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ISOLATION OF UDP-N-ACETYLGALACTOSAMINE-6-SULFATE SULFATASE FROM QUAIL OVIDUCT AND ITS ACTION ON CHONDROITIN SULFATE

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SUMMARY: A sulfatase, which liberates sulfate from UDP-N-acetylgalactosamine-6-sulfate (the nucleotide occurring in quail egg white at high concentration), has been isolated from quail oviduct. The tissue also contained sulfatase activities for UDP-N-acetylgalactosamine-4-sulfate and nitrocatechol sulfate but these activities were removed from the 6-sulfatase fraction during purification. The UDP-N-acetylgalactosamine-6-sulfate sulfatase appears to be very closely related to a sulfatase activity for the non-reducing N-acetylgalactosamine-6-sulfate end group in chondroitin sulfate, i.e. the two activities could not be separated from each other by various fractionation procedures and were affected in a parallel fashion by mild heating. The results, coupled with those of earlier studies on UDP-N-acetylgalactosamine-4-sulfate in hen oviduct, suggest that in avian oviducts a sulfation/desulfation system may exist wherein sulfated sugar nucleotides and sulfated glycosaminoglycans are involved as alternative or competitive substrates.

The enzymatic desulfation of UDP-GalNAc-4-sulfate and UDP-GalNAc-4,6-bissulfate (the nucleotides occurring in hen oviduct and chicken egg white, see Refs. 1-4) was first demonstrated in hen oviduct, and rat liver and kidney extracts (5). The activity in rat liver and kidney extracts appears to be very closely related to an arylsulfatase activity found in such preparations (5). Fluharty et al. (6) have since reported that an arylsulfatase B preparation purified 200-fold from human placenta catalyzed the desulfation of UDP-GalNAc-4-sulfate. In addition, cultured fibroblasts derived from patients with Maroteaux-Lamy disease were shown to be deficient in UDP-GalNAc-4-sulfate sulfatase to the same extent that they were deficient in arylsulfatase B. Although these results suggest a wide distribution of a sulfatase which attacks both UDP-GalNAc-4-sulfate and arylsulfates, the biological significance of desulfation of sugar nucleotides and arylsulfates is not known. Recent studies with human skin fibroblasts (7, 8) and rat skin (9) have led to the postulation that at least in these cells and tissue the enzyme serves as an important adjunct

Abbreviations; UDP-GalNAc, UDP-GalNAc-4-sulfate, UDP-GalNAc-6-sulfate, and UDP-GalNAc-4,6-bissulfate; uridine diphosphate N-acetylgalactosamine, and its derivatives bearing a sulfate at position 4, a sulfate at position 6, and two sulfates at positions 4 and 6 of hexosamine moiety, respectively.

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to proteoglycan metabolism by removing sulfate from the non-reducing N-acetylgalactosamine-4-sulfate end groups in glycosaminoglycan chains. That this may also be the case of the hen oviduct enzyme has been suggested by our recent experiments (unpublished); i.e. following incubation of chondroitin 4/6-[^{35}S]sulfate with a UDP-GalNAc-4-sulfate sulfatase preparation purified 1180-fold from hen oviduct, there was a reduction of 4-sulfate groups at the non-reducing terminal with no detectable change in the amount of the other sulfate groups.

In the course of the comparative studies on sugar nucleotides occurring in various avian eggs (10), we observed that the egg white of quail contains UDP-GalNAc-6-sulfate, an isomer of the 4-sulfated nucleotide present in hen's egg white. In view of the earlier studies on the 4-sulfatase system, it seemed highly probable that a sulfatase system for 6-sulfated sugar nucleotides and glycosaminoglycans might be found in quail oviduct. We report here that the quail oviduct does indeed contain such an enzyme system.

MATERIALS AND METHODS

Preparation of Substrates and Standards. The following materials were prepared by previously described method: UDP-GalNAc and UDP-GalNAc-4-sulfate from hen oviduct (11); UDP-GalNAc-6-sulfate from quail egg white (10); UDP-GalNAc-4,6-bissulfate from UDP-GalNAc-4-sulfate by chemical sulfation with chlorosulfonic acid (12); UDP-GalNAc-4-[^{35}S]sulfate (2.4×10^5 cpm/μmol) from UDP-GalNAc by enzymatic sulfation with 3'-phosphoadenylyl [^{35}S]sulfate (13); chondroitin 4/6-[^{35}S]sulfate (DEAE-cellulose 0.7 M NaCl fraction; 1.2×10^7 cpm/μmol hexuronate) from chick embryo epiphyseal cartilages that had been incubated in [^{35}S]sulfate-containing medium (9); and trisaccharide 4/6-[^{35}S]sulfate (3.3×10^6 cpm/μmol hexuronate) from this labeled chondroitin sulfate preparation by digestion with testicular hyaluronidase and β-D-glucuronidase (9).

UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate was synthesized from UDP-GalNAc-4-sulfate and Cl^{35}SO$_3$H (56.5 mCi/mmol, The Radiochemical Centre) essentially as described by Ishihara et al. (12). Briefly, a sample of UDP-GalNAc-4-sulfate (16 μmol as tri-n-octylamine salt, which had been dried by alternate addition of dry pyridine and evaporation) was dissolved in 0.5 ml of dry pyridine. To this solution was added Cl^{35}SO$_3$H in dry chloroform (3 mCi in 0.1 ml), and the mixture was stirred at room temperature for 3 h. The mixture was then subjected to preparative paper chromatography in n-butyric acid-0.5 M ammonia (5:3). The radioactive zone corresponding to standard UDP-GalNAc-4,6-bissulfate was located by radioautography, eluted with water, concentrated, and further purified and desalted, as described previously (2); yield, 3.6×10^7 cpm (specific activity = 1.9×10^7 cpm/μmol).

UDP-GalNAc-6-[^{35}S]sulfate was prepared from a portion (2.26×10^7 cpm) of the UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate sample by digestion with UDP-GalNAc-4-sulfate sulfatase as described previously (5). From the digest, the compound was isolated by preparative paper electrophoresis and desalted as above; yield, 1.97×10^7 cpm.

1-Phospho-N-acetylgalactosamine 6-[^{35}S]sulfate was prepared from UDP-GalNAc-6-[^{35}S]sulfate by digestion with snake venom phosphodiesterase (Worthington), and N-acetylgalactosamine 6-[^{35}S]sulfate from the 1-phosphate by digestion with *E.coli* alkaline phosphatase (Worthington). The procedures were as described previously (2).

Cerebroside sulfate was kindly provided by Dr. T. Yamakawa, and ascorbate 2-sulfate by Dr. F. Egami.

Crude Enzyme Preparation. All procedures were carried out at 0-4°C. Oviducts (90 g, wet weight) were excised from 14 quails (*Coturnix coturnix Japonica*) and homogenized in 360 ml of 0.02 M Tris-HCl, pH 7.2, by three 1-min treatments in a Polytron homogenizer. The homogenate was centrifuged at 8,000 × g for 15 min and the pellet was retreated with the homogenizer as above. The two supernatants obtained by Polytron homogenization were combined and dialyzed against 0.02 M Tris-HCl, pH 7.2.

Enzyme Assays. All enzymes were dialyzed against 0.02 M Tris-HCl, pH 7.2, prior to activity measurements. Unless otherwise specified, assay procedures for sulfatase activity were carried out by incubating 1,400~6,100 cpm of ^{35}S-labeled substrate, 2.5 μmol of sodium acetate-acetic acid, pH 3.4, and enzyme in a final volume of 50 μl at 37°C for 30~60 min. The reaction was terminated by immersing reaction tube in a boiling water bath for 2 min. An aliquot of the mixture was applied to filter paper together with 0.05 μmol of Na_2SO_4 as an internal marker and subjected to electrophoresis in 0.05 M ammonium acetate-acetic acid, pH 5.0, at 30 volts/cm, which gave separation of inorganic [^{35}S]sulfate from substrates. After drying, the inorganic sulfate marker was located by $BaCl_2$-rhodizonate (14), cut out, placed in a scintillator solution, and counted. Zero time blanks were included in each assay series. One unit of enzyme was defined as the quantity that catalyzed the release of 0.1 nmol of inorganic [^{35}S]sulfate per 30 min.

Arylsulfatase activity was determined using nitrocatechol sulfate (Sigma) as a substrate (15). The reaction was performed for 30 min at 37°C and the liberated nitrocatechol was measured spectrophotometrically at 515 nm.

Sulfatase activity for cerebroside sulfate and ascorbate 2-sulfate was checked by thin layer chromatography of reaction mixtures on a Kieselgel 60 (Merck) plate in chloroform-methanol-water (60:25:4) and 95 % ethanol-1 M ammonium acetate, pH 7.2, (5:2), respectively. On finishing a run the plates were stained with 5 % surfuric acid. The R_F values of standard compounds are: cerebroside sulfate, 0.34; cerebroside, 0.63; ascorbate 2-sulfate, 0.22; and ascorbic acid, 0.39.

RESULTS AND DISCUSSION

In a preliminary test, a 3 ml portion of the crude enzyme preparation was applied to a DEAE-cellulose column (1 × 15 cm). The column was washed with 20 ml of 0.02 M Tris-HCl, pH 7.2, and then eluted with linear NaCl gradient from 0 to 0.4 M in the Tris buffer. Fractions of 1 ml were collected and checked for activity on UDP-GalNAc-4-[^{35}S]sulfate, UDP-GalNAc-6-[^{35}S]sulfate, and nitrocatechol sulfate. The chromatography yielded two fractions with UDP-GalNAc-4-sulfate sulfatase activity, one appearing in the washings and the other between 0.1 and 0.2 M NaCl. Nitrocatechol sulfate sulfatase was eluted in parallel with the 4-sulfatase activity, suggesting that the hydrolysis of nitrocatechol sulfate and UDP-GalNAc-4-sulfate is catalyzed by one enzyme. Besides these 4-sulfatase fractions, a fraction with activity for UDP-GalNAc-6-sulfate appeared in an area before, but overlapping, the earlier eluting component of UDP-GalNAc-4-sulfate sulfatase.

To obtain a large amount of UDP-GalNAc-6-sulfate sulfatase for further characterization, a 174 ml portion of the crude oviduct extract was likewise chromatographed on a DEAE-cellulose column (4.2 × 37 cm), and the 0.02 M

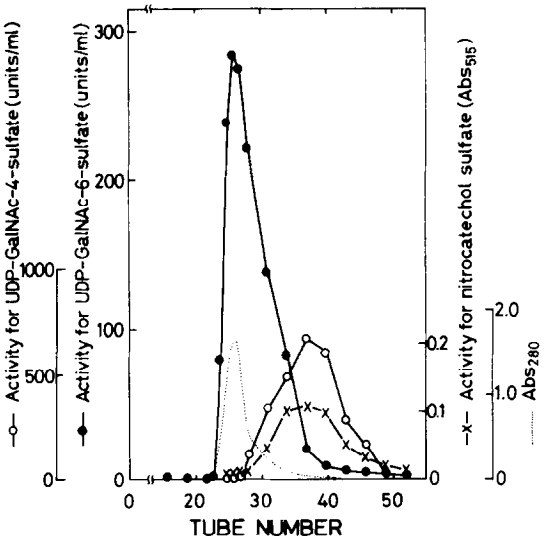

Fig. 1. Rechromatography on a DEAE-cellulose column (2.8 × 81.5 cm) of the 6-sulfatase fraction from the first DEAE-cellulose column (see the text for details). The second column was developed with 0.02 M Tris-HCl, pH 7.2, and fractions of 12.5 ml were collected. Absorbance at 280 nm (·····), UDP-GalNAc-6-sulfate sulfatase activity (●——●), UDP-GalNAc-4-sulfate sulfatase activity (○——○), and arylsulfatase activity (×——×) were recorded.

Tris-HCl washings (268 ml) were pooled, concentrated by ultrafiltration, and subjected to a second chromatography on DEAE-cellulose (Fig. 1). As can be seen, the chromatography yielded a 6-sulfatase fraction separated, with some overlap, from a 4-sulfatase fraction. Fractions 24-28, which were free from 4-sulfatase activity, were pooled and concentrated by ultrafiltration. The preparation contained 1.2×10^4 units of UDP-GalNAc-6-sulfate sulfatase and 83.6 mg of protein.

In view of the known action of UDP-GalNAc-4-sulfate sulfatase on glycosaminoglycans (see "Introduction"), it seemed highly probable that the UDP-GalNAc-6-sulfate sulfatase may also attack some 6-sulfate linkages in chondroitin sulfate. In fact, incubation of the 6-sulfatase preparation with sulfated trisaccharide (prepared from chondroitin 4/6-[^{35}S]sulfate) resulted in the liberation of sulfate at 0.55 nmol/min/mg protein (optimal pH = 3.8), a rate comparable to that of UDP-GalNAc-6-[^{35}S]sulfate hydrolysis (0.5 nmol/min/mg protein). The ratio (1:0.9) of the two activities remained constant during subsequent chromatography of the enzyme preparation on Sephadex G-200 (Fig. 2). Both activities were detected in a single peak (from Tube 89 to 115) with little variation in the ratio.

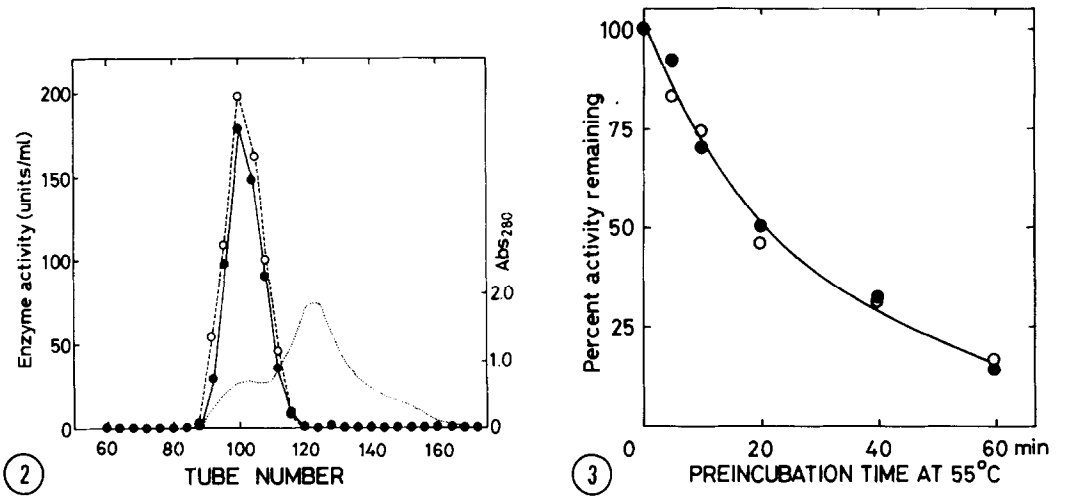

Fig. 2. Gel chromatography of the 6-sulfatase fraction from the second DEAE-cellulose column. The early portion of the 6-sulfatase peak (see Fig. 1, Tubes 24-28) was concentrated to 26 ml by ultrafiltration. A 10 ml portion of this solution was applied to a Sephadex G-200 column (2.8 × 114 cm) equilibrated with 0.02 M Tris-HCl, pH 7.2. The column was eluted at 32 ml/hr in fractions of 4 ml. Absorbance at 280 nm (·····) and sulfatase activities on UDP-GalNAc-6-[^{35}S]sulfate (●——●) and on ^{35}S-labeled trisaccharide (o----o) were recorded.

Fig. 3. Thermal inactivation of sulfatase activities on UDP-GalNAc-6-[^{35}S]sulfate (●) and on ^{35}S-labeled trisaccharide (o). The final 6-sulfatase preparation (see Fig. 2) was concentrated by ultrafiltration to give a protein concentration of 0.56 mg/ml, and assayed, after the indicated intervals of preincubation at 55°C, for the sulfatase activities.

Further evidence to indicate the identity of UDP-GalNAc-6-sulfate sulfatase and chondroitin sulfate trisaccharide sulfatase was obtained when the final enzyme preparation was subjected to heating at 55°C. The two activities were found to be similarly unstable to such treatment (Fig. 3).

To characterize the products of UDP-GalNAc-6-sulfate hydrolysis, 0.2 μmol of a UDP-GalNAc-6-[^{35}S]sulfate sample with relatively low specific radioactivity (1.5×10^5 cpm/μmol) was incubated with 52 units of the final enzyme preparation. Fresh enzyme, 52 units, was added to the mixture every 8 h. After 24 h, the reaction mixture was analyzed by paper electrophoresis. Radioautogram and ultraviolet-absorption print of the electrophoretogram indicated the formation of one radioactive component with the mobility of inorganic sulfate and one ultraviolet-absorbing component corresponding to UDP-GalNAc. On the basis of the radioactivity of the inorganic sulfate and the absorbance (at 262 nm) of the UDP-GalNAc, the hydrolysis products were shown to be present in equimolar amounts. It should be noted that the

reaction started with such a high concentration of substrate (1.4 mM) was not allowed to completion but reached a maximum which is equivalent to about 25 % of the added substrate, due perhaps to an inhibition by the reaction products (see below).

The pH optimum for the desulfation of UDP-GalNAc-6-sulfate was 3.4. At this pH, the Michaelis constant for UDP-GalNAc-6-sulfate was 4×10^{-6} M. Under the standard assay conditions (with 4 units of enzyme), there was no detectable release of $^{35}SO_4^{2-}$ from the following compounds for up to 60 min; UDP-GalNAc-4,6-bis-[6-^{35}S]sulfate, N-acetylgalactosamine 6-[^{35}S]sulfate, and 1-phospho-N-acetylgalactosamine 6-[^{35}S]sulfate. No significant change was observed in the thin layer chromatographic properties of cerebroside sulfate and ascorbate 2-sulfate before and after treatment of the substrates (0.4 μmol) with the 6-sulfatase preparation (27 units) for 6 h. UDP-GalNAc, UDP-GalNAc-4-sulfate, UDP-GalNAc-4,6-bissulfate, and Na_2SO_4 were inhibitory for UDP-GalNAc-6-[^{35}S]sulfate hydrolysis; the concentrations (μM) necessary to produce 50 % inhibition were 80, 20, 2, and 7, respectively. $CaCl_2$, $MgCl_2$, and $MnCl_2$ (10 mM) inhibited the enzyme by 60-80 %.

Although the trisaccharide sample used as substrate is a mixture of 4- and 6-sulfated isomers, it was possible to show that hydrolysis of ester sulfate by the enzyme is limited to 6-sulfate group occurring at the non-reducing terminal. As shown in Table I, chondroitinase-ABC digestion and chromatography of untreated trisaccharide showed N-acetylgalactosamine 4-sulfate and 6-sulfate derived from the non-reducing terminal and unsaturated disaccharide 4-sulfate and 6-sulfate derived from the remaining portion. Following incubation with UDP-GalNAc-6-sulfate sulfatase, there was a reduction of N-acetylgalactosamine 6-sulfate and a corresponding appearance of inorganic sulfate with no detectable change in the amount of the other components.

Intact chondroitin 4/6-[^{35}S]sulfate was shown to be attacked by the enzyme (optimal pH = 5.1) in a similar way. In this case, maximum release of $^{35}SO_4^{2-}$ corresponded to only 0.3 % of the added radioactivity, suggesting that hydrolysis occurs at only suitably located 6-sulfate linkages. Subsequent analyses of the sulfatase-treated chondroitin sulfate with chondroitinase-ABC indicated that 6-sulfate cleavage had occurred only on non-reducing residues (data not shown).

The substrates tested in this study range from free and nucleotidyl N-acetylgalactosamine 6-sulfate to chondroitin sulfate. The available data indicate that both UDP-GalNAc-6-sulfate and chondroitin 6-sulfate serve as good substrates with comparable efficiency whereas free N-acetylgalactosamine 6-sulfate fails to show such activity. A similar specificity has been

TABLE I. Chondroitinase degradation of ^{35}S-labeled trisaccharide before and after treatment with quail oviduct 6-sulfatase

Material	% of product				
	SO_4^{2-}	GalNAc-6-SO_4	GalNAc-4-SO_4	ΔDi-6S	ΔDi-4S
Untreated control	0	17.8	35.3	20.3	26.6
Sulfatase-treated trisaccharide	13.5	1.7	36.4	22.4	26.0

^{35}S-Labeled trisaccharide, 2,660 cpm, was incubated at pH 3.8 with 15 units of the most purified 6-sulfatase preparation for 90 min. Control contained heat-inactivated enzyme. Following incubation, digestion was carried out with chondroitinase-ABC (16) followed by paper-chromatographic and paper-electrophoretic analyses (9) for ^{35}S-labeled inorganic sulfate, monosaccharides (GalNAc-4-SO_4 and GalNAc-6-SO_4), and unsaturated disaccharides (ΔDi-4S and ΔDi-6S).

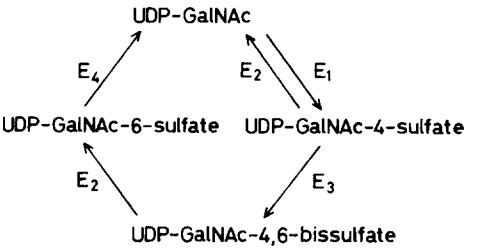

Fig. 4. Proposed interconversion of UDP-GalNAc derivatives by enzymatic sulfation and desulfation. The enzymes involved are: E_1, UDP-GalNAc 4-sulfotransferase (13); E_2, UDP-GalNAc-4-sulfate sulfatase (5); E_3, UDP-GalNAc-4-sulfate 6-sulfotransferase (18); and E_4, UDP-GalNAc-6-sulfate sulfatase; all of which have been isolated and characterized.

observed with rat skin N-acetylgalactosamine 6-sulfatase (9). In human skin fibroblasts, however, the occurrence of a sulfatase which is active for both free N-acetylgalactosaminitol 6-sulfate and chondroitin 6-sulfate has been suggested by DiFerrante *et al.* (17). At present, we have no information regarding the factors responsible for the difference in activity for free monosaccharides.

The four distinct enzymes ($E_1 \sim E_4$) responsible for the sulfation and desulfation of sugar nucleotides have now been identified (Fig. 4). The fact that the sulfatases, E_2 and E_4, can act on the non-reducing terminal N-acetylgalactosamine 4-sulfate (or 4,6-bissulfate) and N-acetylgalactosamine 6-sulfate, respectively, in glycosaminoglycans suggests that *in vivo* a mechanism may operate whereby the sulfated sugar nucleotides are able to influence glycosaminoglycan metabolism by acting as competitive substrates. Similarly, the sulfotransferases, E_1 and E_3, might function, under

appropriate conditions, as a sulfation system for glycosaminoglycan acceptors, forming 4-sulfated and 4,6-bissulfated N-acetylgalactosamine residues, respectively, on the non-reducing termini. Presumptive evidence for such a role of sulfotransferase E_3 has already been obtained in this laboratory and will be described elsewhere.

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