Desulfation of Sulfated Polyanions by Rat Gastric Mucosa[1] (37516)

Y. H. Liau and Martin I. Horowitz
(Introduced by W. Pigman)
Department of Biochemistry, New York Medical College, Valhalla, New York 10595

It has been shown that injected or ingested (1–3) labeled sulfated polyanions may be extensively desulfated as evidenced by the excretion of labeled inorganic sulfate. However, the administration of antibiotics prevented the extensive degradation of the labeled polyanions, thus leading investigators (1, 4) to infer that bacterial enzymes were probably responsible for the observed degradation. Nevertheless, the existence and location of mammalian enzymes which degrade sulfated polyanions continues to be a subject of active investigation (5–8).

We report here the presence in the rat gastric mucosa of sulfatases and depolymerases which are directed at sulfated polyanions isolated from rat stomach, duodenum and rib cartilage.

Materials and Methods. Preparation of substrate. Five adult male Sprague–Dawley rats weighing 100–200 g were injected intraperitoneally with 150 μCi $Na_2{}^{35}SO_4$. The animals were fasted and sacrificed 18 hr later. The stomach and first 12 cm of small intestine were removed and rinsed with cold water and scraped. The scrapings were homogenized and extracted with chloroform–ethanol (2:1), dried and weighed. Then the material was suspended in 0.1 *M* acetate buffer, pH 5.5 containing 6.36 m*M* cysteine and 4.28 m*M* EDTA and digested with papain (100 mg; Mann Biochemical, Orangeburg, New York) at 60°. The material solubilized with papain was dialyzed in a size 20 cellulose dialysis membrane (Union Carbide) against distilled water for 3 days at 5°. Polyanions were precipitated from the retenate by cetylpyridinium chloride (CPC) and fractionated with aqueous 0.125, 0.7, and 1.4 *M* $MgCl_2$ (9).

^{35}S-labeled chondroitin sulfate was obtained from rat rib cartilage after *in vitro* incubation in a medium containing $^{35}SO_4{}^{2-}$ as described by Yamagata *et al.* (10) followed by isolation according to Perlman *et al.* (11). The proportion of chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate in this preparation was determined (12) with the enzyme kit and sulfated disaccharides purchased from Miles Laboratories (Kankakee, Illinois). Radioactive heparin bearing ^{35}S in the sulfamido group at an activity of 1.75 mCi/g was purchased from Calatomic (Los Angeles, Calif.).

Preparation of enzyme extracts. Rats were sacrificed as described earlier. The stomachs were chilled, irrigated with cold distilled water, scraped and homogenized in 20 ml of acetate buffer (0.1 *N* sodium acetate–acetic acid, saturated with toluene, pH 4.0) per 1.5 g wet weight of stomach tissue in a VirTis homogenizer. This homogenate constituted the crude enzyme. This procedure was applied first to the stomachs of 5 rats and later to batches of 100 frozen stomachs (Pel-Freeze Biologicals, Rogers, Arkansas).

Extreme care was taken to prevent the proliferation of microorganisms with the attendant production of bacterial enzymes implicated in the studies by others (1, 4) of polyanion degradation in urine. All tissue sources used here were promptly frozen or processed immediately after removal from the animal, enzyme fractionation was carried out quickly at 0–2° and incubations with substrate were performed in media contain-

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ing toluene and a complete mixture of antibiotics.

Ammonium sulfate fractionation. The stomach homogenate was clarified by centrifugation (16,000*g*, 30 min, 5°). To the supernatant was added graded amounts of $(NH_4)_2SO_4$ with stirring at 0–2°. The precipitates were collected by centrifugation (25,000*g*, 30 min, 5°) and were dissolved in 0.1 *N* acetate buffer at pH 4. The resulting solutions and the 72% $(NH_4)_2SO_4$ supernatant were each dialyzed thoroughly against the acetate buffer saturated with toluene. Protein in the dialyzed solutions was determined by the method of Lowry *et al.* (13).

Liberation of sulfate. Crude enzyme or enzyme fraction obtained by salting-out (1–3 ml) were incubated in 0.1 *N* acetate buffer at pH 4 with ^{35}S-labeled substrate at 37°. Toluene and an antibiotic mixture of streptomycin 100 μg/ml, penicillin 100 units/ml, acromycin 10 μg/ml, and mycostatin 25 units/ml were included. This incubation mixture was placed in a dialysis bag (size 8, cellulose membrane) immersed in 25 ml of buffer containing antibiotics and toluene.

Aqueous $BaCl_2$ was added to the dialyzate in the presence of 0.04 m*M* of carrier sulfate and the resulting precipitate was collected by centrifugation at 2,000 rpm at 5° for 20 min. Incubation mixture containing boiled enzyme and treated in all other respects as the experimental mixture served as a control. The radioactivity in the $Ba^{35}SO_4$ precipitated from the control dialyzate was subtracted from the corresponding radioactivity of the experimental precipitate. Radioactivity in the dialyzate not precipitable by $BaCl_2$ was precipitated by this reagent after oxidation of the supernate with a mixture of HNO_3 and $HClO_4$ (14) or after hydrolysis in 2 *N* HCl for 2 hr at 100°. Aryl sulfatase activity was determined spectrophotometrically using *p*-nitrocatechol sulfate (Sigma Chemical Co., St. Louis, Mo.) as substrate (15).

Electrophoresis. Paper electrophoresis was performed using Whatman 3MM paper and a 0.3 *M* pyridinium–formate buffer of pH 3 at 15 mA per cell for 3 hr in examination for inorganic sulfate and dialyzable organic sulfate and usually for 5 hr for polyanions. Chondroitin 4-sulfate (gift of Dr. L. M. Morrison, Institute for Arteriosclerosis Research, Los Angeles, Calif.), Chondroitin 6-sulfate (purchased from Miles Laboratories), heparin (gift of Dr. I. Danishefsky, New York Medical College, N.Y.C., and hyaluronic acid (16) were used as reference compounds.

Determination of radioactivity. Counting was performed by liquid scintillation spectrometry using a solvent of toluene containing 0.5% of 2,5-diphenyloxazole (PPO) and 0.03% of *p*-bis-[2-(5-phenyloxazolyl)] benzene (POPOP). Radioactivity in precipitated $BaSO_4$ was determined by counting the salt suspended with Cabosil (14). Sufficient counts were accumulated to insure a standard deviation not greater than 1%. The efficiency of counting of $Ba^{35}SO_4$ suspended in Cabosil was 80% and the counts were corrected accordingly. After electrophoresis, paper strips were cut into 1-cm segments, suspended in toluene-PPO-POPOP and counted. Reference strips were stained for inorganic sulfate by rhodizonate (17) and for polyanions by alcian blue (18).

Results. Fractions soluble in (A) 0.125 *M*, (B) 0.7 *M*, and (C) 1.4 *M* aqueous $MgCl_2$ were obtained from the CPC precipitation–fractional salt extraction procedure applied to the papain digest of stomach and duodenum scrapings. The following partition of radioactivity was found for these fractions: (A) 130,000, (B) 1,000,000, and (C) 105, 000 cpm, respectively.

The preparation of rib chondroitin sulfate was obtained in a yield of 8 mg/g (wet wt) cartilage and exhibited a specific activity of 9.1×10^5 cpm/mg of preparation. The preparation contained 84% chondroitin 4-sulfate, 8% chondroitin 6-sulfate and 8% other polyanion sulfates.

Crude homogenate of mucosal scrapings was tested against fraction B (0.7 *M* $MgCl_2$) from the mucosa and both inorganic sulfate and dialyzable organic ^{35}S were liberated and counted. The optimum pH for liberation of radioactivity was pH 4.0.

The radioactive constituent in the precipitated barium salt was investigated further by radioelectrophoresis after dissociation and solubilization in the presence of Dowex 50

TABLE I. Cleavage of ^{35}S-Polyanions by Gastric Enzymes.[a]

Substrate	Dialyzate $^{35}SO_4^{2-}$ (inorg.) (cpm)	Dialyzate $^{35}SO_4^{2-}$ (organic) (cpm)	Retentate ^{35}S (cpm)	Radioactivity liberated (%)
Rib chondroitin sulfate	46,000	30,000	5000	94
^{35}S-*N*-heparin	5200	500[b]	80,000	7
Mucosal fraction A	4800	6000	3000	78
Mucosal fraction B	2100	1000	7500	29
Mucosal fraction C	520	420	3400	22

[a] Incubation for 17 hr, pH 4 at 37° with 1 mg of enzyme in dialysis bag.
[b] As sulfamido—^{35}S.

(Na^+) with heating (19, 20). The radioactivity migrated like $^{35}SO_4^{2-}$.

Prior to fractionation with $(NH_4)_2SO_4$, the homogenate was clarified by centrifugation. The supernate contained 5 times the activity present in the sediment. The supernate was fractionated by the addition of $(NH_4)_2SO_4$, and the fractions, after dialysis against pH 4 acetate buffer, were assayed for activity against mucosal substrate B and rib chondroitin sulfate. The most active fraction precipitated at 60–72% saturation with $(NH_4)_2SO_4$, and was obtained in a yield of 150 mg of protein from 100 rats. This fraction was designated as the partially purified enzyme and represented a 164-fold purification of the enzyme. The partially purified enzyme was tested against the labeled mucosal substrate fractions, chondroitin sulfate, and ^{35}S-*N*-heparin, and the counts liberated as inorganic sulfate and organic ^{35}S are shown in Table I. This enzyme preparation also exhibited 10 units of aryl sulfatase/mg protein.

The dialyzable organic ^{35}S (not precipitable by $BaCl_2$) was hydrolyzed in 2 *N* HCl for 2 hr at 100°, and the ^{35}S was liberated as $^{35}SO_4^{2-}$ precipitable by $BaCl_2$.

The labeled chondroitin sulfate preparation was analyzed before and after incubation with enzyme as shown in Table II. All three components of the substrate were attacked with the liberation of inorganic and organic sulfate.

The dialyzate was subjected to paper electrophoresis and two radioactive bands were found. One migrated like the sulfated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-glucose (12), and the other like inorganic sulfate.

In all, 160 μg of the chondroitin sulfate preparation were degraded in 17 hr at 37° by 1 mg of enzyme protein. The enzyme preparation retained sulfatase activity after 2-month storage in pH 4 acetate buffer under toluene at 5°.

Discussion. Rat stomach scrapings contain a variety of hydrolases directed against sulfated polyanions. Both inorganic and organic sulfates were liberated from chondroitin sulfate and from gastric and duodenal sulfated polyanions indicating that sulfatases and probably glycosidases were present. Since chondroitin 4-sulfate and chondroitin 6-sulfate as well as other sulfated substrates (gastric-duodenal and rib) were desulfated, several different sulfatases probably are present. Sulfamidase activity and arylsulfatase activity also were found.

Mammalian sulfatases directed against sulfated polyanions have been previously re-

TABLE II. Action of Gastric Enzymes on Rib Chondroitin Sulfate.[a]

	Composition (%)	
	Before	After
Chondroitin 4-sulfate	84	4
Chondroitin 6-sulfate	8	<0.25
Other polyanion sulfate	8	2
Dialyzable organic sulfate	—	37
Inorganic sulfate	—	57

[a] 160 μg of rib ^{35}S-chondroitin sulfate was incubated with 1 mg of enzyme for 17 hr at 37°.

ported: a keratan sulfatase (7) and a cerebroside sulfatase from rat and pig kidney (21), a sulfatase hydrolyzing oligosaccharide sulfates from rat liver (5), a chondroitin 4-sulfatase from cattle aorta (6) and a sulfamidase from rat spleen and intestine (8, 22). This is the first report of sulfatases present in the stomach, and it is of interest to note the variety of activities present.

The observation that both inorganic and low molecular weight organic sulfates were released, prompts the following question: does the liberation of organic sulfate and inorganic sulfate proceed independently of one another, or does one process precede the other? Additional kinetic studies, preferably with more highly purified enzymes, may help solve this problem.

The sulfate-cleaving and depolymerizing enzymes from the gastric mucosa may be lysosomal enzymes as is suggested by the low pH optimum. The localization of this enzyme and the conditions which stimulate its release are of interest owing to the possible role of the enzyme in degradation of mucosal polyanions.

Summary. Rat stomach mucosal scrapings contain enzymes which attack chondroitin 4- and 6-sulfates, heparain, sulfated polyanions from gastric and duodenal mucosa and aryl sulfates. Inorganic and organic sulfates were liberated from the sulfated polyanions. Fractionation with $(NH_4)_2SO_4$ afforded enrichment of the sulfatase and depolymerase enzymes in the precipitate obtained at 60–72% saturation. Enzyme activities were stable for at least two months when stored in pH 4.0 acetate buffer under toluene at 5°.

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