ABSENCE OF HYALURONIDASE IN CULTURED HUMAN SKIN FIBROBLASTS

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SUMMARY:The in vitro degradation of [^{35}S]chondroitin sulfate was investigated in human fibroblasts and rat liver. In rat liver, preparations of chondroitin sulfate were shown to be degraded by the concerted action of endoglycosidase and exoglycosidases. However, with human skin fibroblast preparations, hyaluronidase activity was not detected and chondroitin sulfate was degraded by exoglycosidase action.

Degradation of certain glycosaminoglycans is currently believed to involve an endoglycosidase (hyaluronidase) cleavage of the polysaccharide chain to produce oligosaccharides, which are then attacked by exoglycosidases (β-N-acetylhexosaminidase, β-glucuronidase) and sulfatases to produce monosaccharides and inorganic sulfate (1). Previous studies failed to demonstrate hyaluronidase activity in fibroblast extracts by the viscometric method using hyaluronic acid as substrate (2, 3). Using [^{35}S]chondroitin sulfate (CS) as a model substrate, we investigated the possible pathways of chondroitin sulfate catabolism (endoglycosidase, exoglycosidase, or a combination of the two) in cultured human skin fibroblasts. These cells were selected because of their extensive use in detecting genetic disorders of polysaccharide metabolism.

MATERIALS AND METHODS

All skin fibroblasts utilized in these studies were isolated from biopsies obtained of normal individuals by Dr. Reuben Matalon and were grown under conditions described (4, 5).

Preparation of [^{35}S]Chondroitin Sulfate.

[^{35}S]Chondroitin sulfate was prepared from the femoral and tibial epiphyses of 13 day old chick embryos as previously described (4) with the modification

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that alkali digestion (0.5 M-NaOH for 2 days at 4° C [6]) was substituted for papain digestion.

Enzyme Preparation and Incubation.

Mitochondrial-Lysosomal (M and L) fractions were prepared from cultured skin fibroblasts (4) and from rat liver using differential centrifugation. The rat liver was minced, homogenized in 0.25 M sucrose with 2 strokes of a Potter--Elvehjem homogenizer and centrifuged as previously described (4).

Incubation mixtures consisted of 20μl of 0.1 M Na acetate buffer, pH 4.5, 5μl CS (20,000 cpm [specific activity 5000 cpm/μg]), 5μl H_2O and 70μl enzyme containing 5-7 mg protein/ml solution. β-glucuronidase was inhibited by replacing the water in the above incubation with 3.0 M NaCl (9). Samples were incubated at 37° for 18 hr. in a shaking water bath. After incubation, aliquots were electrophoresed for 1 hr. at 400 v on 1 inch paper strips (Whatman No. 3) to determine the amount of inorganic sulfate released (7). The remainder of the incubation mixture was centrifuged at 9,000 x g for 10 min. and the supernatant solution which contained 80-90% of the counts was subjected to gel filtration on a column (1 x 200 cm) of Sephadex G-50 (Fine). Three ml fractions were collected by elution with 0.2 M pyridine acetate buffer, pH 5.0 and 1 ml aliquots counted in a Packard Tricarb Liquid Scintillation Spectrometer as previously described (8). Molecular weights of CS fractions were estimated by gel chromatography on a column (1 x 200 cm) of Sephadex G-200 (12). Other chemicals used were reagent grade.

RESULTS and DISCUSSION

After incubation of [^{35}S]chondroitin sulfate with rat liver M and L fraction nearly all of the label was eluted in the included volume of a column of Sephadex G-50 (Fig. 1a and Table 1). 30% of the total label was identified as inorganic sulfate (Table 1). These results are consistent with a mechanism of degradation in which CS oligosaccharides produced by the action of hyaluronidase are degraded by the concerted action of β-glucuronidase, sulfatase and β-N-acetylgalactosaminidase to produce [^{35}S]sulfate.

To inhibit β-glucuronidase activity rat liver M and L fractions were incubated with [^{35}S]chondroitin sulfate in the presence of NaCl. Under these conditions, compared to incubations without NaCl, higher molecular weight oligosaccharides were produced and considerably less inorganic sulfate was

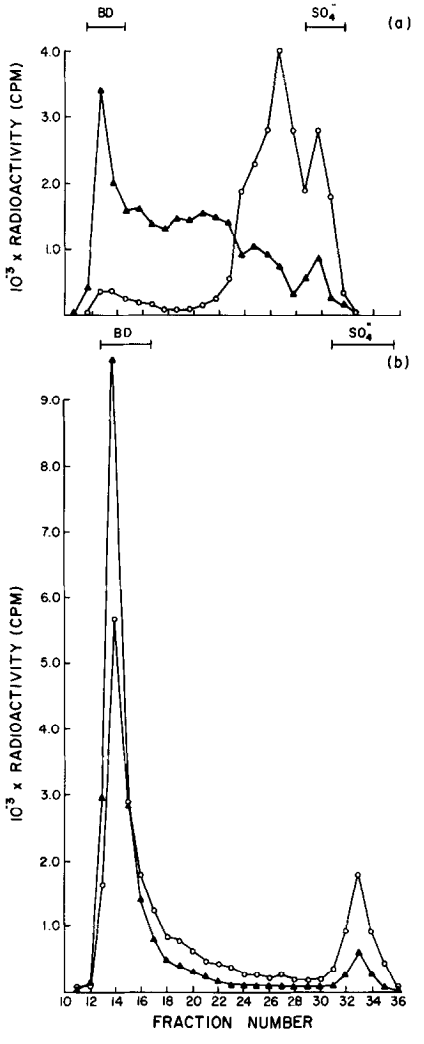

Fig. 1: Elution profiles from a column of Sephadex G-50 of the products obtained after incubation of [^{35}S]chondroitin sulfate with M and L fractions from a)rat liver and b) cultured human skin fibroblasts. Profiles of radioactivity for incubation mixtures with (▲---▲) and without (0---0) NaCl are shown. BD indicates the void volume of the column measured with Blue Dextran (Pharmacia). SO_4 indicates the elution volume of inorganic sulfate measured with [^{35}S]sulfate

released (Table I and Fig. 1a). These findings indicate that degradation of CS in the presence of NaCl results primarily in the accumulation of oligosaccharides from the action of hyaluronidase.

Whereas when M and L fractions from cultured skin fibroblasts was used

Table I: Distribution of [^{35}S]Chondroitin Sulfate Degradation Products on Sephadex G-50

M and L fractions from rat liver and cultured human skin fibroblasts were incubated with [^{35}S]chondroitin sulfate as described under Methods. β-Glucuronidase activity was inhibited in incubations containing NaCl (9). Each incubation mixture was fractionated by chromatography on a column of Sephadex G-50 (figs. 1a and 1b). Boiled blank reaction mixture contained M and L fraction from rat liver heated at 100° C for 10 minutes before incubation with [^{35}S]chondroitin sulfate as described for the other reaction mixtures. [^{35}S] chondroitin sulfate substrate gave a similar distribution of radioactivity on Sephadex G-50 to the boiled blank. Fractions pooled to give inorganic sulfate values as shown in Figs. 1a and 1b. Values for included materials were obtained by pooling fractions between the void and sulfate peaks (figs. 1a and 1b.).

	Void	Included	Inorganic Sulfate
		percent of total counts	
Rat Liver			
With NaCl	23.5	68.5	8.0
Without NaCl	4.4	65.5	30.0
Human Fibroblasts			
With NaCl	82.9	12.1	5.0
Without NaCl	53.5	25.5	21.0
Boiled Blank	88.5	11.5	0.0

as the enzyme source, quite a different profile of radioactivity was obtained on Sephadex G-50. In the presence of NaCl there was 5.0% inorganic sulfate released (Table I) with essentially no change in the average molecular weight of the CS substrate (Fig. 1b and 2). This profile of radioactivity would be expected in a system in which there was incomplete inhibition of β-glucuronidase. In the absence of NaCl there was a 21% release of inorganic sulfate (Table I) and a shift in the average molecular weight of the CS substrate (Fig. 2). The decrease in average molecular weight (from 27,000 to 18,000) with the fibroblast fractions corresponds well with inorganic sulfate released con-

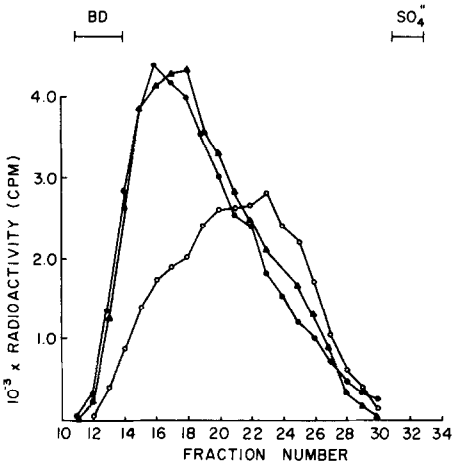

Fig. 2: Elution profile from a column of Sephadex G-200 of the components in the void-volume peak (Fractions 13 to 24) from Sephadex G-50 shown in Fig. 1b. Radioactivity profiles obtained for boiled blank (▲---▲), incubation mixtures with (●---●) and without (0---0) NaCl are shown.

Table II: The Effect of Molecular Weight of [^{35}S]Chondroitin Sulfate Substrate on Lysosomal Degradation Ability

[^{35}S]chondroitin sulfates of different average molecular weights were prepared by pooling fractions of the chick [^{35}S]chondroitin sulfate collected from a column of Sephadex G-200. Molecular weights shown were calculated from the average elution volume of each pool from Sephadex G-200 (12). [^{35}S]chondroitin sulfate shown with a molecular weight of 2000 was isolated from a testicular hyaluronidase digest of the chick [^{35}S]chondroitin sulfate (4). Each chondroitin sulfate fraction was incubated with M and L fraction from cultured human skin fibroblasts as described under Methods. Sulfate released was determined by fractionation of each incubation mixture by chromatography on a column of Sephadex G-50 as shown in Fig. 1b.

Molecular Weight	$\%SO_4$ Released
38,000	29
27,000	19
12,500	16
2,000	14

sequent to exoglycosidase degradation with no hyaluronidase involvement in either the β-glucuronidase inhibited or non-inhibited incubation. The absence of oligosaccharides in the fibroblast digestion compared with liver supports the contention that hyaluronidase was not active in the degradation of chondroitin sulfate.

When CS of varying average molecular weight was incubated with the cultured human fibroblast M and L fraction, the higher molecular weight components were found to be the better substrates (Table II). In contrast to this finding Tudball and Davidson (10) reported that CS oligosaccharides but not intact CS were substrates for CS sulfatase isolated from rat liver. The reasons for the difference are not clear.

Since Cashman et al. (9) were able to isolate hyaluronidase from rat skin, we were interested in whether this activity was lost from fibroblasts during manipulation in tissue culture or was species specific, since we were unable to demonstrate hyaluronidase activity in cultured human fibroblasts. Using whole homogenates of rat skin and whole homogenates of the M and L fraction from cultured rat fibroblasts, in the presence or absence of 0.15 M NaCl at pH 3.0 and 4.5, we were able to demonstrate substantial (7-30%) release of inorganic sulfate with no formation of oligosaccharides indicating that skin and cultured rat skin fibroblast degradation of CS proceeds by exoglycosidase activity alone. Mixtures of fibroblast and liver M and L fractions in β-glucuronidase-inhibited incubations produced no diminution of oligosaccharide production ruling out the possibility of a fibroblast hyaluronidase inhibitor. These results suggest that if hyaluronidase is present in skin it is there in such low amounts to make it insignificant in the degradation of CS as measured under the conditions described above. In skin, therefore, CS is degraded by the concerted action of exoglycosidases and sulfatases. These results support the conclusion that degradation of CS proceeds by two different pathways. In hyaluronidase-containing tissues such as liver, degradation occurs by way of hyaluronidase followed by serial action of exoglycosidases and sulfatases, while in fibroblasts, degradation is confined to the action of exoglycosidases and sulfatases.

The small amount of sulfate (5-8%) released in the β-glucuronidase inhibited incubation (Table I) could result from incomplete inhibition of this enzyme, assuming a stepwise degradation from the non-reducing end with the

sequential action of β-glucuronidase, 4 or 6-O-sulfatase, and N-acetylhexosaminidase. Another possible explanation for this small amount of sulfate release could be that β-glucuronidase is completely inhibited and the sulfate is cleaved from an internal position, possibly, close to, but not necessarily at the non-reducing terminal N-acetylgalactosamine sulfate.

This latter explanation is supported by the observation that lysosomal fractions from fibroblasts with Sandhoff-Jatzkewitz disease (deficient in β-N-acetylhexosaminidase) release the same amount of inorganic sulfate as do normal β-glucuronidase inhibited lysosomal fractions (4). Experiments with an oligosaccharide prepared from CS indicate that internal sulfates are more susceptible to cleavage by the fibroblast 4 and 6-O-sulfatases than the non-reducing terminal sulfate (11). Since only 5-8% of the sulfates are cleaved when stepwise degradation of the CS chain is inhibited, it is possible that only specific sulfate linkages are susceptible; possibly only those 2 or 3 hexosamines from the non-reducing terminus.

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