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THE COMMON IDENTITY OF UDP-*N*-ACETYLGALACTOSAMINE 4-SULFATASE, NITROCATECHOL SULFATASE (ARYLSULFATASE), AND CHONDROITIN 4-SULFATASE

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Summary

Evidence is presented indicating that three sulfatase activities towards UDP-*N*-acetylgalactosamine 4-sulfate, nitrocatechol sulfate, and chondroitin 4-sulfate are functions of the same hen oviduct enzyme. Using chondroitin [^{35}S]sulfate from chick embryo cartilage, it is shown that hydrolysis of ester sulfate by this enzyme is limited to 4-sulfate groups occurring in the non-reducing terminal *N*-acetylgalactosamine 4-sulfate and *N*-acetylgalactosamine 4,6-bissulfate residues.

Introduction

We previously described [1] the occurrence in hen oviduct of a sulfatase which catalyzes the following two reactions

$$\text{UDP-GalNAc-4-sulfate} + H_2O \rightarrow \text{UDP-GalNAc} + SO_4^{2-}$$

$$\text{UDP-GalNAc-4,6-bissulfate} + H_2O \rightarrow \text{UDP-GalNAc-6-sulfate} + SO_4^{2-}$$

A sulfatase activity towards UDP-GalNAc-4-sulfate was also found in rat liver

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Abbreviations: UDP-GalNAc, UDP-GalNAc-4-sulfate, UDP-GalNAc-6-sulfate, and UDP-GalNAc-4,6-bissulfate, UDP-*N*-acetylgalactosamine and its derivatives bearing a sulfate at position 4, a sulfate at position 6, and two sulfates at positions 4 and 6, respectively, of the hexosamine moiety; ΔGlcUA-GalNAc(4-SO4), ΔGlcUA-GalNAc(6-SO$_4$), and ΔGlcUA-GalNAc(4,6-bis-SO$_4$), Δ^4-glucuronosyl(β1 → 3)-*N*-acetylgalactosamine derivatives bearing a sulfate at position 4, a sulfate at position 6, and two sulfates at positions 4 and 6, respectively of the hexosamine moiety.

and kidney, but available evidence indicated that the liver and kidney activities are due to the action of an arylsulfatase, for which *p*-nitrophenyl sulfate also is an efficient substrate. Since the enzyme from hen oviduct did not exhibit such an activity on *p*-nitrophenyl sulfate, we considered that the oviduct enzyme is a sulfatase specific for the sulfated nucleotides and differs in this respect from the arylsulfatase in rat liver and kidney.

Fluharty et al. [2] have since reported that cultured fibroblasts derived from patients with Maroteaux-Lamy syndrome were deficient in UDP-*N*-acetylgalactosamine 4-sulfatase to the same extent that they were deficient in arylsulfatase B for nitrocatechol sulfate. This deficiency is consistent with the diminution in activity of chondroitin sulfate (or dermatan sulfate) *N*-acetylgalactosamine 4-sulfatase described by O'Brien et al. [3] and Matalon et al. [4], suggesting that at least in the fibroblasts the sulfatase activity for UDP-GalNAc-4-sulfate, nitrocatechol sulfate, and chondroitin 4-sulfate are due to one, common enzyme. The information derived from the study with human fibroblasts prompted us to re-investigate the UDP-*N*-acetylgalactosamine 4-sulfatase in hen oviduct in the hope that more conclusive and detailed knowledge of its specificity might be obtained.

Materials and Methods

Unless otherwise indicated, the materials are the same as those employed in previous papers [1,5].

UDP-GalNAc-6-[^{35}S]sulfate ($1.9 \cdot 10^7$ cpm/μmol) was prepared from chemically synthesized UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate by digestion with oviduct 4-sulfatase [6], *N*-acetylgalactosamine 6-[^{35}S]sulfate from UDP-GalNAc-6-[^{35}S]sulfate by digestion with nucleotide pyrophosphatase and alkaline phosphatase [7], proteochondroitin [^{35}S]sulfate Type H ($2.5 \cdot 10^7$ cpm/μmol hexuronate) from 12-day old chick embryo epiphyseal cartilages that had been incubated in $^{35}SO_4^{2-}$-containing Krebs medium (sulfate concentration = 0.15 mM) for 2 h [8], and chondroitin 4/6-[^{35}S]sulfate ($2.35 \cdot 10^7$ cpm/μmol hexuronate) from proteochondroitin [^{35}S]sulfate by alkaline β-elimination followed by protease treatment [8]. GlcUA-GalNAc(4-$^{35}SO_4$) and GlcUA-GalNAc(6-$^{35}SO_4$) were prepared by chondroitinase-ABC digestion of a tetrasaccharide ($2 \cdot 10^7$ cpm/μmol hexuronate) isolated from hyaluronidase digests of chondroitin 4/6-[^{35}S]sulfate [9,10], and ΔGlcUA-GalNAc(4,6-bis-$^{35}SO_4$) ($1.2 \cdot 10^7$ cpm/μmol) by chondroitinase-ABC digestion of chondroitin [^{35}S]sulfate Type E [11] from squid cartilage that had been incubated in $^{35}SO_4^{2-}$-containing Krebs medium (with no added inorganic sulfate carrier) for 6 h.

The following commercial materials were used: [*N*-^{35}S]heparin ($8.4 \cdot 10^7$ cpm/μmol hexuronate) from Calatomic, Los Angeles; cytochrome *c*, potassium 4-nitrocatechol sulfate, bovine epididymis β-*N*-acetyl-D-hexosaminidase, and bovine liver β-D-glucuronidase (further purified by Sephadex G-200 chromatography to remove arylsulfatase B contaminant) from Sigma Chemical Co., St. Louis; *p*-nitrophenyl sulfate from Nakarai Kagaku Co., Kyoto; chymotrypsinogen, hen's egg albumin, bovine serum albumin, and aldolase (molecular weight standards) from Boehringer Mannheim Yamanouchi, Tokyo; CM-cellulose from Brown Co., Keene, NH; Sephadex G-200 from Pharmacia Japan, Tokyo; and

Diaflo PM-10 membrane for ultrafiltration from Amicon Corp., Lexington.

Generous gifts of the following materials are acknowledged: sturgeon notochord chondroitin 4-sulfate from Dr. M.B. Mathews, The University of Chicago; ascorbate 2-sulfate from Dr. F. Egami, Mitsubishi-Kasei Institute for Life Science; and cerebroside sulfate from Dr. T. Yamakawa, University of Tokyo.

Enzyme assays. All sample enzymes were dialyzed against 0.02 M sodium acetate-acetic acid, pH 4.6, prior to activity measurements. Zero time blanks were included in each assay series.

The previously described assay system (measurement of enzymatically liberated $^{35}SO_4^{2-}$ on electrophoresis strips) [1] was used to assess the sulfatase activity for UDP-GalNAc-4-[^{35}S]sulfate. A unit of activity is defined as the amount of enzyme which yields 0.1 nmol (=150 cpm) of $^{35}SO_4^{2-}$ in the assay period (30 min). Note that the definition of enzyme unit differs from that given in the previous paper [1] (1000 cpm/30 min).

The sulfatase activity for ^{35}S-labeled poly-, oligo-, and monosaccharides was assayed as follows. The incubation mixture contained, in 50 μl, 3 μmol of sodium acetate-acetic acid buffer, pH 4.8, $4 \cdot 10^5$ cpm of substrate, and enzyme. After incubation at 37°C for 30 min, the reaction was stopped by immersing the reaction tube in a boiling water bath for 1 min. The mixture was clarified by centrifugation. An aliquot of the supernatant solution was subjected to paper electrophoresis, and the amount of liberated $^{35}SO_4^{2-}$ was estimated on dried electrophoresis strips as described previously [1]. A unit of activity is defined as the amount of enzyme which yield 0.1 nmol of $^{35}SO_4^{2-}$ in the assay period.

When UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate and *N*-acetylgalactosamine 4,6-bis-[6-^{35}S]sulfate were used as substrate, the criterion used for the demonstration of enzyme activity was the formation of UDP-GalNAc-[6-^{35}S]sulfate and *N*-acetylgalactosmine [6-^{35}S]sulfate, respectively, as a product, which were detected on electrophoresis strips by cutting the strips into 1 cm wide pieces and counting these in a liquid scintillation spectrometer.

Arylsulfatase activities were assayed with *p*-nitrophenyl sulfate or nitrocatechol sulfate as substrate [12]. The incubation mixture contained, in 50 μl, 10 μmol of sodium acetate-acetic acid buffer, pH 5.6, 0.16 μmol of substrate, and enzyme. After incubation at 37°C for 30 min, the reaction was stopped by immersing the reaction tube in a boiling water bath for 1 min. The mixture was clarified by centrifugation. An aliquot of the supernatant solution was mixed with 0.1 M NaOH and the amount of liberated phenolate ions were estimated spectrophotometrically at 400 nm (*p*-nitrophenolate ion) or at 515 nm (nitrocatecholate ion). A unit of activity is defined as the amount of enzyme which yields 0.1 nmol of phenolate ions in the assay period.

Other methods. Paper electrophoresis was carried out in a CCl_4-cooled apparatus on 60-cm strips of Toyo No. 51A paper in 0.05 M ammonium acetate-acetic acid buffer, pH 5.0, at 30 V per cm. Protein concentrations of column effluents were determined by absorption at 280 nm, and protein contents of purified enzyme preparations by the method of Lowry et al. [13]. Details of the other experimental procedures are given in the legends to figures and tables.

Results

Demonstration of three sulfatase activities

With 1.5 l of the crude extract (prepared from 470 g of fresh isthmi) as starting material, a purification of UDP-*N*-acetylgalactosamine 4-sulfatase as far as the stage for CM-cellulose chromatography ('Step 4', see Ref. 1) was done. A 110-fold increase in specific activity was achieved. In agreement with our previous observations [1], this enzyme preparation failed to exhibit sulfatase activity towards *p*-nitrophenyl sulfate and chondroitin 4-sulfate (unlabeled sample prepared from sturgeon notochord); i.e. when the enzyme, 1130 units, was incubated at pH values ranging from 3.0—9.0 with 0.5 μmol (as sulfate) of substrate at 37°C for 3 h, there was no detectable liberation of free *p*-nitrophenol (from *p*-nitrophenyl sulfate) or inorganic sulfate (from chondroitin 4-sulfate), as judged by the spectrophotometric [12] or turbidimetric assay method [14], respectively, used in the previous work. NaCl, 1—200 mM, had no effect. When, however, the enzyme preparation was incubated with nitrocatechol sulfate and chondroitin 4/6-[^{35}S]sulfate as substrates, it was apparent that the enzyme preparation has significant sulfatase activities towards these compounds. Thus, incubation for 3 h with the same amount of enzyme as above resulted in the quantitative liberation of nitrocatechol from 0.5 μmol of nitrocatechol sulfate or the liberation of $6 \cdot 10^3$ cpm (about 0.26 nmol) of $^{35}SO_4^{2-}$ from $4 \cdot 10^5$ cpm of chondroitin 4/5-[^{35}S]sulfate. The efficient hydrolysis of nitrocatechol sulfate suggests the presence of an arylsulfatase which, unlike the arylsulfatase in rat liver and kidney [1], has very little activity towards *p*-nitrophenyl sulfate. The small amount of sulfate release from chondroitin sulfate would explain why the turbidimetric assay failed to detect sulfatase activities towards glycosaminoglycans. At any rate, it leaves little doubt that the enzyme preparation from hen oviduct contains sulfatase activities towards UDP-GalNAc-4-sulfate, nitrocatechol sulfate, and chondroitin sulfate.

Fractionation studies

The partially purified enzyme preparation ('Step 4 enzyme') was rechromatographed on CM-cellulose (Step 5). When each fraction was assayed with UDP-GalNAc-4-[^{35}S]sulfate, chondroitin 4/6-[^{35}S]sulfate, and nitrocatechol sulfate as substrates, about 4-fold increase in specific activity could be achieved with each sulfatase activity but there was no indication that the three activities were separated from one another (the elution profile not shown). The peak fractions (protein = 22 mg) were pooled, concentrated on a Diaflo PM-10 membrane in an Amicon ultrafiltation cell, and subjected to gel filtration on a Sephadex G-200 column, 3.0 × 110 cm, equilibrated with 0.02 M sodium acetate-acetic acid, pH 4.6, containing 0.2 M NaCl (Step 6). Again, a close parallelism among the three sulfatase activities was indicated (K_{av} = 0.56); about 2-fold increase in specific activity was achieved with little change in the proportion of the three activities. At this stage, about 1180-fold purification from the crude extract was achieved to a specific activity of 1200 units per mg protein. The apparent molecular weight of the enzyme protein estimated from the K_{av} value relative to those for standard proteins was 47 000 ± 5000.

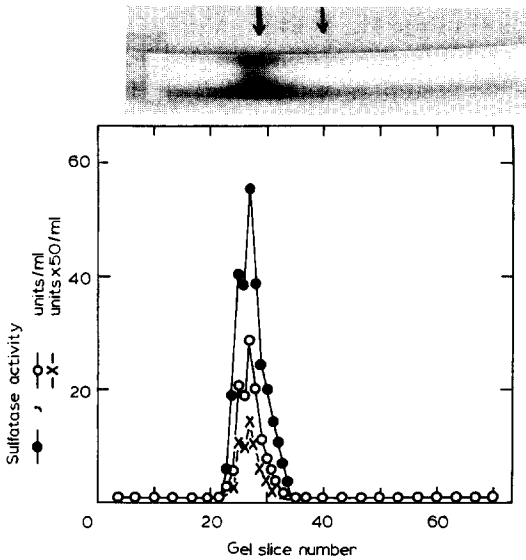

Fig. 1. Polyacrylamide gel electrophoresis of the Step 6 enzyme fraction from Sephadex G-200. In each case, the enzyme fraction equivalent to 20 μg of protein was subjected to gel electrophoresis according to Davis [15] with a operative pH of 4.0. The gels were stained for proteins with Coomassie brilliant blue R 250 or were frozen by placing in a dry ice bath. The frozen gels were sliced and the activity was extracted by homogenization of the slices in 0.02 M sodium acetate-acetic acid, pH 4.6. The enzyme activity was recovered after centrifugation at 17 500 X *g* for 20 min in the supernatant. Activities for UDP-GalNAc-4-[^{35}S]sulfate (○———○), nitrocatechol sulfate (●———●), and chondroitin 4/6-[^{35}S]sulfate (X———X) were recorded.

Upon polyacrylamide gel electrophoresis at pH 4.0, the final preparation ('Step 6 enzyme') gave one major, broad protein band and one minor, faster-moving protein band (Fig. 1), indicating that the preparation is still impure. When, however, slices of the gel were extracted with acetate buffer, and the resulting extracts were assayed for sulfatase activities, coincident migration of the three activities and of the major protein band was revealed.

Thus, the results of the fractionation experiments described strengthen the possibility that the three sulfatase reactions are catalyzed by one enzyme.

Effect of pH on three enzyme activities

The three activities of Step 6 enzyme were studied as a function of pH, using 0.05 M sodium acetate-acetic acid. There was a small difference among the pH optima for the three activities; pH 4.8 for chondroitin 4/6-[^{35}S]sulfate, pH 5.0 for UDP-GalNAc-4-[^{35}S]sulfate, and pH 5.6 for nitrocatechol sulfate. Since the three substrates differ from one another in both quality and quantity of ionic groups, the difference might possibly be due to the maintenance of the substrates in the proper ionic state for enzyme action, rather than a pH effect on the enzyme itself.

Effects of concentration of inorganic ions on three enzymatic activities

The following cations were tested as chloride salts at two to four concentrations in the ranges indicated in parentheses and found to have little effect on

the three activities: Na^+ and K^+ (1—200 mM); Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+} (1—10 mM). Ag^+, a known inhibitor of arylsulfatase A [16], also had little effect on the three activities when tested as nitrate salt at 1—10 mM. Inorganic sulfate and phosphate ions were inhibitor for the three activities; the concentrations (mM) necessary to produce 50% inhibition of UDP-GalNAc-4-sulfate, nitrocatechol sulfate, and chondroitin 4/6-sulfate hydrolysis were 0.05, 1.0, and 3.5 (sulfate ion) and 0.05, 0.1, and 0.5, (phosphate ion), respectively.

Parallel activity and parallel inactivation of three enzymatic activities

No significant loss of the three activities was observed when Step 6 enzyme was stored at —20°C with occasional thawing and refreezing for 6 months. The three enzymatic activities were comparably unstable to heat. At 60°C (pH 4.6) the activities decreased rapidly in a parallel fashion (Fig. 2).

Inhibition of nitrocatechol sulfate hydrolysis of UDP-GalNAc-4-sulfate and chondroitin 4-sulfate

The hydrolysis of both nitrocatechol sulfate and UDP-GalNAc-4-[^{35}S]sulfate was linear with time or protein concentration until about a quarter of the substrate was consumed. The K_m values were 0.42 mM for nitrocatechol sulfate and 0.43 mM for UDP-GalNAc-4-[^{35}S]sulfate.

The hydrolysis of nitrocatechol sulfate by Step 6 enzyme at pH 5.6 was studied as a function of the concentration of UDP-GalNAc-4-sulfate or chondroitin 4-sulfate by measuring the quantity of free nitrocatechol formed during incubation at 37°C for 10 min. Both UDP-GalNAc-4-sulfate and chondroitin 4-sulfate acted as competitive inhibitors of nitrocatechol sulfate hydrolysis. The K_i values were 0.45 mM for UDP-GalNac-4-sulfate and 2.0 mM (as total hexuronate) for chondroitin 4-sulfate. The K_i value for UDP-GalNAc-4-sulfate is comparable to the K_m value determined for UDP-GalNAc-4-sulfate

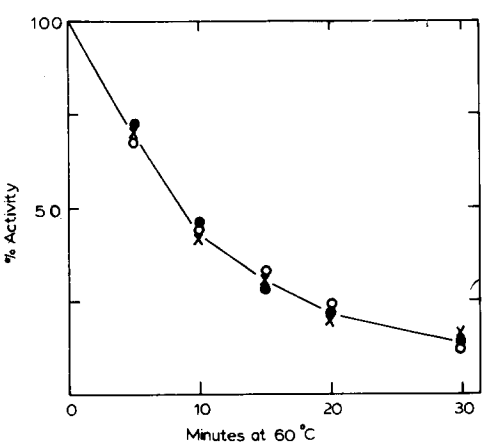

Fig. 2. Thermal inactivation of sulfatase activities for UDP-GalNAc-4-[^{35}S]sulfate (○———○), nitrocatechol sulfate (●———●), and chondroitin 4/6-[^{35}S]sulfate (X———X). A solution of Step 6 enzyme in 0.02 M sodium acetate-acetic acid, pH 4.6, was prepared (protein concentration = 0.89 mg/ml). After the indicated intervals of incubation at 60°C, aliquots of the solution were assayed under the standard conditions. Values obtained with unheated preparation are set as 100% activity.

as sole substrate. The results, taken together, suggest that the three sulfate esters may act as alternate substrates for the same enzyme.

Specificity studies

The ability of Step 6 enzyme to liberate $^{35}SO_4^{2-}$ (or unlabeled SO_4^{2-}) from a number of synthetic or naturally occurring sulfate esters was measured at 37°C for up to 6 h.

None of the following compounds proved to be a substrate for the enzyme: heparan [^{35}S]sulfate, [*N*-^{35}S]heparin, keratan [^{35}S]sulfate, GlcNAc-(6-$^{35}SO_4$)-Gal(6-$^{35}SO_4$)-GlcNAc-(6-$^{35}SO_4$)-Gal, GlcUA-GalNAc(4-$^{35}SO_4$), GlcUA-GalNAc(6-$^{35}SO_4$), ΔGlcUA-GalNAc(4-$^{35}SO_4$), ΔGlcUA-GalNAc(6-$^{35}SO_4$), ΔGlcUA-GalNAc(4,6-bis-$^{35}SO_4$), *N*-acetylgalactosamine 6-[^{35}S]sulfate, UDP-GalNAc-6-[^{35}S]sulfate, ascorbate 2-sulfate, and cerebroside sulfate (dissolved in 3 μl of 5% Triton X-100 and added to a 100 μl assay mixture). The failure of the enzyme to degrade cerebroside sulfate and ascorbate 2-sulfate indicates that the hen oviduct enzyme is distinctly different from arylsulfatase A.

Under the same conditions, the enzyme released $^{35}SO_4^{2-}$ from dermatan [^{35}S]sulfate, proteochondroitin [^{35}S]sulfate Type H [8], *N*-acetylgalactosamine 4-[^{35}S]sulfate, and GalNAc(4,6-bis-$^{35}SO_4$)-[GlcUA-GalNAc(4-$^{35}SO_4$)]$_3$; the amounts of liberated $^{35}SO_4^{2-}$; were 1.3, 3.3, 100, and 24%, respectively, of the added radioactivity. The enzyme also catalyzed the quantitative conversion of UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate and *N*-acetylgalactosamine 4,6-bis-[6-^{35}S]-sulfate to the corresponding mono-[6-^{35}S]sulfate derivatives that were readily separated from the parent compounds on paper electrophoresis (see 'Enzyme assays' for the assay procedure). In no case, radioactivity was found in the SO_4^{2-} region on the electrophoresis strip (note that the substrates have no labeled 4-sulfate residues).

The results, taken together, suggest that the enzyme requires the monosaccharide structure, *N*-acetylgalactosamine 4-sulfate, free or in non-reducing terminal glycoside linkage.

Further information on the specificity of the sulfatase was obtained by examination of chondroitin 4/6-[^{35}S[sulfate before and after digestion with Step 6 enzyme. As shown in Table I, chondroitinase digestion and chromatography of this glycosaminoglycan demonstrate GalNAc 4-[^{35}S]sulfate, GalNAc-6-[^{35}S]sulfate, and GalNAc-4,6-bis-[^{35}S]sulfate derived from the non-reducing terminal and unsaturated [^{35}S]disaccharides derived from the internal repeating units. Following incubation with Step 6 enzyme, there is a reduction of GalNAc-4-[^{35}S]sulfate and GalNAc-4,6-bis-[^{35}S]sulfate and a corresponding increase of inorganic [^{35}S]sulfate and GalNAc-6-[^{35}S]sulfate with no detectable change in the amount of the other components. The results indicate that hydrolysis of ester sulfate by this enzyme is limited to 4-sulfate groups occurring in the non-reducing GalNAc-4-sulfate and GalNAc-4,6-bis-sulfate end groups.

Chondroitin 4/6-[^{35}S]sulfate, $4.0 \cdot 10^4$ cpm, was incubated with excess of Step 6 enzyme. The results shown in Fig. 3 indicate that 1.5% of the added radioactivity was released as inorganic [^{35}S]sulfate within 5 min, and thereafter no sulfate release was observed. Addition of β-*N*-acetyl-D-hexosaminidase at 10 min had little effect, but addition of β-D-glucuronidase caused a significant

TABLE I

CHONDROITINASE-ABC DIGESTION OF CHONDROITIN 4/6-[^{35}S]SULFATE BEFORE AND AFTER TREATMENT WITH HEN OVIDUCT 4-SULFATASE

Chondroitin 4/6-[^{35}S]sulfate, $3 \cdot 10^6$ cpm, was incubated at 37°C with Step 6 enzyme (equivalent to 44 μg of protein). Control contained no enzyme. After 6 h, each reaction mixture was separated into two equal portions, one being subjected to electrophoretic determination of enzymatically liberated $^{35}SO_4^{2-}$. To the other portion was added 500 μl of water and 0.7 μmol (as hexuronate) of chondroitin 4-sulfate as carrier, and glycosaminoglycans were precipitated with 3 vols of ethanol containing 1 mM potassium acetate. The precipitation with ethanol from water was repeated five times to ensure complete removal of $^{35}SO_4^{2-}$. The final precipitate was subjected to chondroitinase-ABC digestion followed by paper chromatography and paper electrophoresis, as described previously [5].

Product	% of product	
	From untreated control	From 4-sulfatase-treated sample
SO_4^{2-}	0	1.5
GalNAc-4-SO_4	1.5	0.3
GalNAc-6-SO_4	0.3	0.6
GalNAc-4,6-bis-SO_4	0.6	0.2
ΔGlcUA-GalNAc(4-SO_4)	38.9	39.0
ΔGlcUA-GalNAc(6-SO_4)	53.3	53.0
ΔGlcUA-GalNAc(4,6-bis-SO_4)	0.2	0.2
Unidentified products *	5.2	5.2

* Including saturated disaccharides, higher oligosaccharides, and undigested polysaccharides.

increase in the extent of sulfate release. Still more increase in both rate and extent of sulfate release was observed when β-D-glucuronidase and β-*N*-acetyl-D-hexosaminidase were added simultaneously at 10 min. The results can be interpreted as indicating that hydrolysis by this enzyme occurs only on non-

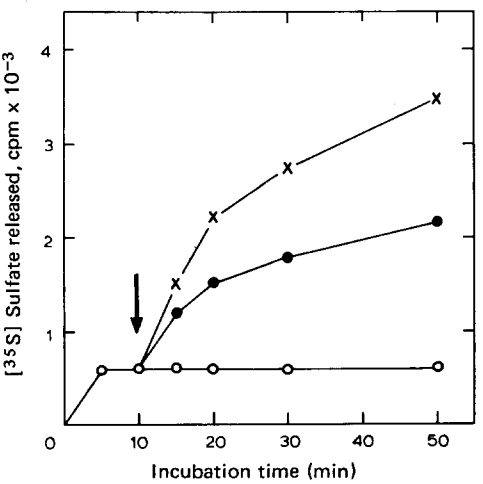

Fig. 3. Time course of [^{35}S]sulfate liberation from chondroitin 4/6-[^{35}S]sulfate by oviduct sulfatase. Assay mixtures contained, in 50 μl, 3 μmol of sodium acetate-acetic acid (pH 4.8), Step 6 enzyme (925 units, 18 μg as protein), and $4 \cdot 10^4$ cpm of chondroitin 4/6-[^{35}S]sulfate. Incubation was carried out at 37°C. At the time indicated by the arrow, 10 μl each of a solution containing 440 units of β-*N*-acetyl-D-hexosaminidase (Sigma) in 100 μl of 0.02 M acetate buffer was added to one set of tubes (○), 10 μl each of a solution containing 150 units of β-D-glucuronidase (arylsulfatase B-free preparation) in 100 μl of 0.02 M acetate buffer to a second set (●), and 10 μl each of a solution containing 440 units of β-*N*-acetyl-D-hexosaminidase and 150 units of β-D-glucuronidase in 100 μl of 0.02 M acetate buffer to a third set (X).

reducing residues and internal sulfate groups can be attacked only when they are exposed by the removal of non-reducing glycosyl end groups by digestion with appropriate exoglycosidases.

Discussion

The combined results of the variety of experiments described constitute strong evidence for the catalysis of the UDP-GalNAc-4-sulfatase, nitrocatechol sulfatase, and chondroitin 4-exosulfatase reactions by one enzyme.

The liberation of SO_4^{2-} from UDP-GalNAc-4-sulfate was first demonstrated in rat liver and kidney as well as in hen oviduct [1]. In the early experiments we observed that the hen oviduct enzyme had little or no activity towards *p*-nitrophenyl sulfate whereas the rat liver and kidney enzymes were very closely associated with an arylsulfatase activity for *p*-nitrophenyl sulfate. This difference in substrate specificity raised the possibility that the oviduct enzyme might represent a functionally distinct sulfatase. However, it is obvious from general properties of arylsulfatases that such criteria for distinguishing different sulfatases must be treated with great caution unless the ionic composition of the reaction mixture is rigorously defined. Webb and Morrow [17], for example, pointed out that *p*-nitrophenylsulfate was hydrolyzed by beef liver arylsulfatase B only extremely slowly but it was hydrolyzed at much higher rates, when Cl^- ions were present in the reaction mixture. No comparable effect was obtained with nitrocatechol sulfate and the hydrolysis of this by beef liver arylsulfatase B is, in fact, slightly inhibited by Cl^- ions. Since the arylsulfatase nature of the hen oviduct enzyme has been established with nitrocatechol sulfate, a possibility must be considered that the failure of this enzyme to cleave *p*-nitrophenyl sulfate is due to the absence of Cl^- ions in the reaction mixture.

A comparison was made, therefore, between the activities of hen oviduct sulfatase towards *p*-nitrophenyl sulfate and nitrocatechol sulfate at various NaCl concentrations (1—200 mM). However, no evidence was seen that NaCl had any effect on the activities on two different substrates. It thus appears that the oviduct sulfatase represents a subclass of arylsulfatase B differing from the mammalian liver enzyme with respect to *p*-nitrophenyl sulfate-hydrolyzing activity and Cl^- sensitivity.

Mention must be made of the difference reported here and in the previous paper [1] regarding the reactivity of the oviduct enzyme towards chondroitin sulfate. In the previous study, we used to turbidimetric method [14] to estimate enzymatically liberated SO_4^{2-} and obtained negative results with various glycosaminoglycan substrates including chondroitin 4- (and 4,6-bis-)sulfate. With this assay method, however, the minimal detectable quantity of SO_4^{2-} is about 50 nmol which corresponds to 10% of the total sulfate of glycosaminoglycan substrate added to the incubation mixture. Therefore, the result of our early experiments could not rule out the possibility that only a small percentage (less than 10%) of total sulfate may be released as SO_4^{2-} because of a high degree of selectivity of the enzyme. That this is indeed the case has been shown by the present work using a much more sensitive method to detect enzymatically liberated SO_4^{2-}. The reason why the maximal release of SO_4^{2-} is so small

(only 1.5% of the added radioactivity) has been explained by the high degree of specificity of the enzyme for non-reducing *N*-acetylgalactosamine 4-sulfate end groups (Table I and Fig. 3).

As both UDP-GalNAc-4-sulfate and chondroitin 4-sulfate are normal constituents of hen oviduct [18,19], it is very likely that in vivo a metabolic system may exist wherein the nucloetide and polysaccharide are involved as alternative or competitive substrates for the common sulfatase. If this being the case, then obviously the whole question of the function of sulfated sugar nucleotides [20—23] must be considered in a new light and a role for them in proteoglycan metabolism would become highly probable. Interest in the problem has been intensified by our recent findings that UDP-*N*-acetylgalactosamine 6-sulfate, an isomer of the 4-sulfated nucleotide in hen oviduct, occurs in the oviduct and egg white of quail [24]. Soon after this, a sulfatase for the 6-sulfated sugar nucleotide has been demonstrated in the oviduct of quail. Furthermore, the enzyme has been shown to cleave 6-sulfate not only in the sugar nucleotide but also in the non-reducing *N*-acetylgalactosamine 6-sulfate end group of chondroitin sulfate [6]. Although the in vivo interaction of the sulfatases with sulfated sugar nucleotides and proteoglycans, and the relevance of such interaction to proteoglycan metabolism remain indefinite, it seems clear that these studies do provide an interesting base for further investigation of the potential roles of sulfated sugar nucleotides in the control of proteoglycan metabolism.

A few comments are appropriate at this point regarding the chemical structure of the chondroitin 4/6-[^{35}S]sulfate sample used as substrate. This sample was prepared from 12-day old chick embryo epiphyseal cartilages that had been incubated in $^{35}SO_4^{2-}$-containing Krebs medium for 2 h [8]. The analysis by digestion with chondroitinase-ABC (Table I) indicates that, although most of the internal repeating units bear one sulfate residue at either position 4 or position 6 of the hexosamine moiety, a large proportion of the non-reducing sulfated *N*-acetylgalactosamine end groups have a distinct structure bearing two sulfate residues at positions 4 and 6. The reason for the high proportion of 4,6-bissulfated units at the non-reducing terminal is not clear, but may be relevant to our recent finding that in connective tissues there is a sulfotransferase capable of catalyzing the introduction of sulfate into position 6 of a non-reducing *N*-acetylgalactosamine moiety already bearing a sulfate residue at position 4 [25]. The occurrence of this type of sulfotransferase, coupled with the efficient hydrolysis of the 4,6-bissulfated end group by 4-sulfatase (Table I), suggests the possibility that in vivo a mechanism may operate whereby the non-reducing *N*-acetylgalactosamine 4-sulfate end group of a chondroitin sulfate chain is converted to a 6-sulfated form via a 4,6-bissulfated form. Presumptive evidence to indicate such a processing of chondroitin sulfate chain has been obtained by our recent experiments and will be reported elsewhere.

Another obvious feature of the oviduct sulfatase is the use to which it may be put as a reagent in studying the structure of glycosaminoglycans. By virtue of its high degree of specificity for a non-reducing *N*-acetylgalactosamine 4-sulfate end group, the enzyme can be used to determine the presence or absence of such a grouping in a glycosaminoglycan chain. Furthermore, the inability of this enzyme preparation to attack any of the sulfate residues within a glycosaminoglycan chain, coupled with its lack of glucuronidase and hexos-

aminidase activity, permits the use of this preparation as an end group reagent. A given chondroitin sulfate or proteoglycan molecule can be modified by this enzyme to give a molecule with little or no 4-sulfate residues at the non-reducing terminal. Such a chondroitin sulfate or proteoglycan may prove to be useful for studies on the mechanism of elongation and sulfation of glycosaminoglycan chain, a process in which sulfate grouping at the non-reducing terminal has been proposed to play a crucial role [26,27].

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