Enzymatic Sulfation of Mucopolysaccharides in Hen Oviduct

I. TRANSFER OF SULFATE FROM 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE TO MUCOPOLYSACCHARIDES

Sakaru Suzuki* and Jack L. Strominger†
From the Department of Pharmacology, Washington University School of Medicine, St. Louis 10, Missouri

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The oviduct of the laying hen is an organ histologically differentiated into four areas, each with a unique function in the biosynthesis of the egg (2). It contains high concentrations of a number of unusual nucleotides, including uridine diphosphateglactosamine and uridine diphosphoglactosamine-(4')-sulfate (3). More recently, it has been observed that uridine diphosphoglactosamine sulfate, previously isolated from whole oviduct, is localized within a 2-cm segment of oviduct, termed the isthmus.1 S35-inorganic sulfate given to hens is taken up by the oviduct only in the isthmus and in the vagina, the terminal 3-cm region of oviduct. Furthermore, the isthmus is concerned physiologically with the synthesis of the inner egg shell membranes. These membranes contain a sulfated mucopolysaccharide, the exact structure of which has not yet been established.1 These observations have led to the present study of the biosynthesis of sulfated mucopolysaccharides in the isthmus of oviduct.

The experiments reported in the present papers are concerned with enzymatic mechanisms of mucopolysaccharide sulfation in the isthmus. In the first paper an enzymatic reaction will be described in which sulfate is transferred from phosphoadenosine 5'-phosphosulfate ("active sulfate" of Robbins and Lipmann (4)) to a number of mucopolysaccharide acceptors. Enzymes which catalyze the synthesis of phosphoadenosine 5'-phosphosulfate from inorganic sulfate have also been isolated from isthmus. Data presented in the following two papers (5, 6) have led to the elucidation of the mechanism of the sulfation reaction. The transfer of sulfate to polysaccharides from phosphoadenosine 5'-phosphosulfate by extracts of embryonic chick cartilage (7) and from p-nitrophenyl sulfate by extracts of the snail, Charonia variegata (8), have been previously investigated.

EXPERIMENTAL

Methods and Materials

Preparation of Enzymes—Isthmi (approximately 15 g obtained from three laying Rhode Island Red hens) were removed and immediately treated in a Waring Blendor for 1 minute in 60 ml of 0.25 M sucrose at 5°. The homogenate was centrifuged at 24,000 X g for 25 minutes. The supernatant solution, referred to as Fraction A, contains the sulfate-activating enzymes, ATP sulfurylase, and APS-kinase (9, 10). The precipitate was homogenized again and centrifuged as above. The supernatant solution was discarded. The precipitate was resuspended in 15 ml of 0.01 M phosphate buffer, pH 7.2, and treated in a Raytheon 10-ke. sonic oscillator for 3 minutes, then spotted on Whatman No. 3 MM filter paper, and chromatographed overnight in solvent A. After drying, the area at the origin where the incubation was originally spotted (2 sq. cm) was cut out, placed in a sample pan, and counted directly in an infinitely thin layer. To correct for absorption of radioactive products of enzymatic activity, previous investigators have employed precipitation of mucopolysaccharide with alcohol or detergents followed by appropriate washing or dialysis, or both. The usefulness of this method for measuring the incorporation of radioactivity into mucopolysaccharides is limited by its requirement for relatively large quantities of the sample and by minor technical errors. The following procedure permits the determination of as low as 50 c.p.m. S35 per 10 µg of mucopolysaccharide in the presence of more than 107 c.p.m. of PAPS35 and S35-inorganic sulfate, and is based on the paper chromatographic immobility of mucopolysaccharides in several solvents. The incubation mixture (see below, 50 µl) was heated in boiling water for 3 minutes, then spotted on Whatman No. 3 MM filter paper, and chromatographed overnight in solvent A. After drying, the area at the origin where the incubation was originally spotted (2 sq. cm) was cut out, placed in a sample pan, and counted directly in a windowless gas flow counter. Inorganic sulfate (RF = 0.35), PAPS (RF = 0.42), APS (RF = 0.49), UDP-GalNAc-S (RF = 0.20), and small molecular weight radioactive compounds which were formed in the incubation were essentially completely removed by this procedure from the mucopolysaccharides employed. It has been established that counting by this procedure is linearly related to the amount of radioactivity in the paper disk, and that the efficiency of counting S35 in these disks is 35% of counting in an infinitely thin layer. To correct for absorption by the paper, all radioactivity measurements obtained by counting paper disks have been divided by the factor, 0.35. A radioautograph of the chromatogram (usually 24 to 48 hours' exposure

* Present address, Department of General Education, Nagoya University, Nagoya, Japan.
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1 S. Suzuki and J. L. Strominger, unpublished observations.
2 The abbreviations used are: UDP-GalNAc-S, uridine diphosphoglactosamine sulfate; PAPS, 5'-phosphoadenosine 5'-phosphosulfate; APS, adenosine 5'-phosphosulfate.
to Kodak blue brand x-ray film) was frequently made by which means all the substrates and products of the reaction could be detected.

Preparation of APS\(^{35}\) and PAPS\(^{35}\)—The soluble extract of isthmus (Fraction A), rich in the sulfate-activating system, has been used in a simplified method for the preparation from \(^{35}\)S-inorganic sulfate of PAPS\(^{35}\) with specific activity of about \(2 \times 10^9\) c.p.m. per pmole. The incubation mixture contained, in 10 ml of a mixed buffer consisting of an equal volume of 0.02 M citrate-phosphate buffer (pH 6.5) and 0.02 M Tris-acetate buffer (pH 6.5), 35 pmoles of ATP, 20 pmoles of MgCl\(_2\), 12 mc of carrier-free \(^{35}\)S-H\(_2\)SO\(_4\) (obtained from the Oak Ridge National Laboratory and neutralized with KOH), and 15 mg of protein from Fraction A. The amount of PAPS\(^{35}\) reached a maximum after a 3-hour incubation at 37\(^\circ\). The components of the incubation mixture were then separated by electrophoresis in a buffer containing equal parts of 0.05 M citrate-phosphate (pH 6.5) and 0.1 M Tris-acetate (pH 6.5) in the apparatus described by Markham and Smith (11). A 10 x 57 cm strip of Whatman No. 3 MM filter paper was wetted with the buffer, hung up by one end, and lightly blotted to remove surface moisture. The incubation mixture, 3.3 ml, was slowly applied with a pipette as a band from edge to edge 9 cm from one end of the strip during about 5 minutes. One to 2 cm of the end of the strip nearest to the applied band was placed in the buffer at the cathode and the other end in the buffer at the anode. Negatively charged compounds migrated toward the anode during 2 hours of electrophoresis at 750 volts (14 volts per cm). The strip was then removed, allowed to dry, and placed on x-ray film overnight at -18\(^\circ\) (to minimize hydrolysis) (Fig. 1). A faint ultraviolet-absorbing band, which corresponded to the radioactive band containing PAPS\(^{35}\) and which was much more prominent when the preparation was carried out with 70 pmoles of K\(_2\)SO\(_4\) replacing carrier-free \(^{35}\)S-inorganic sulfate, was occasionally seen. PAPS\(^{35}\) was eluted from the paper strip with water. In a typical preparation \(2.6 \times 10^8\) c.p.m. of PAPS\(^{35}\) were obtained from the three electrophoresis

![Fig. 1a. Ultraviolet absorption print (left) and radioautogram (right) of paper electrophoretic strip used for preparation of PAPS\(^{35}\).](image.png)
minutes at 37°C. The suspension was then cooled to 5°C, treated inorganic sulfate and incubated in a flask with shaking for 90 minutes at 37°C. Omission of phosphate from the buffer also favored APS accumulation.

Preparation of UDP-GalNAc-S35—Four grams of slices of isthmus were suspended in 5 ml of Krebs-Ringer isotonic medium (14) (Ca++ and SO4²⁻ free) containing 1.7 mc of carrier-free S35-inorganic sulfate and incubated in a flask with shaking for 90 minutes at 37°C. The suspension was then cooled to 5°C, treated for 2 minutes in a Virtis homogenizer, and centrifuged at 24,000 X g for 10 minutes. The clear supernatant fluid was subjected to paper electrophoresis in 0.05 M Tris-acetate buffer (pH 6.0) as described for the preparation of PAPS. UDP-GalNAc-S (mobility, 12 cm per hour), which is present in the slice at high concentrations, was easily located by quenching of ultraviolet light. The principal radioactive band corresponded exactly to this ultraviolet-absorbing band. UDP-GalNAc-S35, 6 x 10⁶ c.p.m., was eluted from the paper with water. The specific activity, based on uridine, was 2.1 x 10⁶ c.p.m. per pmole.

Further, the compound was hydrolyzed to UDP and GalNAc-S35 by treatment with 0.01 N HCl at 100°C for 5 minutes. UDP was identified by paper chromatography in Solvents A and B, and GalNAc-S35 was identified by paper chromatography in Solvent E and by paper electrophoresis in 0.05 M acetate buffer, pH 5.2. A single radioactive compound was obtained both with the intact and with the hydrolyzed preparations corresponding in mobilities to UDP-GalNAc-S and synthetic GalNAc-S35 (3), respectively. With the intact preparation, ultraviolet-absorbing material and radioactivity coincided in Solvents A, B, C, D, and E.

No PAPS35 was observed in the slice experiment. Two other diffuse radioactive bands were observed which corresponded to polysaccharides in their electrophoretic mobilities (5 and 8.5 cm per hour) and in having RP values equal to zero on paper chromatography in Solvents A, B, C, D, and E.

Paper Chromatography—Descending paper chromatography was carried out on Whatman No. 3 MM filter paper in the following solvents: A, isobutyric acid, 0.5 N ammonia (5:3) (15); B, ethanol, 1 M ammonium acetate, pH 7 (7.5:3) (16); C, n-propanol, ammonia, water (6:3:1) (15); D, n-propanol, 0.1 M phosphate, pH 6.8 (containing 60 g of ammonium sulfate per 100 ml) (2:100) (17); E, n-butanol, acetic acid, water (5:1:3:5) (18).

Polysaccharides—Generous gifts of polysaccharides are acknowledged as follows: sodium chondroitin sulfate A (prepared from the crystalline calcium salt from bovine tracheal cartilage (21)) and chondroitin sulfate B from bovine lung (22) (from Dr. Roger Jeanloz), bovine corneal chondroitin and keratosulfate and chondroitin sulfate C from chondrosarcoma (from Dr. K. Meyer) (23), chondroitin sulfate from shark cartilage (24, 25) and charonin sulfates (26) (from Dr. F. Egami), and sulfated hexasaccharide from the liver of a patient with Hurler's syndrome (27) (from Dr. D. Brown). Chondroitin was also prepared by chemical desulfation of chondroitin sulfate A (28). Hyaluronic acid, glycogen, and heparin were commercial preparations.

Results

Enzymatic Transfer of Sulfate from PAPS35 to Water and Other Acceptors—When Fraction B was incubated for 3 hours with PAPS35 as described below but in the absence of phosphate and fluoride, 90 to 95% of the PAPS35 was hydrolyzed to inorganic sulfate. The reaction was followed by paper chromatography of the incubation mixture in solvent A and radioautography of the resulting chromatogram. The product of the reaction had the RP of inorganic sulfate and was further identified as inorganic...
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sulfate by its resistance to hydrolysis by concentrated HCl and by its rapid, characteristic electrophoretic mobility. However, in the presence of 0.01 M fluoride and 0.01 M phosphate only 1 to 2% of the PAPS was converted to inorganic sulfate. Qualitatively, the rate of enzymatic hydrolysis of APS35 to S35-inorganic sulfate seemed faster than that of PAPS35, and, since PAPS35 was also slowly hydrolyzed to APS35 by the enzyme preparation, either or both of these compounds might be substrates for the sulfatase reaction. Sulfate, 0.01 M, and 0.01 M fluoride inhibited the sulfatase to about the same extent as phosphate and fluoride. Some nonenzymatic hydrolysis of PAPS35 (usually less than 1%) also occurred, both during its preparation and during incubation.

Transfer reactions were demonstrable only in the presence of phosphate and fluoride because of the high activity of the PAPS sulfatase. When Fraction B was incubated with PAPS35, phosphate and fluoride, at least 8 unidentified compounds were formed in addition to inorganic sulfate (Fig. 3). With APS35 as substrate, inorganic sulfate was the only product detected.

Since various phenols are substrates for liver phenol sulfotransferase, the effects of addition of p-nitrophenol, tyramine, and tyrosine were examined. With addition of p-nitrophenol or tyramine, in each case formation of an intense radioactive area was stimulated. The addition of tyrosine, however, had no observable effect on the reaction products. The spot formed on addition of tyramine (Rs 0.52 in solvent A) contained approximately 5,400 c.p.m.; that formed on addition of p-nitrophenol (Rs 0.81) contained approximately 7,500 c.p.m. No further work has been done on compounds with Rs greater than 0.5. Their formation was reduced and in some cases eliminated by preliminary fractionation of the system (see below).

Two radioactive compounds with small chromatographic mobilities in solvent A, characteristic of nucleotide or oligosaccharide sulfates, were also formed in the reaction and are designated a (Rs 0.14) and b (Rs 0.20). Both compounds were hydrolyzed to a new sulfate ester by 0.01 M HCl for 5 minutes at 100°C. Exhaustive digestion of the acid hydrolysis products of both a and b by the incompletely purified hyaluronidase preparation yielded a radioactive compound with the chromatographic and electrophoretic mobility of acetylgalactosamine monosulfate (5). The amounts of these compounds were greatly increased by the addition of boiled enzyme. Further characterization is in progress.

Although the mobility of UDP-GalNAc-S is identical to that of b in solvents A and C, these two compounds can be distinguished by electrophoresis and by paper chromatography in solvents B and D. If UDP-GalNAc-S was added as carrier either before or after incubation with PAPS35 and then separated by chromatography and electrophoresis, no radioactivity was found in the UDP-GalNAc-S. Addition of UDP-GalNAc and substitution of APS35 for PAPS35 did not result in incorporation of S35 into UDP-GalNAc-S. It is remarkable that, although UDP-GalNAc-S35 is the predominant radioactive compound formed by slices of ishmu incubated with S35-inorganic sulfate, no trace of incorporation has been detected by the sensitive methods used from APS35, PAPS35, or ATP (or an ATP-generating system) plus S35-inorganic sulfate with the use of homogenates, Fraction A or Fraction B.

Enzymatic Transfer of Sulfate from PAPS35 to Mucopolysaccharides—Although in the experiments described above no radioactivity remained at the origin, occasionally a light spot was observed in this position on the radioautogram. The formation of a compound remaining at the origin in solvent A was greatly stimulated by the addition of several mucopolysaccharides (Fig. 3); the formation of the other products, particularly a and b, was concomitantly reduced. This observation led to development of the method described above for measurement of incorporation of S35 into mucopolysaccharide. The assay system contained, in 50 μl of 0.01 M citrate-phosphate buffer, pH 6.6, 0.2 μmole of PAPS35 (3.8 × 10⁶ c.p.m.), 0.1 μmole of MgCl₂, 1 μmole of KF, 50 μg of mucopolysaccharide as acceptor, 18 μg of protein

![Fig. 2. Effects of ATP and Mg²⁺ concentrations on PAPS and APS formation.](http://www.jbc.org/)

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Fig. 2. Effects of ATP and Mg²⁺ concentrations on PAPS and APS formation. Incubation mixtures contained in 50 μl of 0.02 M citrate-phosphate buffer, pH 6.8, 1.1 × 10⁵ c.p.m. S35-inorganic sulfate, and 0.15 mg of protein from Fraction A. The effect of ATP concentration was determined with 0.1 μmole of MgCl₂ (2 μmole per ml) added and the effect of Mg²⁺ concentration was determined with 0.2 μmole of ATP (4 μmole per ml) added. After 60 minutes at 37°C, the incubation mixtures were subjected to paper electrophoresis, compounds were located by radioautography, and paper disks cut out for counting.

2 For example, electrophoretic mobilities at pH 6.0 of b, a, UDP-GalNAc-S and acetylgalactosamine sulfate are 15, 13.5, 12 and 8.7 cm per hour, respectively.
from Fraction B, and supernatant solution from boiled, centrifuged Fraction B (an amount equivalent, before boiling, to 45 μg of protein), and was incubated for 30 minutes at 37°. The requirements for PAPS\(^5\), acceptor, boiled supernatant solution, and Fraction B have been documented in a preliminary note (1). In the absence of Mg\(^{++}\) the incorporation was reduced about 50%.

A number of mucopolysaccharides served as acceptors in the reaction, viz. chondroitin sulfates A, B, or C, chondroitin (prepared from chondroitin sulfate A by chemical desulfation or isolated from bovine cornea), and the sulfated heptasaccharide isolated from the liver of a patient with Hurler's syndrome (possibly equivalent to heparitin sulfate) (29a). However, glycogen, heparin, keratosulfate, hyaluronic acid, and charonin sulfates (low sulfate content or high sulfate content) did not stimulate the reaction. The relationship between mucopolysaccharide concentration and reaction velocity is shown in Fig. 4. It is evident that both maximum velocities and Michaelis constants differ among the various acceptors. The high \(V_{max}\) with chondroitin sulfate C (containing 6-substituted sulfate residues (23)) as acceptor as compared to chondroitin sulfate A or chondroitin sulfate B (containing 4-substituted sulfate residues (23)) is compatible with data to be presented in the following papers (5, 6) that the primary position of sulfation by the oviduct enzyme may be at the 4-position of acetylgalactosamine, the 6-position being sulfated only at a lower rate or perhaps only after sulfation of the 4-position. \(V_{max}\) with corneal chondroitin was almost 3 times that with chemically prepared chondroitin; the explanation of this difference is not apparent. Under the usual conditions of assay (18 μg of enzyme protein, 30 minutes' incubation) about 3,000 c.p.m. were incorporated into mucopolysaccharides (1) or approximately 1% of S\(^{35}\) from added PAPS\(^{35}\). In absolute terms this represents the transfer of an extremely small amount of sulfate, about 1.5 μmoles to
about 1 μmole of acceptor, the measurement of which was possible only because of the high specific activity of the substrate. Neither APS₃⁵, p-nitrophenyl S₃⁵-sulfate, tyramine S₃⁵-sulfate, or S₃⁵-inorganic sulfate could substitute for PAPEV.

To obtain a greater percentage of transfer of sulfate from PAPS₃⁵, an incubation with chondroitin sulfate A as acceptor was carried out for 4 hours with a large amount of an ammonium sulfate-fractionated enzyme (92 μg of protein) which contained little sulfate activity (see below). After separation of the products of incubation by paper chromatography in solvent A, the distribution of radioactivity was as follows: (a) in a control with boiled enzyme: PAPS₃⁵, 378,000 c.p.m.; S₃⁵-inorganic sulfate, 47,000 c.p.m.; and chondroitin sulfate A, 40 c.p.m. (amounts essentially identical to those found in the substrate itself); (b) in the sample: PAPS₃⁵, 140,000 c.p.m.; S₃⁵-inorganic sulfate, 42,000 c.p.m.; and chondroitin sulfate A, 243,000 c.p.m. Thus, 64% of sulfate from added PAPS₃⁵ was transferred to acceptor. Although this still represents a small net transfer (120 μmole), the experiment insures that the reaction observed was not due to a trace radioactive impurity in the PAPS₃⁵ preparation.

Identification of Radioactive Mucopolysaccharides—A number of procedures have been employed to identify the products formed on addition of mucopolysaccharides as radioactive mucopolysaccharide. Metachromatic staining with toluidine blue indicated that the added mucopolysaccharide, like the radioactive product, remained at the origin in solvent A. Appropriate control incubations further indicated that small molecular weight S₃⁵-labeled compounds, such as inorganic sulfate, APS, PAPS, and UDP-GalNAc-S, were completely removed from mucopolysaccharides by the chromatographic procedure.

A large scale incubation was carried out with chondroitin sulfate A as acceptor. The radioactive compound at the origin was eluted from the paper with water, and aliquots were subjected to various procedures. After paper electrophoresis a single radioactive compound was detected on radioautography (Fig. 5). This radioactive spot coincided with chondroitin sulfate A, located by metachromatic staining. Furthermore, the ratio of radioactivity to glucuronic acid (determined by the carbazole reaction [30]) in the compound eluted from the electrophoresis strip (54,500 c.p.m. per μmole of glucuronic acid) was identical to the ratio obtained before electrophoresis (55,800 c.p.m. per μmole).

Samples were hydrolyzed at 100° in 0.01 N, 0.04 N, and 1 N HCl for 10, 60, and 60 minutes, respectively. The hydrolysates were taken to dryness under reduced pressure and chromatographed in solvent A. The phosphosulfate linkage of PAPS is rapidly hydrolyzed in 0.01 N HCl and N-sulfate linkages (but not O-sulfate linkages) are hydrolyzed in 0.04 N HCl (31). After hydrolysis in 0.04 N HCl no degradation of the radioactive compound was detected. In 0.04 N HCl degradation products were formed with chromatographic mobilities of oligosaccharides. Only about 20% of the S₃⁵ was liberated as sulfate in 0.04 N HCl, and in 1 N HCl the compound was hydrolyzed completely to inorganic sulfate (Fig. 6). Therefore, the acid stability of the radioactive product was that of a sulfate ester.

Samples were hydrolyzed enzymatically with testicular hyaluronidase and with a preparation from Flavobacterium heparinum containing both a chondroitinase and a chondrosulfatase. In each case compounds (observed after chromatography in solvent A) were formed with the mobility of oligosaccharides. Inorganic sulfate was formed only with Flavobacterium enzyme. Identification of the radioactive oligosaccharides obtained from testicular hyaluronidase digests is considered in a subsequent paper (6).

In the case of chondroitin the radioactive product had an electrophoretic mobility, a few millimeters greater than the polymer detected with toluidine blue (Fig. 5). However, this product was considerably slower electrophoretically than chondroitin sulfate A or chondroitin sulfate C. This result is compatible with the fact that, although a large amount of radioactivity was incorporated into the polymer the net transfer of sulfate to polymer was extremely small. Apparently, the amount transferred was sufficient to result in a small increase in electrophoretic mobility. The products of digestion of this radioactive product by testicular hyaluronidase will also be considered in a following paper (6).

The radioactive product obtained from incubations containing the heparitin-like heptasaccharide coincided with polysaccharide in electrophoretic mobility, and moved slower than the product obtained with chondroitin sulfate A as acceptor (Fig. 5). The radioactivity was quantitatively converted to S₃⁵-inorganic sulfate by treatment with 0.1 N HCl for 4 hours. Like the heptasaccharide, the radioactive product was not hydrolyzed by testicular hyaluronidase, but both oligosaccharides and inorganic sulfate were formed after incubation with enzyme from Flavobacterium heparinum (Fig. 6). Therefore, the observed suitability of the heptasaccharide as acceptor could not be due to contamination of the heptasaccharide with any sulfated mucopolysaccharide susceptible to testicular hyaluronidase (chondroitin, chondroitin sulfate A, and chondroitin sulfate C).

It is clear, therefore, that the radioactive products formed on addition of mucopolysaccharide acceptors have properties similar to the acceptors themselves.
FIG. 5. Paper electrophoresis of enzymatically synthesized radioactive polysaccharides. Electrophoresis was carried out at 750 volts for 2.5 hours on Whatman No. 1 paper in 0.05 M phosphate buffer, pH 7.0. A radioautogram (a) of the electrophoretic strip was prepared and the polysaccharides were then located by metachromatic staining with toluidine blue (b). The diffuseness of electrophoretic mobility of the heparitin-like heptasaccharide may indicate some heterogeneity of degree of sulfation or of molecular size. ChS-A, chondroitin sulfate A.

**Effects of Boiled Enzyme and Nucleotides on Reaction Velocity**—The stimulation of activity by boiled enzyme (1) suggested that some other compound might be essential to the reaction, possibly a uridine nucleotide. However, the following compounds added alone or in combinations were ineffective, either in replacing boiled enzyme or in stimulating activity in the presence of boiled enzyme: UDP-glucose, UDP-glucuronic acid, UDP-acetylgalactosamine, UDP-acetylgalactosamine, UDP-GalNAc-S, ATP, UTP, CTP, GTP, DPN, and DPNH. UDP-acetylgalactosamine and UDP-GalNAc-S inhibited the reaction. An amount of UDP-GalNAc-S sufficient to produce 50% inhibition (0.02 
mu mole) was included in an incubation after which 0.1 
u mole of UDP-GalNAc-S was added as carrier. UDP-GalNAc-S was then isolated by chromatography in solvent A and electrophoresis; it contained no detectable radioactivity. Therefore, free UDP-GalNAc-S could not have been an intermediate in the synthesis of the radioactive polysaccharide synthesized in this partially inhibited condition.

The observation that boiled enzyme in the absence of added acceptor stimulated the formation of α and β, and that the addition of mucopolysaccharide resulted in a striking decrease in the amounts of α and β formed (Fig. 3) suggested that these unidentified compounds might be intermediates in the sulfate transfer reaction. However, when α or β was substituted for PAPS in the incubation, no formation of radioactive mucopolysaccharide occurred. Other experiments suggest that the mucopolysaccharide-induced decrease in the amounts of α and β is due to a competition between several transferases for PAPS.

UDP-GalNAc-S35, isolated after incubation of slices of isthmus with 35S-inorganic sulfate, and 3H-UDP-GalNAc-S, prepared by the Wilzbach procedure (32), also did not transfer radioactivity to mucopolysaccharides. Since the specific activity of these substrates was low (about 10^5 c.p.m. per 
u mole) compared to the specific activity of PAPS, incubations were carried out with 150 μ g of enzyme protein for 4 hours.

Finally, a number of observations suggest that the effect of boiled enzyme is due to stabilization of the enzyme. The effect of boiled enzyme was marked only at **low enzyme concentrations** (Fig. 7) where the concentration of protein during incubation was about 0.3 mg per ml. At such low protein concentrations boiled enzyme had a marked effect on the stability of the enzyme at various temperatures. Enzyme incubated under assay conditions without substrates at -18º for 7 days lost 20% of its initial activity in the presence of boiled enzyme and 91% in its absence; at 37º for 1 hour, values were 53% loss of activity in the presence of boiled enzyme and 95% loss in its absence. Both the factor which stimulated activity and the factor which protected the enzyme were nondialyzable and were destroyed by...
Properties of Enzyme—Purification of the preparation has been difficult because of instability of the enzyme and the limited amounts of starting material obtainable. About 4-fold purification has been realized by precipitation between 45 and 65% ammonium sulfate at pH 5.5, followed by chromatography on diethylaminoethyl cellulose with a pH gradient from 7.0 to 5.7 in 0.01 M phosphate buffer to elute the enzyme. Enzyme purified by ammonium sulfate fractionation did not catalyze any significant hydrolysis of PAPS under the conditions of assay. Yields in each step were about 40% and recombination of fractions failed to result in increase in the amount of activity recovered. Attempts at purification are being continued. The variation of activity of Fraction B with pH and linearity with time of incubation and enzyme concentration are shown in Fig. 7.

Fig. 6. Hydrolysis of enzymatically sulfated chondroitin sulfate A (ChS-A) and heparitin-like heptasaccharide. Radioautograms of chromatograms developed in solvent A are shown. a, Acid hydrolysis of radioactive chondroitin sulfate A: 1, by 0.01 N HCl; 2, by 0.04 N HCl; and 3, by 1 N HCl. b, Hydrolysis of radioactive heptasaccharide: 1, by testicular hyaluronidase; 2, by acetone powder extract of Flavobacterium heparinum, and 3, by 0.1 N HCl for 4 hours. In unhydrolyzed preparation of both compounds only the radioactive spot at the origin of the chromatogram is seen (X = artifact).
A number of transfer reactions of PAPS have been previously identified including transfer to phenols (13), to two different types of steroids (catalyzed by separate enzymes) (33), and to tyrosine derivatives (34). Preliminary investigation of transfer reactions leading to formation of choline sulfate (35), of aryl-sulfamates (36), and of cerebroside sulfate (37) have also been reported, but the mechanisms of these sulfation reactions have not been clarified.

In previous work on the transfer of sulfate from PAPS to mucopolysaccharides in embryonic cartilage extracts (7), a transfer of 4% of S35 from added PAPS35 was demonstrated. In appropriately supplemented preparations S35-inorganic sulfate and C14-acetate could also be incorporated. No dependence of the reactions on added acceptor was demonstrated (presumably a consequence of the presence of acceptor in the enzyme extract) and the nature of the product formed was not established. Comparison of data suggests that the preparations employed here may be of considerably higher activity. In any case, the demonstrated transfer of 64% of sulfate from PAPS35 eliminates the possibility that the observed reaction was due to a trace impurity in the PAPS35 preparation. The reaction had an absolute dependence on added acceptor and the radioactive products formed were shown to have properties similar to the particular acceptor employed. Among the acceptors which were active in this system, chondroitin, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C all contain acetylgalactosamine residues, although chondroitin sulfate A and chondroitin sulfate B contain acetylgalactosamine-4-sulfate residues whereas chondroitin sulfate C is believed to contain acetylgalactosamine 6-sulfate residues (23). However, the heparitin-like heptasaccharide which was active as an acceptor contains only acetylglucosamine. This heptasaccharide was obtained in large quantity from the liver of a patient with Hurler’s syndrome and has not been isolated from normal liver. Although the location of the sulfate is unknown, fractions of varying degrees of sulfation have been isolated (27). The enzymatic sulfation of these materials is being examined in greater detail. It is noteworthy that hyaluronic acid, which also contains acetylglucosamine, is inactive as an acceptor.

Although the transfer of sulfate from p-nitrophenyl sulfate to a glucan (charonin sulfate) by extracts of Charonia lampas was closely related to the aryl sulfatase content of the preparations, it is not certain that the sulfatase participates directly in the transfer reaction (8). In the experiments reported here, there are several differences between the transfer of sulfate from PAPS35 to mucopolysaccharide and the hydrolysis of PAPS35 by the enzyme preparation. Thus, the pH optimum for sulfate transfer was 6.5 and that for hydrolysis was 7.3. The sulfatase was greatly inhibited by phosphate and fluoride; however, these compounds activated the sulfate transfer reaction due to protection of the substrate from hydrolysis. Finally, in some preparations sulfate transfer has been carried out in the presence of phosphate and fluoride without significant enzymatic hydrolysis of the substrate. Whether or not some catalysis of transfer of sulfate to water is a property of the enzyme which catalyzes transfer of sulfate to mucopolysaccharide is a question which can be answered only after extensive purification, but it seems probable that the transfer reaction is distinct from the major PAPS sulfatase activity of the preparation.

In the experiments reported here, no data were obtained to support the idea that the sulfate transfer reaction being studied was a multistep reaction involving the addition of sugar units as well as sulfate to the polymer chain. In the following two papers, evidence will be presented to support the conclusion that the enzyme or enzymes under investigation catalyze a direct transfer of sulfate from PAPS to mucopolysaccharides.

SUMMARY

1. An enzyme has been solubilized from the isthmus of hen oviduct which catalyzes the transfer of S35-sulfate from 3',5'-phosphoadenosine 5'-phosphosulfate (PAPS) to added mucopolysaccharide acceptors. The reaction has an absolute dependence on added acceptor. A number of mucopolysaccharides, including chondroitin, chondroitin sulfates A, B, or C and the heparitin-like sulfated heptasaccharide from the liver of a patient with Hurler’s syndrome, are active in the system. Sixty-four percent of sulfate from PAPS has been transferred to mucopolysaccharide with the preparation employed.

2. Chemical and enzymatic analysis of the radioactive prod-
ucte formed with chondroitin, chondroitin sulfate A, and the heparitin-like heptasaccharide as acceptors have shown that a different radioactive product is formed in each case and that these radioactive products have properties similar to those of the acceptors employed.

3. Despite the presence of uridine diphosphoacetylgalactosamine sulfate in the isthmus, no evidence that there were any intermediates in the transfer of sulfate from PAPS to chondroitin sulfate A could be obtained.

4. The preparation employed contains a PAPS-sulfatase and a number of other sulfotransferases, including a tyramine sulfotransferase. These reactions have not been fully characterized.

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