

O-Sulfate Esters of Hydroxy Amino Acids in Hydrolyzates of Proteoglycans¹

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Proteoglycans, macromolecules isolated from connective tissues, contain large proportions of sulfated glycosaminoglycans covalently attached to protein (1). Amino acid (AA) analyses of acid hydrolyzates of proteoglycans by the methods of Spackman, Stein, and Moore (2) contain variable amounts of unidentified ninhydrin-reactive materials which elute before aspartic acid. Some of these materials appear near the position of cysteic acid in the analyses even when precautions are taken to prevent its formation (3). It seemed possible that these peaks represent products of reactions between hydroxy amino acids and/or hexosamines with sulfate residues released during acid hydrolysis of the proteoglycans. This suggestion is reinforced by the report of Murray and Milstein (4), who observed the formation of the *O*-sulfates of serine and threonine when proteins were hydrolyzed in the presence of small amounts of sulfate. Further examination of the literature revealed that, earlier, Moore (5) had cautioned against preparing hydrolyzates in the presence of appreciable quantities of sulfate or phosphate because he found that significant amounts of serine were converted to serine *O*-sulfate in a model system containing inorganic sulfate. Indeed, more than 25 years ago, Reitz *et al.* (6) noted that mild treatment of proteins with sulfuric acid led to the formation of sulfate esters of the hydroxy amino acids.

This paper describes the results of experiments which attempt to identify these ninhydrin-positive materials. *O*-Sulfate esters of hydroxyamino acids were synthesized and a chromatographic system was developed for separating and quantitating them.

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METHODS AND MATERIALS

(1) *Preparation of O-Sulfate Esters of Hydroxyamino Acids*

Amino acids and galactosamine·HCl were purchased from Schwarz/Mann Research Laboratories. Potassium L-serine O-sulfate, potassium L-hydroxyproline O-sulfate, and potassium L-tyrosine O-sulfate were prepared by the method of Tudball (7). In the preparation of potassium L-threonine O-sulfate, 500 mg of L-threonine was dissolved in 1 ml of H₂SO₄ (sp.gr. 1.84) at room temperature and then set aside *in vacuo* over CaCl₂ for 1 hr. Instead of pouring the reaction mixture into 100 ml of a 7% solution of Ba(OH)₂·8H₂O at 4°C to remove excess H₂SO₄ as in the preparation of the other sulfate esters, the reaction mixture was poured into 200 ml of cold methyl ethyl ketone, as suggested by Dodgson *et al.* (8). The syrup which separated at the bottom of the beaker was repeatedly triturated in cold methyl ethyl ketone and ethyl ether. After removal of the residual ether *in vacuo* the material was dissolved in 10 ml of water.

For each sulfate ester, the concentrate was passed through a 1 × 20 cm column of Dowex 50(H⁺), and the column was washed with 30 ml of water (7). The unreacted amino acids were retained on the column. Each of the eluates was combined with the respective water wash and the pH was adjusted to 7.0–7.2 with a 5% solution of KOH. The neutralized solutions were reduced in volume to 3–5 ml in a rotary evaporator at 45°. The potassium salts of the esters were then precipitated by the dropwise addition of absolute ethanol at room temperature.

After 3–4 hr at 4° the solid samples were isolated by filtration, using 15 ml funnels with medium-porosity sintered-glass plates. They were washed repeatedly with ethanol and then with ethyl ether. The samples were dried overnight *in vacuo* over CaCl₂ and for 4 days *in vacuo* over P₂O₅ before analysis.

(2) *Formation of O-Sulfate Esters of Hydroxy Amino Acids in Presence of Potassium Sulfate under Conditions Simulating Acid Hydrolysis of Proteins*

5 mg of L-serine, L-threonine, L-hydroxyproline, and L-tyrosine were each mixed with 50 mg of K₂SO₄ and 2 ml of 6 N HCl in 16 by 150 mm test tubes. After evacuating and sealing, the tubes were heated at 110°C for 24 hr. Samples of the amino acids in the absence of K₂SO₄ were similarly treated with 6 N HCl. The simulated hydrolyzates were evaporated to dryness in an evacuated desiccator at room temperature, using CaCl₂/NaOH as the desiccant. Each residue was dissolved in citrate buffer, pH 2.2, and aliquots were analyzed for the amounts of the

O-sulfate ester and of the free amino acid present, using the long column on a Beckman amino acid analyzer (2).

The extent to which the *O*-sulfate esters could form under the conditions of hydrolysis and drying as above was also determined when 2 or 1 mg of each of the amino acids was mixed with 25 mg of K_2SO_4 .

Ikawa and Snell (9) suggested that *O*-sulfate esters of amino acids may form during the evaporation procedure rather than during hydrolysis. This possibility was checked in two ways. In one case, 5 mg samples of L-serine were heated with 50 or 100 mg K_2SO_4 and 2 ml of 6 *N* HCl, as above. Then, one-half of each was dried at room temperature over $CaCl_2/NaOH$ in an evacuated desiccator and the other half was brought to dryness in a flash evaporator at 45°. In the second case, 5 mg of L-serine and 5 mg of L-threonine were each mixed with 50 mg of K_2SO_4 and dissolved in 2 ml of 6 *N* HCl. Immediately thereafter, the solutions were set aside to evaporate at room temperature over $CaCl_2/NaOH$ in an evacuated desiccator. All the residues were dissolved in citrate buffer, pH 2.2, and analyzed as above.

(3) *Separation and Determination of O-Sulfate Esters of Hydroxyamino Acids and Cysteic Acid on Dowex 1-X10 Resin*

Fine particles of Dowex 1-X10 resin in the chloride form were obtained by stirring portions of the resin (200–400 mesh) in distilled water and allowing the slurry to settle for 5 min. Fine particles which had not settled in this period of time were used to prepare a 0.9 by 60 cm column. It was equilibrated with a citrate buffer of pH 2.20; 10 liters of this buffer contained 196.0 gm sodium citrate·2H₂O, 170 ml concentrated HCl, 25 ml thiodiglycol, and 3 ml pentachlorophenol. The pH was adjusted to 2.20 with either concentrated HCl or 10 *N* NaOH, if necessary.

With the column at 56°C and the flow rates adjusted to 68 ml/hr for the buffer and 34 ml/hr for ninhydrin, the effluent volume in which each of the *O*-sulfate esters and cysteic acid appeared was determined. This was done by using the *O*-sulfate esters singly and in combination with each other as well as in the presence of cysteic acid. The absorbance for hydroxyproline *O*-sulfate in the analytical system was greater at 440 nm than at 570 nm as is typical for imino acid derivatives.

(4) *Observations on Hydrolyzates of Proteoglycans*

10 mg portions of proteoglycan isolated from bovine nasal cartilage and designated as PGS (10), were hydrolyzed with or without the addition of 25 mg of K_2SO_4 for 20 hr at 110°C in evacuated and sealed tubes; 2 ml of 6 *N* HCl was used for each sample. The hydrolyzates were evaporated to dryness either at 25°C over $CaCl_2$ and NaOH pellets in an

evacuated desiccator or at 45° in a flash evaporator. Solutions of the residues were analyzed on columns of UR-30 resin with a Beckman amino acid analyzer according to Spackman, Stein, and Moore (2) or by the use of a column of Dowex 1 resin as in 3 above.

Because galactosamine constitutes about 25% of PGS (10), a control experiment was done in which 5 mg portions of galactosamine·HCl were hydrolyzed with or without the addition of 25 mg of K₂SO₄. The hydrolyzates were treated as above and analyzed on the UR-30 resin as described for PGS.

RESULTS

(1) *Synthesis of Reference O-Sulfate Esters*

In a typical experiment, starting with 500 mg of an amino acid, 340 mg of potassium L-serine *O*-sulfate, 760 mg of potassium L-threonine *O*-sulfate, 610 mg of potassium L-hydroxyproline *O*-sulfate, and 810 mg of potassium L-tyrosine-*O*-sulfate were obtained. Each preparation yielded only one component on the long column of a Beckman amino acid analyzer. There was no indication that any of the preparations contained any of the respective parent amino acid.

The analytical values for the sulfate esters are summarized in Table 1. The observed values are in reasonable agreement with expected values for the monopotassium salts. Furthermore, the infrared spectra of these esters confirm that they are *O*-sulfate esters. There was no absorption in the region of 3320 cm⁻¹, where the unsubstituted hydroxyl group of the parent amino acids would absorb, whereas, there were strong absorp-

TABLE 1
Composition of *O*-Sulfate Esters of Hydroxyamino Acids
(As monopotassium salts)

Element	Serine <i>O</i> -sulfate KC ₃ H ₆ O ₆ SN		Threonine <i>O</i> -sulfate KC ₄ H ₈ O ₆ SN		Hydroxyproline <i>O</i> -sulfate KC ₅ H ₈ O ₆ SN		Tyrosine <i>O</i> -sulfate KC ₉ H ₁₀ O ₆ SN	
	Theory	Found	Theory	Found	Theory	Found	Theory	Found
	%	%	%	%	%	%	%	%
N	6.28	6.35	5.91	5.96	5.62	5.52	4.68	4.64
S	14.35	14.43	13.50	13.53	12.85	13.02	10.70	10.90
K	17.49	16.91	16.46	16.74	15.66	16.06	12.96	13.17

Before analysis the samples were redried at 100°C *in vacuo* over P₂O₅ for 3 hr. Nitrogen was determined by a micro-Kjedahl procedure. Sulfur was determined as barium sulfate following hydrolysis in 0.1 *N* HCl for 2 hr. Values for potassium were calculated from weight of the ash (K₂SO₄) remaining after incineration in a muffle furnace at 500°.

tion bands in the ranges of 1210–1250 cm^{-1} and 770–810 cm^{-1} , characteristic of *O*-sulfate esters (11).

The *O*-sulfate esters were eluted from a 0.9×69 cm column of UR-30 resin (Beckman) with a citrate buffer at pH 3.25 between 17.0 and 21.5 min and could not be readily distinguished from cysteic acid with an elution time of 18.5 min (Table 2).

(2) *Formation of O-Sulfate Esters during Simulated Hydrolysis and Evaporation*

After simulated hydrolysis of serine, threonine, hydroxyproline, or tyrosine with 6 *N* HCl in the presence of potassium sulfate for 24 hr at 110°C and subsequent evaporation over $\text{CaCl}_2/\text{NaOH}$ in an evacuated desiccator at room temperature, different amounts of the respective *O*-sulfate esters were found. The amount of an ester found did not seem to vary consistently as the concentration of either the amino acid or potassium sulfate was varied (Table 3). The formation of *O*-sulfate serine and *O*-sulfate threonine occurred more readily than *O*-sulfate hydroxyproline or *O*-sulfate tyrosine. Comparable results were obtained if an amino acid was mixed with potassium sulfate and 6 *N* HCl and the mixture was then allowed to evaporate over $\text{CaCl}_2/\text{NaOH}$ in an evacuated desiccator at room temperature without the simulated hydrolysis step. On the other hand, if either a simulated hydrolyzate or the mixture of an amino acid, potassium sulfate, and 6 *N* HCl was quickly evaporated to dryness, using a rotary evaporator at 45°, little or no *O*-sulfate ester was detected, as is shown for serine in Table 4. Further, if acid hydrolyzates of proteoglycans are rapidly evaporated at elevated temperatures, less ninhydrin-reactive material is present in the region of cysteic acid.

Treatment of the amino acids with 6 *N* HCl in the absence of potas-

TABLE 2
Comparison of Elution Time of *O*-Sulfate Esters and Respective Hydroxy Amino Acids

Amino acid	Elution time, min	<i>O</i> -Sulfate ester	Elution time, min
Hydroxyproline	44.5	Hydroxyproline <i>O</i> -SO ₄	17.0
Serine	56.5	Serine <i>O</i> -SO ₄	18.0
Threonine	52.5	Threonine <i>O</i> -SO ₄	18.0
Tyrosine	160.0	Tyrosine <i>O</i> -SO ₄	21.5
Cysteic acid	18.5		

Analyses were carried out as suggested in "Procedures Manual," Beckman, 1966, for analysis of protein hydrolyzates, except that, as soon as a sample was delivered onto the column of UR-30 resin, the recorder was started. Citrate buffer of pH 3.25 was changed to the citrate buffer of pH 4.30 at 85 min.

TABLE 3
Formation of O-Sulfate Esters of L-Serine, L-Threonine, L-Hydroxyproline, and L-Tyrosine during Simulated Acid Hydrolysis in Presence of Potassium Sulfate

Serine mg (μM)	K ₂ SO ₄ mg (μM)	Serine O-SO ₄ %	Threonine mg (μM)	K ₂ SO ₄ mg (μM)	Threonine O-SO ₄ %	HO-proline mg (μM)	K ₂ SO ₄ mg (μM)	HO-proline O-SO ₄ %	Tyrosine mg (μM)	K ₂ SO ₄ mg (μM)	Tyrosine O-SO ₄ %
5 (47.5)	50 (287)	4.2	5 (42.0)	50 (287)	14.2	5 (38.2)	50 (287)	3.5	5 (27.6)	50 (287)	0
2 (19.0)	25 (143)	5.0	2 (16.8)	25 (143)	8.6	2 (15.3)	25 (143)	0	2 (11.0)	25 (143)	1.0
1 (9.5)	25 (143)	4.0	1 (8.4)	25 (143)	12.6	1 (7.6)	25 (143)	0	1 (5.5)	25 (143)	1.2

The amounts of amino acid and potassium sulfate were mixed with 2 ml 6 N HCl and heated in sealed evacuated tubes at 110°C for 24 hr. The reaction mixtures were then evaporated at room temperature, 23–25°, over CaCl₂/NaOH in an evacuated desiccator. The residual material was analyzed on a Beckman amino acid analyzer. The amount of an amino acid converted to the O-sulfate ester is given as per cent of amount of amino acid used.

TABLE 4

Effect of Conditions of Evaporation of Simulated Hydrolyzates Containing Serine, Potassium Sulfate, and 6 N HCl on Presence of L-Serine O-Sulfate

Serine mg (μM)	K ₂ SO ₄ mg (μM)	Serine O-sulfate (%)	
		Evap. at 45°*	Evap. at 25°†
5 mg (47.5)	50 (287)	0	2.0
5 mg (47.5)	100 (574)	0	7.7

The indicated amounts of serine and potassium sulfate were heated with 2 ml 6 N HCl for 24 hr at 110°C in evacuated sealed tubes. The reaction mixtures were then evaporated to dryness *in vacuo* in a rotary evaporator* at 45° or over CaCl₂/NaOH in a desiccator† at 25°. The fraction of serine converted to serine O-sulfate is given in columns 3 and 4.

sium sulfate gave no ninhydrin-positive material in the effluent at 17 to 21 min. In the case of hydroxyproline an additional small peak was invariably found in the effluent at 54 min. This was subsequently shown to be *cis*-4-hydroxy-D-proline (12). Such material was not detectable if the samples of hydroxyproline were not heated.

(3) Separation of O-Sulfate Esters of Hydroxyamino Acids from Cysteic Acid

The O-sulfate esters of serine, threonine, hydroxyproline, and tyrosine can be separated from cysteic acid on an amino acid analyzer by the use of a 0.9 × 60 cm column of very fine particles of Dowex 1-X10. This is shown in Figure 1. The pattern is reproducible from run to run when dealing with a mixture of the O-sulfate esters and cysteic acid. The recoveries of each is of the order of ±2%, except for hydroxyproline O-sulfate, which can be recovered with a variance of ±8%.

(4) Observations on Hydrolyzates of Proteoglycans

When acid hydrolyzates of PGS were analyzed using a column of UR-30 resin according to Spackman, Stein, and Moore (2) the patterns of ninhydrin-reactive materials in the effluent for the first 50 min are as shown in Fig. 2. Three major 570 nm positive peaks (I, II, III) and one 440 nm positive peak (IV) are observed between 18 and 30 min. The amount of material in peaks I, II, and III is substantially reduced if the hydrolyzate is flash evaporated at 45° (Fig. 2A) instead of being slowly evaporated at room temperature in a desiccator (Fig. 2B).

The O-sulfate esters of the hydroxyamino acids chromatograph as the material in peak I (see Table 2). Indeed, when the hydrolyzates of PGS

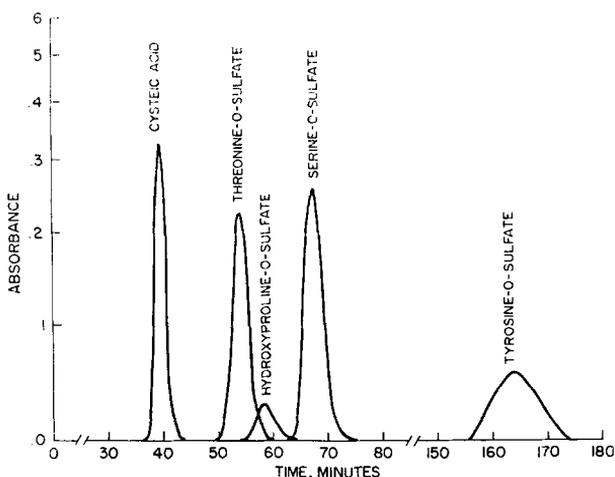


Fig. 1. Separation of *O*-sulfate esters of serine, threonine, hydroxyproline, and tyrosine from cysteic acid using Dowex 1-X10 resin. The components were eluted from a Dowex 1-X10 resin as described in "Materials and Methods." A composite of five separate chromatograms is shown but a comparable separation of the *O*-sulfate esters and cysteic acid was observed when mixtures were used. The peak for hydroxyproline *O*-sulfate is as seen at 440 nm.

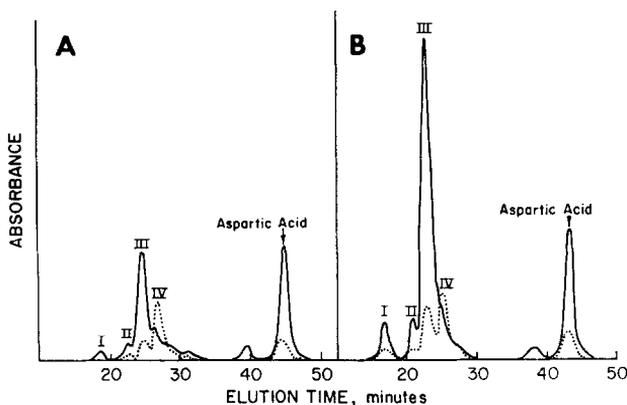


Fig. 2. Tracings of segments of chromatograms of hydrolyzates of PGS: (A) which had been evaporated to dryness in a flash evaporator at 45° and (B) which had been evaporated slowly at 25° in a desiccator containing CaCl₂ and NaOH. The amounts of ninhydrin-positive material at about 18 min and at about 25 min is less in A than in B. Analyses were with a column of UR-30 resin used according to Spackman, Stein, and Moore (2).

TABLE 5
Effect of Conditions of Evaporation of Hydrolyzates of PGS on Presence of Serine *O*-Sulfate and Threonine *O*-Sulfate

Sample	Treatment of hydrolyzate	Serine <i>O</i> -sulfate (%)	Threonine <i>O</i> -sulfate (%)
PGS	DD	13	3
PGS + 25 mg K ₂ SO ₄	DD	36	12
PGS	FE	Trace	Trace
PGS + 25 mg K ₂ SO ₄	FE	10	4

Hydrolyzates of PGS with or without added sulfate were dried as indicated (DD, dried in desiccator; FE, flash evaporated). The contents of serine and threonine in each were determined according to Spackman *et al.* (2) and the contents of serine *O*-sulfate and threonine *O*-sulfate as described in the text.

were chromatographed on Dowex 1 resin as described in section 3, serine *O*-sulfate and threonine *O*-sulfate were found in the hydrolyzates that were slowly evaporated but were barely detected in the hydrolyzates that were flash evaporated (Table 5). This table also indicates that added sulfate in the hydrolyzates of PGS greatly increases the yield of the sulfate esters.

Peaks II and III in Fig. 2A-B are reaction products, in part at least, between galactosamine and sulfate. When *D*-galactosamine was "hy-

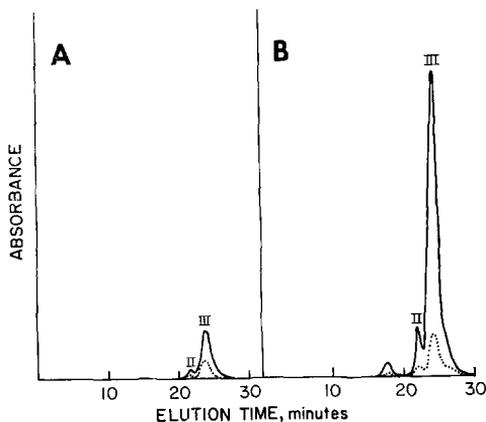


FIG. 3. Tracings of segments of chromatograms of "hydrolyzates" of *D*-galactosamine and K₂SO₄: (A) which had been evaporated to dryness at 45° in a flash evaporator and (B) which had been evaporated slowly at 25° in a desiccator containing CaCl₂ and NaOH. The amount of ninhydrin-positive material at about 25 min is markedly less in A than in B. Analyses were with a column of UR-30 resin used according to Spackman, Stein, and Moore (2).

drolyzed" in the presence of sulfate and flash evaporated (Fig. 3A) or desiccated (Fig. 3B), subsequent analyses on UR-30 resin (2) revealed the presence of these two components. As in the case of the *O*-sulfate esters of hydroxy amino acids, it is noteworthy that these materials were present in smaller amounts in the "hydrolyzates" which were flash evaporated than in the "hydrolyzates" which were desiccated. Neither component was formed after "hydrolysis" of galactosamine alone. Presently, they remain uncharacterized, as does the 440 nm positive peak IV in Fig. 2A-B.

DISCUSSION

In the analysis of proteoglycans, which are predominantly sulfated heteropolysaccharides covalently linked to protein, the observed contents of serine, threonine, and tyrosine may be underestimated unless precautions are taken to account for artifacts and destruction. It is particularly pertinent in the case of serine and threonine if exact values for these amino acids are required in making estimates of the numbers of these which might be involved in the linkage regions between the polysaccharide chains and the core protein (13-18). On the basis of the observations noted here, the possibility of observing less serine and threonine, and tyrosine, because of the formation of *O*-sulfate esters can be minimized if acid hydrolyzates of proteoglycans are evaporated to dryness rapidly and at elevated temperatures.

The urines of animals, including man, contain many sulfate esters (19,20). The nature of these is for the most part unknown. The method suggested for the separation and quantitation of serine *O*-sulfate, threonine *O*-sulfate, hydroxyproline *O*-sulfate, and tyrosine *O*-sulfate may be applicable as such or after further modification, in assessing kinds and amounts of additional sulfate esters.

SUMMARY

In the preparation of hydrolyzates of proteoglycans for analysis of amino acids, ninhydrin-positive materials other than free amino acids can be generated. Some of these are *O*-sulfate esters of the hydroxy amino acids and some unknown sulfate derivatives of the hexosamines.

The amounts of the *O*-sulfate esters of the hydroxy amino acids and of the unknown derivatives of the hexosamines can be minimized if the hydrolyzates are evaporated to dryness in a flash evaporator at 45°C.

A method is described for the separation and quantitation of the *O*-sulfate esters of serine, threonine, hydroxyproline, tyrosine, and cysteic acid.

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