Inhibition of Mucopolysaccharide Sulfation

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Abstract □ A method of measuring the inhibition of sulfation in mucopolysaccharides employed the coupled enzyme system of Wortman. This procedure utilized the phenol and mucopolysaccharide sulfotransferases of beef cornea extract. Compounds tested against this system included amino acids, salicylates, 2-mercaptoethylamine and a derivative, heterocyclic ethanethiosulfuric acids, arylsulfonamidoethanethiosulfates, and several other compounds. In general, the salicylates and thiosulfates were inhibitory, with sodium 2-(4-acetamidobenzenesulfonamido)-ethanethiosulfate being the most inhibitory and 2-mercaptoethylguanidine trithiocarbonic acid the second most inhibitory. Salicylamide and allopurinol, which have been employed in kidney stone therapy, were inhibitors. Kidney stones have a mucopolysaccharide sulfate matrix, which is involved in their growth.

Keyphrases □ Mucopolysaccharides—inhibition of sulfation by salicylates and thiosulfates □ Salicylates—inhibition of mucopolysaccharide sulfation □ Thiosulfates—inhibition of mucopolysaccharide sulfation

Mucopolysaccharide sulfates play a role in the calcifying mechanism of most kinds of renal calculus formation (1). Boyce and Garvey investigated over 1200 renal calculi and found a matrix of mucopolysaccharide sulfates in all of them, to the extent of ~2.5% of the total weight (2). Furthermore, kidney stone formers generally have more highly sulfated mucopolysaccharides in their urine than normal (3). Other pathological conditions that are known to be associated with abnormal amounts or degrees of sulfation of mucopolysaccharides (4) include some malignant tumors (5). Abnormal amounts of mucopolysaccharide sulfates also have been observed in virus-transformed cells (6).

It therefore was considered important to find compounds that might inhibit the sulfation of mucopolysaccharides and to develop a method of measuring this inhibition. Previous studies (7) on anti-inflammatory agents showed that salicylate exerts an inhibitory effect on the sulfation of mucopolysaccharides. It also was found that several metal-binding agents, including substituted salicylates, cysteine, and 2-mercaptoethylamine and some of its derivatives, inhibited sulfation (8). Other inhibitors of this process are propylthiouracil (9), 4-methylumbelliferyl β-xyloside (10), and *p*-nitrophenyl β-xyloside (10). All of these studies were carried out with intact cartilage. However, the use of the enzyme systems involved appeared to be a more convenient and reliable method of measuring inhibition of mucopolysaccharide sulfation since use of intact cartilage requires embryonic cartilage not yet fully sulfated.

ENZYME ASSAY

Sources of the enzyme systems known to catalyze formation of mucopolysaccharide sulfates are found in various cartilaginous tissues, including the cornea, as well as in the liver. Trials with extracts of rabbit liver, dog cornea, and beef cornea revealed that the beef corneal extracts gave the most reproducible results. Wortman (11) showed the presence of both phenol and mucopolysaccharide sulfotransferase activities in beef cornea epithelial extracts and made use of the phenol sulfotransferases (Schemes I and II) as a feeder system for the assay and characterization of mucopolysaccharide sulfotransferase (Scheme III). Although both phenol and mucopolysaccharide are sulfated in this combined assay, it appeared to be suitable for measuring mucopolysaccharide sulfation and allowed the use of a spectrophotometric procedure.

p-nitrophenol sulfate + 3′,5′-diphosphoadenosine
→ *p*-nitrophenol + 3′-phosphoadenosine 5′-phosphosulfate
Scheme I

3′-phosphoadenosine 5′-phosphosulfate + phenol
→ phenol sulfate + 3′,5′-diphosphoadenosine
Scheme II

3′-phosphoadenosine 5′-phosphosulfate + chondroitin
→ chondroitin sulfate + 3′,5′-diphosphoadenosine
Scheme III

Phenol sulfotransferase presumably carries out the reactions shown in both Schemes I and II. The overall rate of sulfate transfer is measured spectrophotometrically by the appearance of *p*-nitrophenol at 400 nm. The participation of mucopolysaccharide sulfotransferase, which also utilizes 3′-phosphoadenosine 5′-phosphosulfate as a coenzyme, assists in driving the overall reaction to the right. This process results in an increase in the change of the optical density and provides a coupled assay method for measuring mucopolysaccharide sulfation.

RESULTS AND DISCUSSION

The activity of the coupled enzyme system in beef cornea extract over 5 hr is shown in Fig. 1. The reaction rate decreased somewhat after the 1st hr, but the reaction still was measurable after 9 hr. The changes in the optical density observed for the phenol sulfotransferase assay (Schemes I and II) and the coupled enzyme assay (Schemes I–III) over 2 hr are shown in Table I. The protein concentration of the extract varied from 0.24 to 0.84 mg/ml. The enzyme activity decreased slowly over 2 weeks; a change in the optical density (Δo.d.) of 0.104 at 2 hr was observed with freshly prepared extract, but after storage at −10° for 2 weeks, Δo.d.

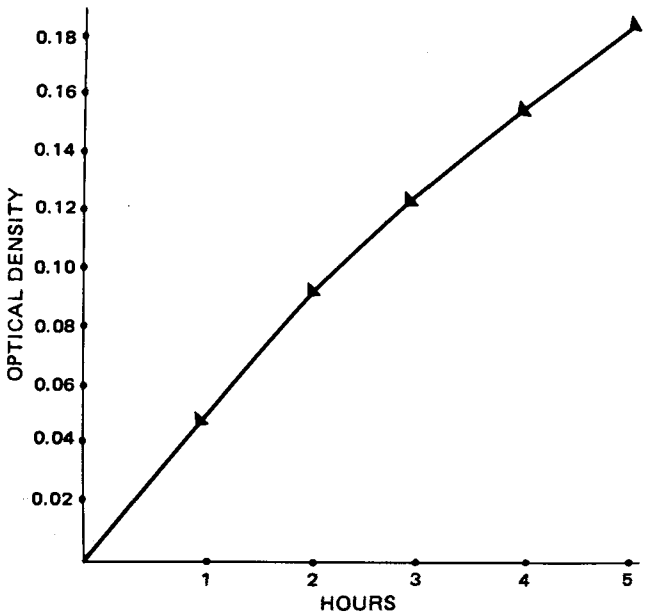

Figure 1—*Activity of the coupled enzyme system in beef cornea extract.*

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Table I—Sulfate Transfer Activity of Beef Cornea Extract

Reaction[a]	Δo.d. at 1 hr	Δo.d. at 2 hr
Schemes I and II	0.040	0.070
Schemes I–III	0.044	0.078

[a] Measured at pH 7.0 and 37° for transfer to chondroitin sulfate. Optical density was recorded at 400 nm.

was 0.021 at 2 hr. However, the enzyme showed no change in activity for 24 hr.

It has been found that various tissues containing sulfotransferases also contain some 3′,5′-diphosphoadenosine (12). A 3′,5′-diphosphoadenosine concentration of 2 μM was reported as the saturation level for phenol sulfotransferase (13), whereas concentrations above 20 μM began to be inhibitory. In this study, the decrease in the reaction rate where added 3′,5′-diphosphoadenosine was omitted from the assay system indicated the presence of 3′,5′-diphosphoadenosine in the extract. The concentration of added 3′,5′-diphosphoadenosine should not be more than 10^{-4} M to prevent inhibition of the enzyme.

Beef cornea is known to contain arylsulfatase, which is inhibited by monobasic potassium phosphate and sodium fluoride (14). In the present study, phosphate buffer was used routinely to prevent sulfatase action. Various concentrations of chondroitin added to the enzyme system did not show appreciable differences in Δo.d. in the range of 0.05–0.5 mM. Although the sulfation of chondroitin was a rapid reaction in the rabbit liver system, the reaction persisted for 5 hr in the beef cornea system.

A previous investigation of inhibition of mucopolysaccharide sulfation in veal costal cartilage showed that 2-mercaptoethylamine and 2-mercaptoethylguanidine trithiocarbonate were powerful inhibitors (8), inhibiting sulfation by 90% or more. Since this degree of inhibition may have undesirable effects, particularly on bone growth, compounds with a lower degree of inhibitory activity were sought. A series of 2-mercaptoethylamine derivatives, aromatic and arylsulfonyl aminoethanethiosulfates, which should be less toxic than the thiols, was synthesized. Several salicylates and other compounds used in the previous study also were included, as well as nitrofurantoin and allopurinol, compounds found to be effective in inhibiting kidney stone growth. Results of the inhibition study are shown in Table II.

Of the salicylic acid derivatives tested, salicylic acid and salicylamide showed appreciable inhibition of sulfation and p-aminosalicylic acid showed some inhibition, but salicyl hydrazide showed slight stimulation of the enzyme system. These results indicate that metal-ion complexation is not a factor in the inhibition of this sulfation system. Cysteine and riboflavin both inhibited sulfation by ~20%, but nitrofurantoin gave only 5% inhibition. The latter compound showed much greater inhibition (65%) in the study using veal costal cartilage.

Allopurinol, currently used in kidney stone treatment, gave 26% inhibition. 2-Thiouracil produced 12% inhibition. 2-Mercaptoethylamine gave 27% inhibition, whereas it gave 90% inhibition in the costal cartilage system. 2-Mercaptoethylguanidine trithiocarbonate, which gave 93% inhibition in veal costal cartilage, showed 82% inhibition in the beef cornea system. At the pH (7.0) of the corneal enzyme system, much of the 2-mercaptoethylamine may have been converted to the disulfide during the observation period (2 hr).

Table II—Inhibition of Sulfate Transfer in Beef Cornea Extract

Compound	Activity[a], %
None	100
Cysteine	79
Riboflavin	80
Nitrofurantoin	95
Salicylamide	22
Salicylic acid	38
Salicyl hydrazide	109
p-Aminosalicylic acid	73
2-Mercaptoethylamine	73
2-Mercaptoethylguanidine trithiocarbonic acid (17)	18
Allopurinol	74
2-Thiouracil	88
2-[2-(4,6-Diamino-1,3,5-triazinyl)amino]-ethanethiosulfuric acid	75
Sodium 2-(7-theophyllinyl)ethanethiosulfate	145
Sodium 2-(4-acetamidobenzenesulfonamido)-ethanethiosulfate	0
Sodium 2-(2-naphthalenesulfonamido)ethanethiosulfate	23
Sodium 2-(8-quinolinesulfonamido)ethanethiosulfate	68

[a] Measured after 2 hr of incubation at 37° at a concentration of 5×10^{-3} M of the inhibitory compound, with the exceptions noted for riboflavin, nitrofurantoin, and sodium 2-(7-theophyllinyl)ethanethiosulfate. Activities of different enzyme preparations were adjusted to give a standard value.

The arylaminoethanethiosulfates were, in general, inhibitory. Sodium 2-(4-acetamidobenzenesulfonamido)ethanethiosulfate showed complete inhibition, and sodium 2-(2-naphthalenesulfonamido)ethanethiosulfate gave 77% inhibition. Sodium 2-(8-quinolinesulfonamido)ethanethiosulfate gave 32% inhibition, and 2-[2-(4,6-diamino-1,3,5-triazinyl)-amino]ethanethiosulfuric acid gave 25% inhibition. However, sodium 2-(7-theophyllinyl)ethanethiosulfate showed marked stimulation of the enzyme system. An increase in the synthesis rate of glycosaminoglycans (chondroitin and dermatan sulfates) was demonstrated in transformed fibroblast cells by the addition of theophylline (15).

It was postulated in the previous study of sulfation inhibition (8) that metal-ion complexation may be a factor since the compounds tested had the common property of metal binding and the enzyme system involved requires magnesium and possibly other metals. Not all of the compounds observed in the present study are known to be metal binding, so this postulation cannot be extended here. No other chemical property common to all of the compounds is apparent.

With compounds having hydroxyl groups, such as the salicylates and allopurinol, the possibility exists that they may act as sulfate acceptors and compete with mucopolysaccharides. It also is possible that the thiol group could function in the same manner, but the thiosulfates could enter only into possible sulfate-exchange reactions. Therefore, direct inhibition of the enzyme system involved by some form of complexation seems more likely.

EXPERIMENTAL[1]

Enzyme Preparation—Wortman's method (11) was followed. Abou 75 fresh, iced beef corneas were washed with tris(hydroxymethyl)aminomethane hydrochloride buffer (0.05 M, pH 7.4) and then soaked in 50 ml of the buffer before homogenization. A blender[2] was used for homogenization for 30 sec, and the volume was brought to 100 ml by addition of the same buffer. The mixture was homogenized for 1 min, and the homogenate was centrifuged at 0° for 1 hr at 34,800×g. The supernate was collected and dialyzed against 2000 ml of 0.005 M phosphate buffer with 0.5 mM ethylenediaminetetraacetic acid (pH 6.5) for 1 hr in a refrigerator. The preparation was stored in a freezer.

Sulfotransferase Assay—The reagents were 0.6 M phosphate buffer (pH 7.0), 5×10^{-3} M potassium p-nitrophenol sulfate, 10^{-5} M 3′,5′-diphosphoadenosine, 10^{-4} M chondroitin sulfate, 5×10^{-3} M phenol, and beef cornea extract.

Method A: Assay of Phenol Sulfotransferase—To a mixture of 0.3 ml of each reagent (except chondroitin sulfate), 0.1 ml of buffer, and 1.5 ml of distilled water in a spectrophotometric cell (S_1) with a 1-cm light path was added 0.5 ml of beef cornea extract to make the total volume 3 ml. After mixing, the optical density at 400 nm was recorded. The mixture was incubated in a water bath at 37° for 2 hr, and the change in the optical density at 400 nm was recorded after each hour of incubation. A blank ($S_{1'}$) was run in which phenol was replaced by water.

Method B: Assay of Mucopolysaccharide Sulfotransferase—The same procedure as in Method A was carried out, except that 0.3 ml of water in S_1 and $S_{1'}$ was replaced by chondroitin sulfate. A spectrophotometric cell (R) filled with 2 ml of phosphate buffer and 2 ml of water was used as the reference cell for sample cells S_1 and $S_{1'}$ to measure the reactions shown in Schemes I and II and Scheme I, respectively.

Inhibition Study—To a mixture of each reagent used in the assays and 0.1 ml of buffer in a spectrophotometric cell (S_2) with a 1-cm light path was added 0.3 ml of a solution of inhibitor [5×10^{-3} M, except that 0.25×10^{-3} M riboflavin and nitrofurantoin and 0.28×10^{-3} M sodium

[1] Melting points were determined with a Mel-Temp block and are uncorrected. IR absorption spectra were obtained with a Perkin-Elmer 457 grating spectrophotometer using potassium bromide pellets. NMR spectra were measured with a Varian T-60 spectrometer with tetramethylsilane as the internal standard. Elemental analyses were done by F. B. Strauss, Oxford, England, or by Carol K. Fitz, Carlisle, Mass. TLC was carried out using silica gel plates, and products were detected by exposure to iodine vapor or UV light. Starting substances for the syntheses were obtained from Aldrich Chemical Co., Eastman Organic Chemicals, and Fisher Scientific Co.

p-Nitrophenol sulfate, 3′,5′-diphosphoadenosine, and chondroitin sulfate were obtained from Sigma Chemical Co. Rabbit liver and beef cornea were obtained from Pel-Freez Biologicals. Nitrofurantoin was a gift of Eaton Laboratories, and allopurinol was a gift of Dr. Edwin L. Prien, Sr.

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2-(7-theophyllinyl)ethanethiosulfate were used]. Beef cornea extract (0.5 ml) was added and mixed to give a total volume of 3 ml. The mixture was incubated in a water bath at 37°. The change in optical density at 400 nm was recorded over 2 hr. A blank ($S_{2'}$) was run in which phenol and chondroitin sulfate were replaced by water. A third cell filled with 1.0 ml of phosphate buffer, 1.7 ml of distilled water, and 0.2 ml of inhibitor (at the given concentrations) was used as a reference cell, and the previous cells (S_2 and $S_{2'}$) were used as sample cells. The change of optical density of each solution was recorded.

Calculations—The calculation of the change in the optical density (Δo.d.) of the coupled enzyme system was done as shown in the following chart, where S_x is the sample cell, $S_{x'}$ is the blank cell, and R_x is the reference cell.

o.d. at 1 hr	o.d. at 2 hr	Δo.d.
$S_1 - S_{1'} = A$	$S_2 - S_{2'} = a$	$a - A$
$S_1 - R_1 = B$	$S_2 - R_2 = b$	$b - B$
$S_{1'} - R_1 = C$	$S_{2'} - R_2 = c$	$c - C$

Since $A = B - C$, $a = b - c$, and $a - A = (b - c) - (B - C) = (b - B) - (c - C)$, $a - A = (b - B) - (c - C)$.

2-[2-(4,6-Diamino - 1,3,5 - triazinyl)amino]ethanethiosulfuric Acid—To 13.44 g (0.075 mole) of sodium 2-aminoethanethiosulfate (16) and 2.0 g (0.05 mole) of sodium hydroxide in 100 ml of ethanol was added 7.28 g (0.05 mole) of 2-chloro-4,6-diamino-1,3,5-triazine suspended in 10 ml of ethanol with stirring over 2 hr. The mixture was refluxed for 48 hr, filtered, and evaporated to a slurry. Neutralization of the slurry with acetic acid followed by addition of ethanol gave 3.0 g (22%) of a white solid, mp 210–215°. Crystals were obtained from 95% ethanol, mp 225–228°, R_f 0.375 (silica gel with benzene–methanol 8:2); IR (KBr): 3390, 3330, 3240–3120, 1200–1180 (S_2O_3), 1028 (ν-ring), and 645 (β-ring) cm^{-1}.

Anal.—Calc. for $C_5H_{10}N_6O_3S_2$: C, 22.55; H, 3.78; N, 31.55. Found: C, 22.65; H, 4.18; N, 31.79.

Sodium 2-(7-Theophyllinyl)ethanethiosulfate Monohydrate—A mixture of 9.71 g (0.04 mole) of 7-(2-chloroethyl)theophylline and 11.0 g (0.045 mole) of sodium thiosulfate pentahydrate in 150 ml of ethanol–water (1:1) was refluxed with stirring for 24 hr. Flash evaporation of the solvent gave a white solid, which was extracted with 200 ml of boiling ethanol. The insoluble material was filtered, and the filtrate yielded white crystals on chilling overnight; after drying over phosphorus pentoxide for 24 hr, 7.31 g (51%) was obtained. Three recrystallizations from absolute ethanol, with drying (phosphorus pentoxide) for 24 hr and at 60° (calcium chloride) for 48 hr, gave a product that melted at 258–261°; IR (KBr): 3500, 3000, 2900, 1695 (ν-ring), 1650 (ν-ring), 1250–1175 (S_2O_3), and 1030 (ν-ring) cm^{-1}.

Anal.—Calc. for $C_9H_{11}N_4NaO_5S_2 \cdot H_2O$: C, 30.00; H, 3.63; N, 15.54. Found: C, 30.30; H, 3.96; N, 15.25.

Sodium 2-(4-Acetamidobenzenesulfonamido)ethanethiosulfate—A suspension of 11.68 g (0.05 mole) of 4-acetamidobenzenesulfonyl chloride in 150 ml of tetrahydrofuran was added over 2 hr to a solution of 2.0 g (0.05 mole) of sodium hydroxide, 6.3 g (0.075 mole) of sodium bicarbonate, and 7.86 g (0.05 mole) of 2-aminoethanethiosulfuric acid (16) in 150 ml of ethanol at 0–5° with stirring. The mixture was stirred at room temperature for 24 hr, and the precipitate was filtered and dried. It was extracted with 300 ml of boiling 95% ethanol and filtered. After cooling overnight, the filtrate yielded a crude product, which was extracted with 100 ml of ethanol–ethyl acetate (1:1) and recrystallized four times from isopropanol. It was dried (calcium chloride) at 60° *in vacuo* for 24 hr, giving 8.65 g (46%) of a white solid, mp 155–158°; IR (KBr): 3280, 3190, 3130, 1325 (SO_2), 1240–1210 (S_2O_3), 1160 (SO_2), 1040 (CH), and 640 (CH) cm^{-1}.

Anal.—Calc. for $C_{10}H_{13}N_2NaO_6S_3$: C, 31.90; H, 3.48; N, 7.44. Found: C, 31.61; H, 3.85; N, 7.00.

Sodium 2-(2-Naphthalenesulfonamido)ethanethiosulfate—A solution of 11.33 g (0.05 mole) of 2-naphthalenesulfonyl chloride in 100 ml of tetrahydrofuran was added, with stirring, over 1 hr to a solution of 2.0 g (0.05 mole) of sodium hydroxide, 6.3 g (0.075 mole) of sodium bicarbonate, and 7.86 g (0.05 mole) of 2-aminoethanethiosulfuric acid (16) in 100 ml of 95% ethanol at 0–5°. The mixture was stirred at room temperature for 24 hr, filtered, and evaporated to a white solid. This solid was stirred with methylene chloride for 1 hr, filtered, and extracted with 100 ml of boiling absolute ethanol. The cooled filtrate gave 12.0 g (65%) of product, which was recrystallized three times from absolute ethanol and charcoal and dried (calcium chloride) at 60° for 24 hr *in vacuo*, mp 184–185°; IR (KBr): 3280, 3060, 1320 (SO_2), 1225–1195 (S_2O_3), 1155 (SO_2), 1025 (CH), and 635 (CH) cm^{-1}; NMR (deuterium oxide-d_2): δ 3.06–3.35 (m, 4H, CH_2), and 7.40–8.33 (m, 7H, ring H) ppm.

Anal.—Calc. for $C_{12}H_{12}NNaO_5S_3$: C, 39.01; H, 3.27; N, 3.79. Found: C, 39.17; H, 3.65; N, 3.62.

Sodium 2-(8-Quinolinesulfonamido)ethanethiosulfate Monohydrate—A solution of 5.69 g (0.025 mole) of 8-quinolinesulfonyl chloride in 200 ml of tetrahydrofuran was added, with stirring, over 2 hr to a solution of 1.0 g (0.025 mole) of sodium hydroxide, 2.2 g (0.026 mole) of sodium bicarbonate, and 3.39 g (0.25 mole) of 2-aminoethanethiosulfuric acid (16) in 50 ml of water at 0–5°. The mixture was stirred at room temperature for 24 hr and evaporated *in vacuo* to a white solid. The solid was extracted with 300 ml of boiling absolute ethanol and filtered. The cooled filtrate yielded 9.1 g (93%), which was recrystallized several times to give an analytical sample, mp 173–176°; IR (KBr): 3540–3500, 3280, 1335 (SO_2), 1230–1200 (S_2O_3), 1175 (SO_2), 1030 (CH), and 645 (CH) cm^{-1}; NMR (deuterium oxide-d_2): δ 3.0–3.40 (m, 4H, CH_2) and 7.45–8.45 (m, 6H, ring H) ppm.

Anal.—Calc. for $C_{11}H_{11}N_2NaO_5S_3 \cdot H_2O$: C, 34.02; H, 3.44; N, 7.26. Found: C, 34.40; H, 3.32; N, 7.20.

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