abstract—When dextran 35S-sulfates (DSs), anticoagulants and antilipemic agents, were intravenously given to rats, the specific radioactivity was five to twenty times higher in the lysosomal than in the other subcellular fractions of liver, but the counts in this fraction were only 10% of total radioactivity distributed to the liver. Therefore, we investigated the biliary excretion of 35S-DSs with a similar sulfur content of 18% and three different average molecular weights (AMW) of 3000, 20,000, and 200,000. The excretion rate was about 2% of the radioactivity given, per hour, after intravenous administration of 35S-DS with AMW of 20,000, 10 mg/kg. At this time, acid phosphatase activity and calcium content of bile also were increased, and well correlated with the amount of the radioactivity excreted to bile. The amount of the radioactivity distributed to the lysosomal fraction is correlated with the enhanced activities of Na+-K+-dependent and Ca2+-activated ATPase in this fraction, indexes of endocytotic and exocytotic transports. DSs significantly enhanced 45Ca levels of the lysosomal, but not the cytosol fraction when 45CaCl2 was given alone or simultaneously with DSs. According to our previous reports that DSs are transferred to the lysosomal fraction of liver and intestinal mucosa by endocytosis, the present results suggest that DSs, especially with high AMW and which are distributed to liver lysosomes are rapidly excreted into bile by exocytosis and accompanied by calcium and lysosomal enzymes.

Macromolecules can enter into cells (1-3). DeDuve and Wattiaux (4) reported that dextran, a high molecular weight polysaccharide, is transported to liver cells and accumulates within endocytotic granules in the cytosol. Most of the granules can be collected in the lysosomal fraction by subcellular fractionation (5). Aronson and Davidson (6) and our group (7) have indicated that acid polysaccharides, chondroitin sulfates and dextran sulfates (DSs), are found in the lysosomal fraction of rat liver after intravenous administration. When 35S-labeled DS with an average molecular weight of 200,000 was given intraduodenally, the lysosomal fraction of intestinal mucosa contained 40% of the tissue radioactivity (8). In the liver lysosomal fraction only 10% of the tissue radioactivity was found after intravenous administration of the DS (7).

Acid polysaccharides distributed to liver lysosomes are eliminated by catabolism (6) and biliary excretion. DeDuve and Wattiaux (4) indicated that dextran which was transferred to liver lysosomes is rapidly excreted to bile. Therefore, the difference in lysosome/tissue ratio of the radioactivity between liver and intestinal mucosa may be due to the rapid biliary excretion of 35S-DSs distributed to liver lysosomes. Thus, we examined the biliary excretion of DSs with a similar sulfur content of 18% and three different average molecular weights of 3000, 20,000, and 200,000. Our findings are reported herein.
MATERIALS AND METHODS

Experimental animal: In all experiments female Donryu rats (Nihon Rat Co., Urawa, Japan), about 200 g, were used after an 18 hr fasting period.

Composition of DSs: DS-L, intrinsic viscosity (IV) 0.027, average molecular weight (AMW) 3000, sulfur content (SC) 18.3%; DS-M, IV 0.082, AMW 20,000, SC 18.1%; DS-H, IV 0.198, AMW 200,000, SC 18.1%. IV was determined in 2 M NaCl at 37°C. All of the DSs were labeled with 35S, and had a specific activity of about 1.5 μCi/mg. These DSs were a gift of Meito Sangyo Co., Nagoya, Japan.

Drug administration: The solution of 35S-DS, 100 mg (150 μCi)/ml in 0.9% saline, was given into a femoral vein in a dose of 10 mg (15 μCi)/kg. In the experiment designed to measure calcium levels of the lysosomal and cytosol fractions of liver, 45CaCl2 1 mmole (5 μCi)/kg was given alone i.v. or simultaneously with unlabeled DSs.

Collection of bile: Rats were anesthetized with ether, fixed on a warm box (37-38°C), and the common bile duct was cannulated. Bile was collected at 1 hr intervals. The volume of the bile collected was measured gravimetrically. The bile was diluted with distilled water, and the samples were used to measure the radioactivity, acid phosphatase activity, calcium, and protein contents.

Subcellular fractionation of liver: Rats were decapitated 1 hr after DS administration. Immediately, the liver was perfused with cold 0.9% saline and subsequently with cold 0.25 M sucrose, and homogenized in 0.25 M sucrose. According to the method of DeDuve et al. (9), the lysosomal and supernatant (cytosol) fractions were obtained as previously described (7). The lysosomal pellet was suspended in distilled water. The samples were used to measure 45Ca content, Na+-K+-dependent and Ca2+-activated ATPase activities, and protein content.

Assays: Acid phosphatase, Na+-K+-dependent and Ca2+-activated ATPase activities were assayed respectively by the methods of Bessey et al. (10), Quigley and Gotterer (11), and Kagawa and Tomizawa (12). Details of incubation conditions are described in the figures. Calcium in bile and the lysosomal fraction was determined by the method of Webster (13) after deproteinization of the samples. Protein was determined by the method of Lowry et al. (14). The radioactivity was measured using a liquid scintillation spectrophotometer (Aloka, model 651) and a toluene scintillator.

RESULTS

Figure 1 shows the biliary excretion of the radioactivity after intravenous administration of 35S-DSs. When 35S-DS-L or 35S-DS-H was given, the maximal excretion was found at 1 hr. The excretion rate of 35S-DS-M, however, gradually increased and reached a peak at 4 hr. None of the DSs used in this experiment had any effect on the volume of bile, within 6 to 8 hr after the administration. In addition, more than 90% of the radioactivity in bile was detected as inorganic 35S-sulfate by paper chromatographic analysis (7) (not included in the data).

To determine whether the radioactivity excreted into bile came from that distributed
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to the lysosomes of liver after 35S-DS administration, we measured the acid phosphatase activity, which is a marker enzyme of lysosomes, in bile. Figure 2 shows a correlation between the radioactivity and acid phosphatase activity in bile. A statistically significant correlation was found in DS-M and DS-H. Although no significant correlation was observed in DS-L through 6 hr, the radioactivity excreted within the early time (1 to 3 hr) after the administration well correlated with the acid phosphatase activity. The result indicates that the biliary excretion of lysosomal contents from liver cells is provoked simultaneously with the excretion of DSs, when DSs are given intravenously.

On the basis of this evidence, it is likely that DSs accumulated by the lysosomes are released into the bile by an exocytotic transport. In general, exocytosis depends on an increase in calcium influx into cells (15). Therefore, we measured calcium levels in the
cytosol and lysosomal fractions of liver 1 hr after intravenous administration of DSs. The result is shown in Fig. 3. All of the DSs used in this experiment significantly increased $^{45}\text{Ca}$ levels in the lysosomal, but not the cytosol fraction. Calcium content and the specific radioactivity in the lysosomal fraction were respectively $3.6\pm1.2$ and 100 for control ($^{45}\text{CaCl}_2$ alone), $4.7\pm1.4$ and 300 for DS-L, $5.3\pm0.7$ and 460 for DS-M, and $4.9\pm1.2$ nmoles calcium/mg of protein (mean $\pm$ S.D., n=4) and 440 dpm/nmole of calcium for DS-H. The result indicates that DSs enhance the transport of calcium not to the cytosol, but to the lysosomal fraction of liver.

According to the data shown in Figs. 2 and 3, there is a possibility that calcium in the lysosomes may be secreted to bile similarly to DSs and lysosomal enzymes. To confirm this possibility, the biliary excretion of calcium was examined and compared with that of the radioactivity after $^{35}\text{S}$-DS administration. The result is shown in Fig. 4. There was a significant correlation between the biliary excretion of the radioactivity and calcium in DS-M and DS-H.

$\text{Na}^+\text{-K}^+\text{-dependent}$ (16) and $\text{Ca}^{2+}\text{-activated}$ (12, 17) ATPase are present in liver plasma membranes, and have been considered to be marker enzymes of endocytosis (18) and exocytosis (19), respectively. Therefore, we examined these ATPase activities of the lysosomes. Figure 5 shows $\text{Na}^+\text{-K}^+\text{-dependent}$ and $\text{Ca}^{2+}\text{-activated}$ ATPase activities and the radioactivity of the lysosomal fraction of liver 1 hr after administration of $^{35}\text{S}$-DSs. Both DS-L and DS-M significantly enhanced these ATPase activities, and this is well correlated with the specific radioactivity. Although DS-H did not stimulate $\text{Na}^+\text{-K}^+\text{-dependent}$ ATPase activity, it appears that there is a good correlation between these two ATPase activities and the radioactivity after DS-H administration. The result indicates that DSs increase $\text{Na}^+\text{-}
DISCUSSION

Monkhouse and Dickies (20) had attributed the elimination of injected acid polysaccharides to excretion by the kidneys, the uptake by reticular endothelial cells, and the distribution to extracellular space. However, the present report demonstrated that large amounts of $^{35}$S-DSs are excreted into the bile. This is supported by our earlier finding (7) that DSs accumulate in the liver (about 2.5% of the radioactivity given per g of liver). There are at least two possible explanations for the biliary excretion of DSs.

First, DS transfer into bile may occur from the cytosol. Most of the radioactivity excreted to the bile was detected as inorganic $^{35}$S-sulfate. In our previous work (7) we found that, when $^{35}$S-DS-L or $^{35}$S-DS-H was given i.v., inorganic $^{35}$S-sulfate was found in the cytosol and lysosomal fractions of liver. Thus, $^{35}$S-DSs appear to be transferred into liver cells, desulfated, and finally the released inorganic $^{35}$S-sulfate excreted partly into the bile. However, DSs and the released inorganic sulfate trapped by the cytosol may not be available for the biliary excretion, because no direct correlation was noted between the time course of the radioactivity distributed in the cytosol (7) and the radioactivity excreted into bile.

Second, there is a possibility that DSs distributed to the lysosomes are excreted by exocytosis into bile.

Meijer and Willighagen (21) have shown that, when dextran with a high molecular weight is given to mice, peribiliary bodies develop in the immediate vicinity of bile canaliculas, which include dextran and later fuse with primary lysosomes containing hydrolytic enzymes, such as acid phosphatase, aryl sulfatase, and dextranase. DeDuve and Wattiaux (4) have shown that endocytotic granules can be separated from nuclear, mitochondrial, and microsomal granules by subcellular fractionation. We demonstrated herein that there is a good correlation between the time course of the radioactivity and acid phosphatase activity.
excreted into bile after intravenous administration of $^{35}$S-DS-M or $^{35}$S-DS-H. One might expect that acid phosphatase in bile would come from the cytosol. This possibility can be excluded as acid phosphatase activity in the cytosol fraction was not increased by DS administration. Therefore, the radioactivity and acid phosphatase activity in bile probably come from the lysosomes.

Although we (7) previously found that the unchanged forms of DSs are transported to the lysosomes, the present report demonstrated that the radioactivity observed in bile was almost all inorganic $^{35}$S-sulfate. This may be due to an enhanced activity of sulfatase in the lysosomes during exocytosis and such is now being investigated.

Secretory processes which are probably the exocytosis of granular materials, such as neurotransmitters, some amines, and secretory hormones, require extracellular calcium (15, 22). However, it is becoming apparent that some tissues, platelets for example, utilize an intracellular calcium pool for their secretory activity (23). Here, we could not obtain the data that the biliary excretion of $^{35}$S-DSs depends on an increase in calcium influx into the cytosol of liver cells, because calcium levels of the cytosol were not at all increased by administration of DSs. DSs, however, significantly increased calcium contents in the lysosomes. We (8) already showed that EDTA and a decrease in extracellular calcium concentration inhibit the DS transfer to the lysosomal fraction of intestinal mucosa, and speculated that an endocytotic transport of DSs requires extracellular calcium which is transferred to endocytotic granules (pinosomes) and finally to lysosomes (secondary lysosomes) accompanying DSs. The present report may support this hypothesis. It is also possible that the endocytotic granules which were formed by administration of DSs take up calcium from the cytosol, because the lysosomal fraction after DS administration had a high activity of Ca$^{2+}$-activated ATPase. As DSs increase calcium contents in the mitochondrial and lysosomal fractions of liver (24), it is likely that the transfer of calcium into the cytosol is enhanced simultaneously with the transport to endocytotic granules during an endocytotic uptake of DSs by liver cells.

Ca$^{2+}$-activated ATPase has been shown to be present in liver plasma membranes (12, 17). It seems likely that the ATPase is localized in the inner surface of liver plasma membranes similar to its localization in erythrocytes (25). Endocytotic granules are developed by an invagination of the plasma membranes which bind DSs and may turn outside in. Thus, Ca$^{2+}$-activated ATPase that is probably localized in the outer membranes of endocytotic granules may enhance the transfer of calcium to the lysosomes which were formed by the fusion of the granules and primary lysosomes. The endocytotic granules that were formed by DS administration probably contain extracellular calcium and further take up calcium from the cytosol. Terepka et al. (26) speculated that calcium is transported transcellularly by a process that involves endocytosis and granular formation. Their hypothesis may support our finding that the endocytotic granules which were formed by DS administration accumulate calcium, and their contents are excreted by exocytosis into bile.

In the present report, DS-L enhanced calcium contents and Ca$^{2+}$-activated ATPase
activity in the lysosomal fraction of liver, while there was no good correlation between the
time course of the radioactivity and calcium excreted into bile. This may be because the
inorganic $^{35}$S-sulfate released from $^{35}$S-DS-L is excreted from the cytosol by a transmembrane
transport and from the lysosomes by an exocytotic transport. On the other hand, the
finding that DS-H did not enhance Na$^+$-K$^+$-dependent ATPase activity of the lysosomes
may be due to less formation of endocytotic granules in liver cells 1 hr after DS-H admin-
istration (7).

In summary, the DSs with high molecular weights that were transferred to the lysosomes
of liver are probably excreted into the bile by exocytosis, and extracellular calcium is probably
necessary for an invagination of plasma membranes, transport to the lysosomes, and excretion
into the bile simultaneously with DSs and lysosomal enzymes.

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