The Enzymatic Desulfation of Sulfated Sugar Nucleotides by a Novel Sulfatase in Hen Oviduct

(Received for publication, September 13, 1973)

MASAHIRO TSUJI,† MASAKO HAMANO, YASUO NAKANISHI, KAZUHIKO ISHIHARA, AND SAKARU SUZUKI

From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya 464, Japan

SUMMARY

A soluble fraction from the isthmus region of hen oviduct catalyzes the liberation of \( \text{SO}_3^- \) from UDP-N-acetylgalactosamine-4-sulfate (the nucleotide that accumulates in the same tissue during egg formation). The enzyme is purified some 88-fold and is shown to catalyze the reactions: (a) UDP-N-acetylgalactosamine-4-sulfate \( \rightarrow \) UDP-N-acetylgalactosamine plus \( \text{SO}_3^- \); and (b) UDP-N-acetylgalactosamine-4,6-disulfate \( \rightarrow \) UDP-N-acetylgalactosamine-6-sulfate plus \( \text{SO}_3^- \). N-Acetylgalactosamine-4,6-disulfate is desulfated in a similar way but at approximately 13% of the rate of UDP-N-acetylgalactosamine-4-sulfate hydrolysis. The enzyme does not act upon \( \text{p-nitrophenyl sulfate}, \text{N-acetylgalactosamine-4- and 6-sulfate, glucose 6-sulfate, galactose 3- and 6-sulfate, glucan sulfate, chondroitin sulfates, dermatan sulfate, keratan sulfate, heparan sulfate, and unsaturated disaccharides mono- and disulfates derived from chondroitin sulfates.}

There are striking differences in the sulfatase activity among the anatomically distinct regions of the oviduct: the specific activity of the isthmus extract being 7 to 12 times greater than the values of the magnum and uterus extracts. A somewhat similar activity is detected in the extracts of rat liver and kidney, but available evidence suggests that the activity in these extracts is due to the action of an arylsulfatase which acts preferentially on these sulfated sugar nucleotides.

We previously described the occurrence in the isthmus of specific sulfotransferases which catalyze UDP-N-acetylgalactosamine-4-sulfate and UDP-N-acetylgalactosamine-4,6-disulfate formation, respectively. The present finding of a sulfatase which acts preferentially on these sulfated sugar nucleotides gives additional support to the idea that these nucleotides have an important biological role.

UDP-GalNAc-4-sulfate is first isolated from hen oviduct by Strominger (2, 3). In subsequent studies of the characterization of this series "Sulfation of Sugar Nucleotides." The preceding paper in this series is Reference 1.

† The work was carried out during the tenure of a Postdoctoral Fellowship from The Japan Society for the Promotion of Science.

1 The abbreviations used are: UDP-GalNAc, UDP-N-acetyl-galactosamine-4-sulfate; UDP-GalNAc-4,6-disulfate, UDP-GalNAc-4-sulfate, N-acetylgalactosamine-6-phosphoglucoaminase; UDP-galactose-sulfate, UDP-N-acetylgalactosamine-6-phosphoglucoaminase, and UDP-N-acetylgalactosamine-4-sulfate, 6-phosphoglucoaminase. At least one of the sulfated sugar nucleotides, UDP-GalNAc-4-sulfate, appears to be secreted into egg white as the egg traverses the isthmus region (5) and so might be considered to play a role in the development of chick embryo. Indirect evidence for a specific biological role of the sulfated sugar nucleotides also comes from our findings that the isthmus region contains two specific sulfotransferases, one catalyzing the introduction of sulfite to position 6 of UDP-GalNAc (6) and the other the introduction of sulfite to position 6 of UDP-GalNAc-4-sulfate (7). Not only these sulfated sugar nucleotides but also various other sugar nucleotides have been shown to occur in the isthmus (4), and it is highly probable that a specific mechanism exists in the isthmus cell to control the accumulation, secretion, and destruction of the particular group of sulfated sugar nucleotides. In this paper we present evidence for the existence of a new type of sulfatase, with a high specificity toward UDP-GalNAc-4-sulfate and UDP-GalNAc-4,6-disulfate.

EXPERIMENTAL PROCEDURE

Materials

The following compounds were prepared by previously described methods: UDP-GalNAc, UDP-GalNAc-4-sulfate, and UDP-GalNAc-4,6-disulfate from hen oviduct (1, 4); N-acetylgalactosamine-4-sulfate from UDP-GalNAc (2); N-acetylgalactosamine-6-sulfate from UDP-GalNAc-4-sulfate (3); chondroitin-4,6-disulfate (type E) from squid cartilage (9); keratan sulfate from bovine cornea (10); chondroitin sulfate (glucuronate) from mollusc (11); AGlcUA-GalNAc (4S), AGlcUA-GalNAc (4S), AGlcUA-GalNAc (4S), and AGlcUA-GalNAc (4S) from chondroitin sulfates (12, 13); PAPS: UDP-GalNAc sulfotransferase from the isthmus (6); [PS]PAPS (2.4 x 10⁶ cpm per pmole) from galactosamine; UDP-GalNAc-4-sulfate, UDP-N-acetylgalactosamine-4-sulfate; UDP-GalNAc-4,6-disulfate, UDP-N-acetylgalactosamine-6-sulfate; UDP-GalNAc-4-sulfate, UDP-N-acetylgalactosamine-4,6-disulfate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; ΔGlcUA-GalNAc (4S), ΔGlcUA-GalNAc (4S), and ΔGlcUA-GalNAc (4S), derivatives of Δ-glucuronopyranosyl-(1→3)-N-acetylgalactosamine bearing a sulfate at position 4, a sulfate at position 6, and two sulfates at position 4 and position 6 on the hexosamine moiety, respectively.
$^{35}$SO$_4^{2-}$ and ATP (14); UDP-GalNAc-4-[${}^{35}$S]sulfate (2.0 $\times$ 10$^8$ cpm per $\mu$mole) from UDP-GalNAc and [${}^{35}$S]PAPS (6); and UDP-GalNAc-4-[${}^{33}$P]sulfate (2.0 $\times$ 10$^8$ cpm per $\mu$mole) from UDP-GalNAc-4-sulfate and [${}^{33}$P]PAPS (7). In the individual experiments described below, the radioactive nucleotides were diluted with the corresponding unlabeled substances to give the indicated specific radioactivities.

We are very grateful to the following individuals for their generous gifts of the substances indicated: $\beta$-galactose-3-sulfate from Dr. M. B. Mathews and heparan sulfate (hog intestinal mucosa) and heparin preparation) from Dr. M. B. Mathews and Dr. H. Jatzkewitz, Max Planck Institut, Miinchen; dermatan sulfate (whale cartilage) and chondroitin-6-sulfate (shark cartilage) from Dr. M. Nomoto, Seikagaku Kogyo Company, Tokyo; and unlabeled PAPS from Dr. M. Honjo, Takeda Chemical Industry, Osaka.

The commercial materials used were: diethylaminoethylcellulose (DEAE) and carboxymethylcellulose (CM) from Brown Company; Diaflo PM-10 from Amicon; nucleotides from Calbiochem; $\beta$-glucose $\alpha$-sulfate and $\beta$-galactose $\beta$-sulfate from Sigma; Na$_2$SO$_4$ (carrier-free) from Radioisotope Association, Tokyo; and snake venom phosphodiesterase (synonym: nucleotide pyrophosphatase) and Escherichia coli alkaline phosphatase from Worthington.

**Methods**

**Analytical Procedures**—The following compounds were determined by the indicated method: p-nitrophenol released from p-nitrophenyl sulfate by the method of Dodgson and Spencer (15); sulfate by the method of Dodgson (16); phosphate by the method of Lowry et al. (17); protein by the method of Lowry et al. (18); and $\alpha$-galactosamine by the chromatographic (19) or colorimetric (20) method. The galactosamine in the nucleotides was determined after conversion to free galactosamine by hydrolysis with $1 \mathrm{~N}$ HCl at 100$^\circ$C for 1 hour.

**Paper Chromatography and Paper Electrophoresis**—The following solvent systems were used by descending chromatography on Toyo No. 51A filter paper (60 cm long): A, n-butyril acid-0.5 $\times$ ammonia (5:3); B, 95% ethanol-1 $\times$ ammonium acetate, pH 7.2 (5:2); C, 1-butanol-ethanol-water (15:3:4); D, ethylacetate-pyridine-water-acetic acid (5:5:3:1) in the atmosphere of the chamber saturated with pyridine-ethylacetate-water (11:40:6).

Paper electrophoresis was carried out on 60 cm strips of Toyo No. 51A paper in the apparatus described by Markham and Smith (21) at a potential gradient of 2.5 volts per cm for the periods indicated in individual experiments. The buffer used for the procedures was 0.05 $\times$ ammonium acetate-acetic acid, pH 5.0.

On a preparative scale, the sample was applied as a thin zone on the paper strip. If necessary, guide strips were cut and stained as described below. The compounds were then eluted from the remainder of the chromatogram or the electrophoretic strip with water.

Nucleotides were examined on paper chromatograms or electrophoretograms under ultraviolet light, reducing sugars by staining with the aniline hydrogen phthalate reagent (22) or with the silver nitrate reagent (23), and $\text{SO}_4^{2-}$ by staining with the BaCl$_2$-rhodizonate reagent (24).

**Measurements of Radioactivity**—Radioactive compounds on paper chromatograms were measured by cutting out the radioactive area and counting in a Horiba liquid scintillation spectrometer, model LS-500 (Horiba Seisakusho, Kyoto), with 6 ml of a solution of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in a liter of toluene. All counts were corrected for quenching in the sample.

Radioactive areas on paper chromatograms were detected by radioautography or by counting small strips of paper in the scintillation spectrometer. Radioautograms were prepared with Fuji x-ray film by leaving chromatograms in contact with the film for 1 to 10 days, depending on the amount of radioactivity present.

**Enzyme Assay**—The assay measures the conversion of UDP-GalNAc-4-[${}^{35}$S]sulfate to $^{35}$SO$_4^{2-}$ by electrophoretic separation. The assay mixture was made up as follows: 3 $\mu$mols of sodium acetate-acetic acid buffer (pH 5.0), 2 $\times$ 10$^8$ cpm (about 13.3 nmoles) of UDP-GalNAc-4-[${}^{33}$P]sulfate, enzyme, and water to a volume of 50 $\mu$l. After incubation at 37$^\circ$C for 30 min, the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. The resultant precipitate was removed by centrifugation at 11,500 $\times$ g for 20 min. An aliquot of the supernatant solution was applied to filter paper together with 0.05 $\mu$mole of inorganic sulfate as an internal marker. Electrophoresis was carried out, which gave separation of $^{35}$SO$_4^{2-}$ from UDP-GalNAc-4-[${}^{33}$P]sulfate within 30 min. After drying, the inorganic sulfate was located by BaCl$_2$-rhodizonate, cut out, placed in a scintillation vial, and counted.

Control mixtures were made up in the same way, except that the reaction mixture was heated at 100$^\circ$C for 1 min immediately after the addition of enzyme. One unit of enzyme is defined as that quantity which yields 1000 cpm (about 0.67 nmoles) of $^{35}$SO$_4^{2-}$ in the assay period (30 min).

**Other Methods**—Hydrolysis of nucleotides with snake venom phosphodiesterase and hydrolysis of phosphate esters with E. coli alkaline phosphatase were carried out as described previously (1).

**RESULTS**

**Purification of Enzyme**

All operations were conducted between 0$^\circ$ and 4$^\circ$. All centrifugations were at 17,500 $\times$ g for 20 min.

**Step 1**—About 70 g of isthmi, obtained from freshly excised oviducts of 28 White Leghorn laying hens, were freed from muscle fibers and connective tissues which cover the outside of the isthmus, placed in 280 ml of 0.02 $\times$ Tris-HCl, pH 7.2, and disrupted for 2 min in a motor driven Potter-Elvehjem glass homogenizer. After centrifugation, the pellet was resuspended in 140 ml of 0.02 $\times$ Tris-HCl, pH 7.2, and the homogenization and centrifugation were repeated. The resultant supernatant fluids were combined and used for further purification of the enzyme. The final pellet was also rich in a sulfatase activity (for the properties of this sulfatase system, see below) but, in the present study, it was not used as a source for further purification.

**Step 2**—Solid ammonium sulfate was added to the supernatant fraction to attain 40% saturation. After standing for 30 min the mixture was centrifuged, and the supernatant solution was collected. The concentration of ammonium sulfate was then raised to 65% saturation. After 60 min, the mixture was centrifuged, and the supernatant solution was discarded. The precipitate was dissolved in 50 ml of 0.02 $\times$ Tris-HCl, pH

---

2 We are indebted to Mr. I. Yamada for his assistance with the preparation of the tissue.
7.2, and dialyzed for 24 hours against four 2-liter changes of the same buffer.

Step 5—A column (4 x 39 cm) of DEAE-cellulose was prepared and equilibrated with 2 liters of 0.02 M Tris-HCl, pH 7.2. The dialyzed ammonium sulfate fraction was applied to the column at the rate of 80 ml per hour. The column was then washed with 650 ml of 0.02 M Tris-HCl, pH 7.2. Most of the activity was recovered in the wash, whereas approximately 85% of the protein was retarded by the column. The pooled DEAE-cellulose fraction was concentrated to approximately 23 ml with a Diaflo PM-10 (membrane filter), and dialyzed for 18 hours against three 2-liter changes of 0.02 M sodium acetate-acetic acid, pH 4.5.

Step 6—A column (2.5 x 35 cm) of CM-cellulose was equilibrated with 1 liter of 0.02 M sodium acetate-acetic acid, pH 4.5. A 20-ml portion of the dialyzed DEAE-cellulose fraction was applied to the column at the rate of 40 ml per hour, and the adsorbent was washed with 250 ml of the same buffer. The column was developed by linear gradient elution with 450 ml of 0.02 M sodium acetate-acetic acid, pH 4.5, in the mixing flask and 450 ml of 0.6 M NaCl in the same buffer in the reservoir. The flow rate was 40 ml per hour and fractions of 8 ml were collected. As shown in Fig. 1, most of the activity was found in the fractions from tubes 90 to 120 as a single peak. The fractions were pooled, concentrated to approximately 8 ml with the membrane filter, and dialyzed for 16 hours against three 2-liter changes of 0.02 M sodium acetate-acetic acid, pH 4.5.

A summary of the purification procedure is shown in Table I. The enzyme retained 95% and 90% of its activity for 2 and 9 months, respectively, when stored at -20°C.

**General Properties of Enzyme and Demonstration of Sulfatase Activity**

The crude extracts from isthmus (see above) catalyzed phosphorylase and phosphomonoesterase reactions which interfere with the assay of sulfatase, i.e. a portion of the UDP-GalNAc-4-sulfate added to assay mixture was cleaved to give rise to N-acetylgalactosamine-4-[36S]sulfate (which is not attacked by the sulfatase, see below). The enzyme purified by CM-cellulose did not catalyze any significant cleavage of the sugar nucleotide at the pyrophosphate bond, and this preparation was used to study the properties of sulfatase described below.

The effect of pH on activity was investigated with sodium acetate-acetic acid buffer (pH 3.0 to 6.2), Tris-maleate buffer (pH 5.0 to 9.5), and Tris-HCl buffer (pH 7.0 to 9.5). The results showed that the optimum lies close to pH 5.0.

Na⁺ and K⁺ had no effect on the enzyme when they were tested as chloride salts at 2 and 10 mM. Ca²⁺, Mg⁴⁺, and Mn⁴⁺, on the other hand, inhibited the enzyme; with 15 mM divalent cation, inhibition being about 50%.

Under the standard assay condition, the velocity of reaction was proportional to protein concentration up to 4 μg of the purified enzyme per 50 μl. Somewhat different results were obtained, however, at higher substrate concentrations. When 2.05 μmoles of unlabeled UDP-GalNAc-4-sulfate were incubated with 0.3 mg (as protein) of the purified enzyme in a final volume of 500 μl, the velocity of SO₄²⁻ liberation was constant for 1 to 10 min, but thereafter decreased markedly. Thus, the reaction was not allowed to go to completion but reached a maximum which is equivalent to about 14% of the added substrate. Addition of more enzyme (0.3 mg as protein) at 60 mm had little effect, but addition of 4.5 ml of 0.06 M acetate buffer (pH 5.0) resulted in a significant increase in SO₄²⁻ liberation. Thus, after 60 min of incubation, the diluted mixture yielded approximately 0.74 μmole of SO₄²⁻ which is equivalent to 29% of the added substrate. It appears therefore that the cessation of SO₄²⁻ liberation could have been due to an inhibition by the products which accumulated during the first 60-min reaction. Supporting this view is the observation (see below) that both SO₄²⁻ and UDP-GalNAc showed a strong inhibition at concentrations greater than 10⁻⁴ M.

A balanced study of the reaction showed that the disappearance of UDP-GalNAc-4-sulfate resulted in the formation of approximately equimolar amounts of SO₄²⁻ and UDP-GalNAc (Table II). The latter product was eluted from the electrophoretic strip with water for further identification. The sample had chromatographic mobilities (in Solvents A and B) identical with those of UDP-GalNAc and contained, for each mole of uridine (estimated from absorbance at 262 nm), about 2 moles of total phosphate and 1 mole of galactosamine. The galactosamine could be distinguished from glucosamine by paper chromatography in Solvent D. In addition, the sample was identical with UDP-GalNAc in its activity with PAPS:UDP-GalNAc sulfotransferase system (6), i.e. using 1 mg (as protein) of fresh sulfotransferase, approximately 3 X 10⁵ cpm of [³⁵S]sulfate could be transferred from 3 X 10⁵ cpm (1.3 X 10⁻⁴ μmoles) of

**Table I**

**Purification of isthmus sulfatase**

For definition of units, see the text. The exceedingly high yield in Step 2 makes it apparent that interfering substances were removed during the purification procedure. When the reaction mixture incubated with the crude extract was examined by paper chromatography in Solvent A, it was apparent that a portion of the added UDP-GalNAc-4-[³⁵S]sulfate had been cleaved, presumably by reactions with phosphorylase and phosphomonoesterase, to give rise to N-acetylgalactosamine-4-[³⁵S]sulfate which is resistant to the sulfatase.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>430</td>
<td>4,200</td>
<td>169</td>
<td>38</td>
</tr>
<tr>
<td>2. Ammonium sulfate</td>
<td>47</td>
<td>1,040</td>
<td>219</td>
<td>210</td>
</tr>
<tr>
<td>3. DEAE-cellulose</td>
<td>23</td>
<td>165</td>
<td>69</td>
<td>1,140</td>
</tr>
<tr>
<td>4. CM-cellulose</td>
<td>8</td>
<td>12</td>
<td>40</td>
<td>3,333</td>
</tr>
</tbody>
</table>
The Ki determined by this plot was $8 \times 10^{-6}$.

It leaves little doubt, therefore, that the nucleotide product is UDP-GalNAc.

### Specificity

As shown in Table III, several compounds related in structure to UDP-GalNAc-4-sulfate were desulfated by the enzyme. UDP-GalNAc-4,6-disulfate was fully active in the system. N-Acetylgalactosamine-4,6-disulfate was also desulfated, but at approximately 13% of the rate of UDP-GalNAc-4-sulfate hydrolysis. No liberation of sulfate was observed with N-acetylgalactosamine-4-sulfate under the standard condition.

That the activities for UDP-GalNAc-4-sulfate and UDP-GalNAc-4,6-disulfate are catalyzed by the same protein is supported by the following observations.

Upon chromatography on CM-cellulose, both activities were detected from tubes 90 to 120 (cf. Fig. 1) with no significant variation in the activity ratio.

When heat labilities were compared at 60°, the inactivation of the two activities took place at the same rate (Fig. 2, Experiment 1).

The activity with UDP-GalNAc-4-[35S]sulfate was inhibited by m-labeled UDP-GalNAc-4,6-disulfate. The inhibition was competitive with respect to UDP-GalNAc-4-sulfate (Fig. 3). The $K_I$ determined by this plot was $8 \times 10^{-4}$ M. From the similarity of the $K_I$ and $K_m$ values for UDP-GalNAc-4,6-disulfate, it would appear that the compound is cleaved by the same enzyme as that for UDP-GalNAc-4-sulfate.

The labeled products from UDP-GalNAc-4,6-[35S]disulfate and N-acetylgalactosamine-4,6-[35S]disulfate were shown to be UDP-GalNAc-6-[35S]sulfate and N-acetylgalactosamine-6-

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$ units/mg protein</th>
<th>$K_m$ $M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. UDP-GalNAc-4-sulfate</td>
<td>4,200</td>
<td>$4 \times 10^{-4}$</td>
</tr>
<tr>
<td>2. UDP-GalNAc-4,6-disulfate</td>
<td>10,500</td>
<td>$6 \times 10^{-6}$</td>
</tr>
<tr>
<td>3. N-Acetylgalactosamine-4-sulfate</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>4. N-Acetylgalactosamine-4,6-disulfate</td>
<td>560</td>
<td>$3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* N.A., negligible activity.

![Fig. 2. Effect of heat on isthmus sulfatase (Exp. 1) and rat liver sulfatase (Exp. 2).](http://www.jbc.org/)

The incubation conditions for each substrate were those for enzyme assay described under "Methods," except that varying amounts of the indicated substrate were used in each case. The radioactive substrates were diluted with the corresponding unlabeled substances to give 2 x $10^6$ cpm per pmole and added to the incubation mixtures containing 6.4 units of enzyme. The assays measured the radioactivities released as $SO_4^{2-}$ (Experiments 1 and 3), UDP-GalNAc-6-sulfate (Experiment 2), or N-acetylgalactosamine-6-sulfate (Experiment 4) after paper-electrophoretic or paper-chromatographic (Solvent A) separation. The products corresponding to UDP-GalNAc-6-sulfate and N-acetylgalactosamine-6-sulfate have not been isolated in substance but as paper-electrophoretic and chromatographic fractions, the identity and purity of which have been supported by the following criteria.

All the radioactivity in the product from UDP-GalNAc-4,6-[35S]disulfate was converted, by digestion with phosphodiesterase and alkaline phosphatase, to a single fraction whose chromatographic mobility in Solvent A was identical with that of authentic N-acetylgalactosamine-6-sulfate ($R_{f}$ = 0.58) but not with the mobility of the 4-sulfate isomer ($R_{f}$ = 0.64). Likewise, the radioactive product from N-acetylgalactosamine-4,6-[35S]disulfate behaved as a single compound on chromatography in Solvent A, the mobility of which was the same as that of authentic N-acetylgalactosamine-6-sulfate.

Inorganic sulfate was determined by the method of Dodgson (18).

---

**Table II**

**Balance study of reaction**

The reaction mixture contained, in a final volume of 500 $\mu$l, 2.65 $\mu$moles of UDP-GalNAc-4-sulfate, 30 $\mu$moles of acetate buffer (pH 5.0), and 1000 units of enzyme; it was incubated at 37° for 60 min, after which 4.5 ml of 0.06 M acetate buffer (pH 5.0) were added and incubation was continued for another 60 min. The reaction was stopped by immersing the tube in a boiling water bath for 2 min. The resultant precipitate was removed by centrifugation at 11,500 x g for 20 min. The supernatant fluid was concentrated to about 50 $\mu$l and submitted, together with 0.05 $\mu$ mole each of $SO_4^{2-}$, UDP-GalNAc-4-sulfate, and UDP-GalNAc (external markers), to paper electrophoresis for 50 min. The zone corresponding to SO$_4^{2-}$ (mobility = 38 cm) was located by staining the external SO$_4^{2-}$ marker with the BaCl$_2$-rhodizonate reagent, and the zones corresponding to UDP-GalNAc-4-sulfate (mobility = 26 cm) and UDP-GalNAc (mobility = 19 cm) by viewing under ultraviolet light. All the radioactivity in the product from UDP-GalNAc-4,6-[35S]disulfate was converted, by digestion with phosphodiesterase and alkaline phosphatase, to a single fraction whose chromatography in Solvent C, in which the compounds had little mobility, the zones at and near the origin were cut out and eluted with water. The nucleotides were measured spectrophotometrically at 262 nm (pH 2) against a blank tube containing the eluate from the adjacent region of paper where the spots were not present. Inorganic sulfate was determined by the method of Dodgson (18).

### Specificity

As shown in Table III, several compounds related in structure to UDP-GalNAc-4-sulfate were desulfated by the enzyme. UDP-GalNAc-4,6-disulfate was fully active in the system. N-Acetylgalactosamine-4,6-disulfate was also desulfated, but at approximately 13% of the rate of UDP-GalNAc-4-sulfate hydrolysis. No liberation of sulfate was observed with N-acetylgalactosamine-4-sulfate under the standard condition.

That the activities for UDP-GalNAc-4-sulfate and UDP-GalNAc-4,6-disulfate are catalyzed by the same protein is supported by the following observations.

Upon chromatography on CM-cellulose, both activities were detected from tubes 90 to 120 (cf. Fig. 1) with no significant variation in the activity ratio.

When heat labilities were compared at 60°, the inactivation of the two activities took place at the same rate (Fig. 2, Experiment 1).

The activity with UDP-GalNAc-4-[35S]sulfate was inhibited by m-labeled UDP-GalNAc-4,6-disulfate. The inhibition was competitive with respect to UDP-GalNAc-4-sulfate (Fig. 3). The $K_I$ determined by this plot was $8 \times 10^{-4}$ M. From the similarity of the $K_I$ and $K_m$ values for UDP-GalNAc-4,6-disulfate, it would appear that the compound is cleaved by the same enzyme as that for UDP-GalNAc-4-sulfate.

The labeled products from UDP-GalNAc-4,6-[35S]disulfate and N-acetylgalactosamine-4,6-[35S]disulfate were shown to be UDP-GalNAc-6-[35S]sulfate and N-acetylgalactosamine-6-

---

**Fig. 2. Effect of heat on isthmus sulfatase (Exp. 1) and rat liver sulfatase (Exp. 2).** In Experiment 1, the purified enzyme (isthmus) from Step 4 (see the text) was assayed, after the indicated intervals of incubation at 60°, with UDP-GalNAc-4-[35S]sulfate ($\bullet----\bullet$) or UDP-GalNAc-4,6-[35S]disulfate ($\bigtriangleup----\bigtriangleup$) as substrate. Assay systems were as given under "Methods" and Table III. In Experiment 2, the purified enzyme (liver) from CM-cellulose (see Table V) was assayed, after the indicated intervals of incubation at 60°, with UDP-GalNAc-4-[35S]sulfate ($\bigtriangleup----\bigtriangleup$) or N-p-nitrophenyl sulfate ($\bigtriangleup----\bigtriangleup$) as substrate. Assay systems were as given in Table V. The heat stability curves obtained with the kidney enzyme essentially the same as the corresponding curves of the liver enzyme. The results with the kidney enzyme, therefore, are not shown here.
Distribution of sulfatase activity in different regions of oviduct

"Extract" (17,500 × g supernatant) and "pellet" were prepared from 20 g each of the indicated tissues essentially as described in the text (see "Purification of Enzyme"). Prior to the assay, the extracts were dialyzed for 6 hours against three 2-liter changes of 0.02 M Tris-HCl, pH 7.2. Assay system for the activity with UDP-GalNAc-4-[35S]-sulfate was as given under "Methods."

### Table IV

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oviduct in active state</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnum</td>
<td>2,000</td>
<td>5.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>5,470</td>
<td>5.2</td>
</tr>
<tr>
<td>Isthmus</td>
<td>1,420</td>
<td>30.4</td>
</tr>
<tr>
<td>Pellet</td>
<td>4,000</td>
<td>22.3</td>
</tr>
<tr>
<td>Uterus</td>
<td>350</td>
<td>3.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>1,440</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Oviduct in inactive state</strong></td>
<td>800</td>
<td>33.0</td>
</tr>
<tr>
<td>Isthmus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>2,280</td>
<td>18.0</td>
</tr>
</tbody>
</table>

* Expressed as the amounts (mg) obtained from 20 g (wet weight) of tissue.

† Excised from egg-laying hens.

‡ Excised from the hens that ceased laying (due to advanced age).

### Inhibition by Products

The reaction with UDP-GalNAc-4-sulfate was inhibited by SO₄²⁻ and UDP-GalNAc. In each case, the inhibition was competitive with respect to UDP-GalNAc-4-sulfate (Fig. 4). The Kᵢ values calculated from Fig. 4 are 8 × 10⁻⁴ M (for SO₄²⁻) and 2 × 10⁻⁴ M (for UDP-GalNAc). The data suggest that there is a regulatory interaction of substrate and products on the enzyme molecule.

**Sulfatase in 17,500 × g Pellet**

Although the above description has been restricted to the soluble preparation from the isthmus, sulfatase activity was also found in the pellet when the homogenate was centrifuged at 17,500 × g (Table IV). Time studies done with the washed pellet (1.4 mg as protein) showed release of [³⁵S]SO₄⁻ from added UDP-GalNAc-4-[³⁵S]sulfate (2 × 10⁴ cpm) at a linear rate for about 20 min. There was no clear lag period in the time curve. Further, the enzyme was not released at all from the pellet by treatment with 0.1 to 1.0% Triton X-100 in 0.25 M sucrose at 0° for 30 min. As far as these properties are concerned, the enzyme is unlikely to be associated with lysosomes or other membranous vesicles.

**Distribution of the Sulfatase Activity in Different Regions of Oviduct**

The distribution of the sulfatase activity was measured among the three anatomically segregated regions (magnum, isthmus, and uteru) of the hen oviduct. As shown in Table IV, the specific activities of both extract and pellet were strikingly higher in the isthmus region than in the magnum and uterus regions. These analyses were made with tissues from 10 hens who had maintained a high level of egg production. To
Additional analyses were made with isthmi obtained from seven active and inactive egg-production (Table IV). Specific activity was observed, however, between the tissues in hens who had stopped egg-laying. No significant difference in producing activity and the activities of isthmus sulfatase, addi-
sulfatase, it was found that extracts from rat liver and kidney see whether any relation may exist between the levels of egg-
also catalyze the formation of SOdp from UDP-GalNAc-4-
liver and kidney extracts were shown to be about 85% of the
therefore represent minimum values for the enzyme fractions.

crude extract of liver contained a factor which caused incomplete
potassium p-nitrophenyl sulfate with the method of Dodgson and
as has been pointed out by these authors, the crude extract of liver contained a factor which caused incomplete recovery of p-nitrophenol. The amounts of p-nitrophenol liberated by the crude extract were therefore corrected for the loss of p-nitrophenol. One unit of enzyme is defined as that quantity which yields 1 m mole of p-nitrophenol in the assay period (30 min).

The purified liver and kidney enzymes both exhibited a pH optimum of 5.0 for UDP-GalNAc-4-sulfate and 0.0 for p-nitrophenyl sulfate (in sodium acetate buffer). Like the isthmus enzyme, the liver and kidney enzymes were able to release the sulfate at position 4 of UDP-GalNAc-4,6-disulfate but not the

<table>
<thead>
<tr>
<th>Enzyme fractions</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UGFDAE on UDP-GalNAc-4-sulfate</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Crude extract</td>
<td>1350</td>
<td>1.2</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>600</td>
<td>17</td>
</tr>
<tr>
<td>0-40%</td>
<td>600</td>
<td>17</td>
</tr>
<tr>
<td>40-65%</td>
<td>500</td>
<td>82</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>16</td>
<td>925</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>450</td>
<td>3.6</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>180</td>
<td>32</td>
</tr>
<tr>
<td>0-40%</td>
<td>180</td>
<td>32</td>
</tr>
<tr>
<td>40-65%</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>8.5</td>
<td>1760</td>
</tr>
</tbody>
</table>

a Note that all the enzyme fractions contained interfering en-
zymes which catalyze the degradation of the substrate to N-acetyl-
galactosamine-4-sulfate. The results presented in this table there-
therefore represent minimum values for the enzyme fractions.

b See Fig. 5. Fractions 52 to 65 were pooled in each case.

see whether any relation may exist between the levels of egg-
producing activity and the activities of isthmus sulfatase, addi-
tional analyses were made with isthmi obtained from seven hens who had stopped egg-laying. No significant difference in specific activity was observed, however, between the tissues in active and inactive egg-production (Table IV).

Occurrence in Rat Liver and Kidney of Sulfatase Catalyzing Desulfation of Sulfated Sugar Nucleotides

In further attempts to determine the distribution of the sulfatase, it was found that extracts from rat liver and kidney also catalyze the formation of SOdp from UDP-GalNAc-4-sulfate. When the extracts and assays were performed as described for the isthmus, the apparent specific activities of the liver and kidney extracts were shown to be about 1.2 and 3.6,
sulfates of the hexose monosulfates, unsaturated disaccharide sulfates, and polysaccharide sulfates described above (see the specificity experiment with the isthmus enzyme). Of interest was the fact that the liver and kidney enzymes were inactive with N-acetylgalactosamine-4,6-disulfate, the compound which was active with the isthmus enzyme.

**DISCUSSION**

The desulfation of sulfated sugar nucleotides by the enzyme described here is remarkable for its uniqueness. It is also of considerable interest that the distribution of the enzyme appears to be very limited; the magnum and uterus extracts had only one-seventh to one-twelfth the activity of the isthmus extract on a protein basis. A somewhat similar cleavage of sulfated sugar nucleotides was observed with the extracts of rat liver and kidney, but the data presented in this paper indicate that the reactions are brought about by an arylsulfatase which is distinct from the isthmus enzyme in its action on p-nitrophenyl sulfate. The physiological implication of this reaction in the isthmus is not immediately apparent, and much additional information may be necessary before its relevance to other metabolic events becomes clear. For example, it will be of considerable interest to investigate the possibility that the enzyme will function when the sulfated sugar nucleotides are synthesized in excess of the rate at which they are secreted for UDP-GalNAc-4-sulfate synthesis (i.e. PAPS : UDP-GalNAc 5-epimerase). Additional information may be necessary before its relevance to other metabolic events becomes clear. For example, it will be of considerable interest to investigate the possibility that the reactions are brought about by an arylsulfatase which is distinct from the isthmus enzyme in its action on p-nitrophenyl sulfate. The physiological implication of this reaction in the isthmus is not immediately apparent, and much additional information may be necessary before its relevance to other metabolic events becomes clear. For example, it will be of considerable interest to investigate the possibility that the enzyme will function when the sulfated sugar nucleotides are synthesized in excess of the rate at which they are secreted.

Whether the observed desulfation of sulfated sugar nucleotides by “aryl sulfatase” may actually occur in vivo is not yet known. The widespread distribution of arylsulfatases and their multiplicity (25) certainly suggest a rather fundamental role, but what this is remains unknown. Thus far, many compounds have been described as substrates for arylsulfatases but none of these is likely to be the physiological substrates. In view of the unexpected observation that the liver and kidney arylsulfatase could catalyze the desulfation of sulfated sugar nucleotides, it is obvious that the problem of their physiological substrate is one requiring much further investigation.

**REFERENCES**

The Enzymatic Desulfation of Sulfated Sugar Nucleotides by a Novel Sulfatase in Hen Oviduct
Masahiro Tsuji, Masako Hamano, Yasuo Nakanishi, Kazuhiko Ishihara and Sakaru Suzuki


Access the most updated version of this article at http://www.jbc.org/content/249/3/879

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/3/879.full.html#ref-list-1