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Original articles

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ROLE OF SULFATION IN THE PROCESSING OF GASTRIC MUCINS

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The role of sulfation in the processing of mucus glycoprotein in gastric mucosa was investigated. Rat gastric mucosal segments were incubated in MEM at various medium sulfate concentrations in the presence of [^{35}S]Na_2SO_4, [^{3}H]glucosamine and [^{3}H]proline, with and without chlorate an inhibitor of PAPS formation. The results revealed that the mucin sulfation attained maximum at 300 μM medium sulfate concentration. Introduction of chlorate into the incubation medium, while having no effect on the protein synthesis as evidenced by [^{3}H]proline incorporation, caused at its optimal concentration of 2 mM a 90% decrease in mucin sulfation and a 40% drop in mucin glycosylation. Evaluation of mucin molecular forms distribution indicated the predominance of the high molecular mucin form in the interacellular fraction and the low molecular mucin from in the extracellular fraction. Increase in medium sulfate caused an increase in the high molecular weight mucin form in both fractions, and this effect was inhibited by chlorate. Also, higher medium sulfate concentrations led to a higher degree of sulfation in the high molecular weight mucin form, the effect of which was inhibited by chlorate. The results suggest that the sulfation process is an early event taking place at the stage of mucin subunit assembly and is required for mucin polymer formation. Hence, the disturbances in mucin sulfation process could be determinal to the maintenance of gastric mucus coat integrity.

Key words: *Gastric mucin, synthesis, effect of sulfation.*

INTRODUCTION

The epithelial surfaces of gastric mucosa are protected from noxious environment of the lumen by copious quantities of viscous mucus which functions as the first line of mucosal defense (1). The functional performance of mucus gel depends mainly on its glycoprotein component, mucin, and the features acquired by this glycoprotein during the synthesis and processing (2, 3). The early stages of mucus glycoprotein assembly involve the ribosomal synthesis of the peptide core, its acylation with fatty acids, translocation into the lumen of endoplasmic reticulum, and the initiation of glycosylation

(2, 4, 5). Further processing of the glycoprotein involves such posttranslational events as elongation of carbohydrate chains, subunit assembly, and sulfation (3, 6). The sulfate transfer to sugar residues from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is a two-step process involving first sulfurylase which catalyses the reaction between sulfate and ATP to form APS, which is then converted to PAPS by APS kinase (7—9), and then the sulfotransferase catalyzing the transfer of sulfate from PAPS to glycoproteins (10).

Thus the availability of sulfate can affect the formation of PAPS and the course of mucin sulfation. Indeed, in vitro studies on glycosaminoglycan synthesis indicate that a decrease in sulfate concentration in the medium leads not only to the reduction of polysaccharide chains sulfation but also their degree of polymerization (11—14). A similar effect on the proteoglycan biosynthesis is observed with chlorate, a potent inhibitor of sulfate adenyltransferase (15, 16). In this study, we have focused on the effect of sulfation on the synthesis and mucin molecular forms distribution in gastric mucosa.

EXPERIMENTAL PROCEDURES

Materials

Radioactive precursors D-[6-^{3}H]glucosamine/HCl (30Ci/mmol), [^{35}S]Na_2SO_4 (520mCi/mmol) and L-[2, 3, 4, 5-^{3}H]proline (112Ci/mmol) were purchased from New England Nuclear, Boston, MA and scintillation solution, Opto-fluor, from Packard Instrument Co., Downers Grove, IL. Joklik modified minimal essential medium (MEM) was obtained from Grand Island Biochemical Corp., Grand Island, NY. Bio-Gel P-30 (100—200), Bio-Gel A-1.5 (50—100 mesh) and Bio-Gel A-50 (50—100 mesh) were obtained from Bio-Rad Laboratories, Rockville Centre, NY, and BCA protein assay kit was purchased from Pierce, Rockford, IL. Male Sprague-Dawley rats (175—200 g) were obtained from Taconic Farms, Germantown, NY.

Tissue incubation

Rats were sacrificed by decapitation and their stomachs removed, immediately washed with cold phosphate buffer-saline (PBS), pH 7.2 and with cold MEM, pH 7.4. The rumen was discarded, and the remainder of the stomach was cut into four segments. The segments, each weighing about 1.4 g, were preincubated with 10 ml of MEM under 95% O_2—5% CO_2 atmosphere at 37°C for 30 min, the medium was discarded and replaced with MEM containing [^{35}S]Na_2SO_4 (50—250 μCi), [^{3}H]glucosamine (0—100 μCi), [^{3}H]proline (0—20 μCi), cold Na_2SO_4 (0—800 μM) and $NaClO_3$ (0—8 mM), and incubated for various periods of time up to 8 h (17).

Mucus glycoprotein isolation

The extracellular and intracellular of mucus glycoprotein fractions were isolated from the incubation mixture as decribed previously (18). The incubation mixture was centrifuged at 500 x g for 10 min and the segments washed three times with MEM. The incubation medium and washes were combined and used for the extracellular mucus glycoprotein isolation. The segments were then suspended in 2 M NaCl—0.05 M phosphate, pH 7.0 and homogenized with Tekmar Tissumizer, and centrifuged at 8000 x g for 30 min. The residue was re-extracted two more times with buffered

2 M NaCl, centrifuged, and the combined supernatants used for isolation of intracellular mucus glycoprotein.

The extracts containing extracellular and intracellular fractions were treated with 2% phosphotungstic acid-20% trichloreacetic acid and the formed precipitates were collected by centrifugation at 800 x g. The precipitates were dissolved in 6 M urea-0.05 M phosphate, pH 7.0, dialyzed against distilled water, and lyophilized. The dried samples were reconstituted in 1 ml of 6 M urea and applied to a Bio-Gel P-30 column (0.7×20 cm) equilibrated and eluted with 6 M urea. Fractions of 1.8 ml were collected and 250 µl aliquots used for scintillation counting. The excluded fraction of each glycoprotein sample was subjected to chromatography with Bio-Gel A-1.5 column (2×170 cm). The fractions (5 ml each) were monitored for protein at 280 nm, and for radioactivity by liquid scintillation spectrometry. The excluded fractions from A-1.5 were pooled, dialyzed, lyophilized, and subjected to chromatography on Bio-Gel A-50 column (0.9×120 cm). The peaks containing the high and the low molecular weight fractions were pooled, dialyzed against water, and lyophilized. Each fraction was analyzed for total incorporation of each radioactive label and for protein content.

RESULTS

The effect of medium sulfate concentration on the incorporation of of [^{35}S]Na_2SO_4 and [^{3}H] glucosamine into gastric mucus glycoprotein is shown in *Fig. 1.* The incorporation of both labels increased with the concentration of sulfate in the medium, attained their maximus at 300 µ M, and then leveled off.

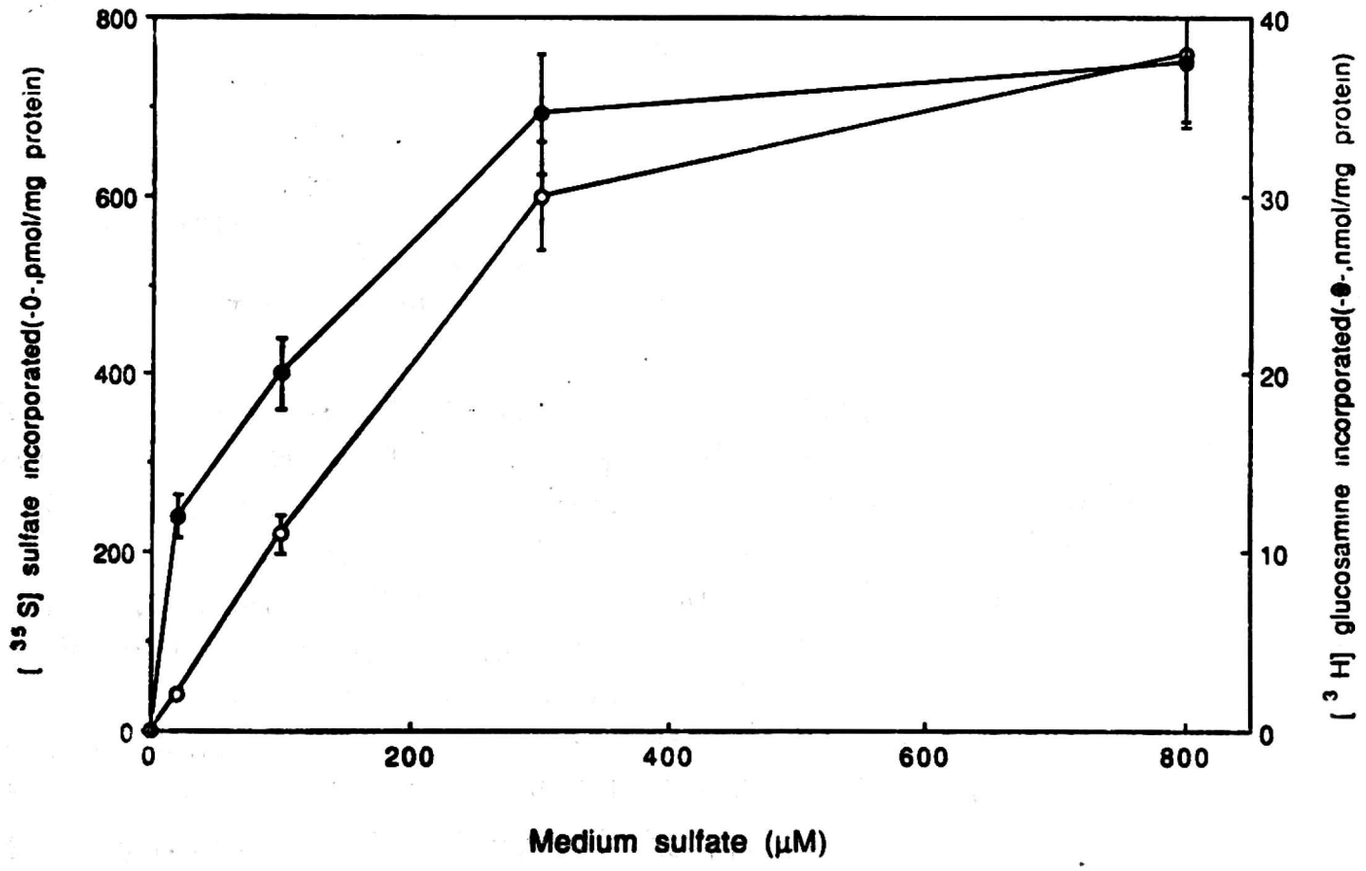

Fig. 1. Effect of medium sulfate concentration on the incorporation of [^{35}S]Na_2SO_4 and [^{3}H]glucosamine into the gastric mucus glycoprotein. Incubation time 4h. The data show the means ±SD of five experiments performed in duplicate.

The effect of chlorate concentration on the incorporation of [^{35}S]Na_2SO_4, [^{3}H] glucosamine and [^{3}H] proline to mucus glycoprotein at 300 μ M medium sulfate is shown in *Fig. 2*. The data indicated that while the incorporation of [^{3}H] proline was not affected by chlorate, the incorporation of glucosamine decreased by 40% and that of sulfate by 90%. The maximum inhibition for the incorporation of sulfate and glucosamine was achieved with 2 mM chlorate concentration. The effect of different concentrations of medium sulfate in the absence and the presence of 2 mM chlorate is presented in *Table 1*.

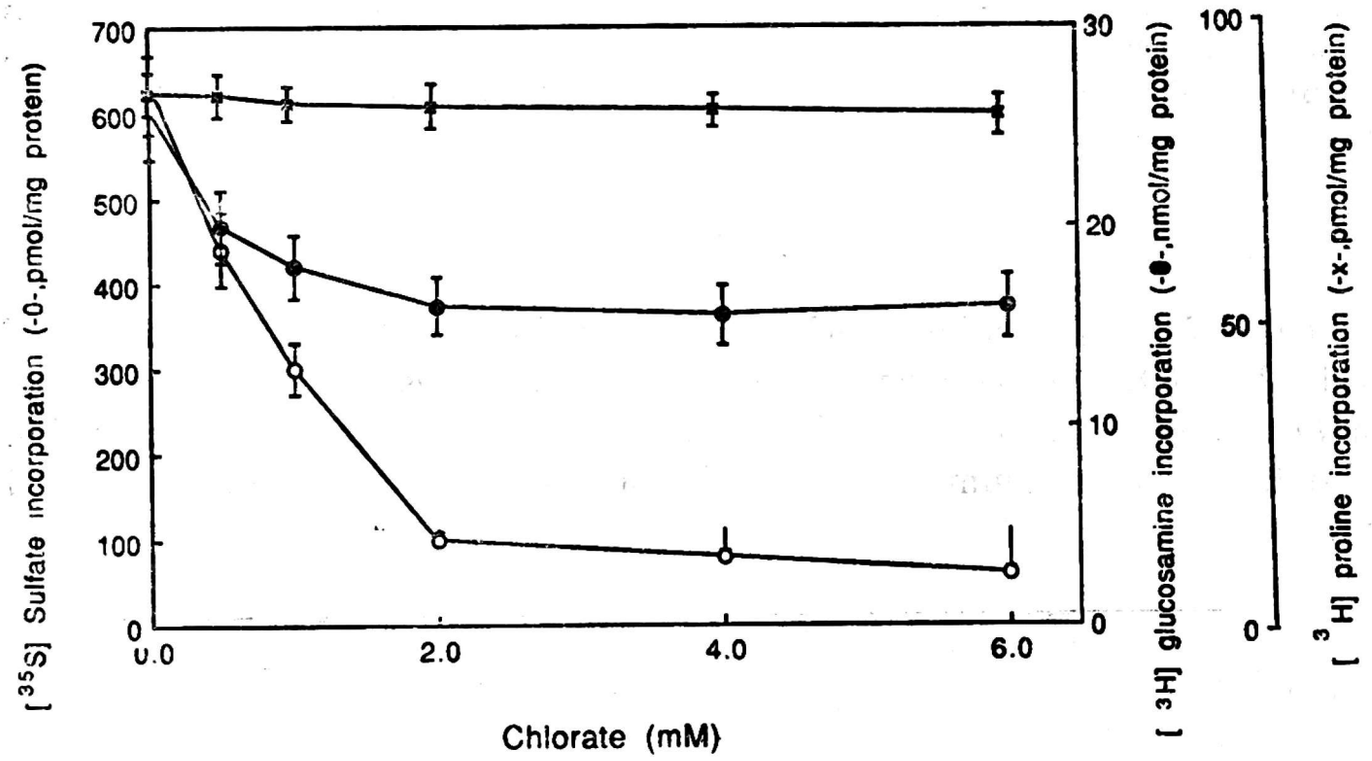

Fig. 2. Effect of chlorate concentration on the incorporation of [^{35}S]Na_2SO_4, [^{3}H]glucosamine and [^{3}H]proline into the gastric mucus glycoprotein. Incubation time 4h at 300 μM medium sulfate concentration. The data show the means ±SD of six separate experiments performed in duplicate.

The incorporation of [^{3}H] proline was not affected by variation in either sulfate or chlorate concentration in the medium. On the other hand, the incorporation of labeled sulfate increased 30-fold when the medium sulfate concentration was increased from 10 to 300 μ M. However, this caused only 3-fold increase in glucosamine incorporation. A significant increase in the incorporation of both markers into the glycoprotein occurred when the medium sulfate concentration was increased from 10 to 800 · M. Introduction of 2 mM chlorate into the incubation mixture in the presence of 10 μ M medium sulfate caused essentially complete inhibition in labeled sulfate incorporation, and 37% inhibition in the incorporation of glucosamine. The inhibitory effect of chlorate on sulfation, however, was partially (20%) abolished at highest concentration of medium sulfate, whereas only barely discernible effect was observed on the incorporation of glucosamine.

Table 1. Effect of medium sulfate concentration on the incorporation of [^{35}S]sulfate, [^{3}H]glucosamine and [^{3}H]proline into mucin in gastric mucosal segments cultured in the absence and the presence of chlorate.

Medium sulfate conc. (μM)	Incorporation (nmol/g wet tissue)			% inhibition by 2 mM $NaClO_3$		
	[^{35}S] sulfate	[^{3}H] glucosamine	[^{3}H] proline	[^{35}S] sulfate	[^{3}H] glucosamine	[^{3}H] proline
10	0.8±0.1	590± 48	3.4±0.3	99±4	37±4	4±3
100	7±0.6	1100± 98	3.6±0.4	92±3	39±5	2±2
300	24±1.9	1900±180	3.6±0.4	86±3	45±3	3±3
800	27±2.5	2100±200	3.8±0.3	80±4	42±4	3±3

The segments were maintained in MEM under 95% O_2 — 5% CO_2 at 37° C for 4h in the absence and the presence of 2 mM chlorate. The data show the means ±SD of five separate experiments performed in duplicate.

The time course of the incorporation of sulfate and glucosamine into the intracellular and extracellular mucus glycoproteins in the presence of 300 mM medium sulfate is illustrated in *Fig. 3*. The results revealed at the onset of the incubation, a rapid increase in the incorporation of both labels into the glycoproteins which reached maximum in 4 h. The amount of radioactive glucosamine incorporated into the extracellular fraction was 42% lower than that incorporated into the intracellular glycoprotein, while the values for sulfate incorporation in the extracellular glycoprotein were 53% lower than those for the intracellular glycoprotein.

The intracellular and extracellular mucus glycoprotein fractions were chromatographed on Bio-Gel A-1.5 followed by Bio-Gel A-50 column. The representative patters of A-50 chromatography are shown in *Fig. 4*. The evaluation of the elution patters indicated that the intracellular mucus glycoprotein fraction contained more of the high molecular weight glycoprotein form and only small amounts of the low molecular weight species were identified. The extracellular glycoprotein fraction upon Bio-Gel A-50 yielded mucin molecular forms in which the low molecular weight glycoprotein predominated. Furthermore, with the increase in medium sulfate, a substantial increase in the high molecular wieght mucin form was observed. This effect of sulfate was, however, drastically reduced in the presence of 2 mM chlorate. In the case of the extracellular fraction, the ratio of the low molecular weight mucin to high molecular weight mucin increased when the sulfate concentration of medium decreased. In the presence od chlorate, the amount of the low molecular weight mucin became much higher than that of the high molecular weight mucin, and at 10 ; M medium sulfate concentrations and 2 mM chlorate, no high molecular weight mucus glycoprotein form was present.

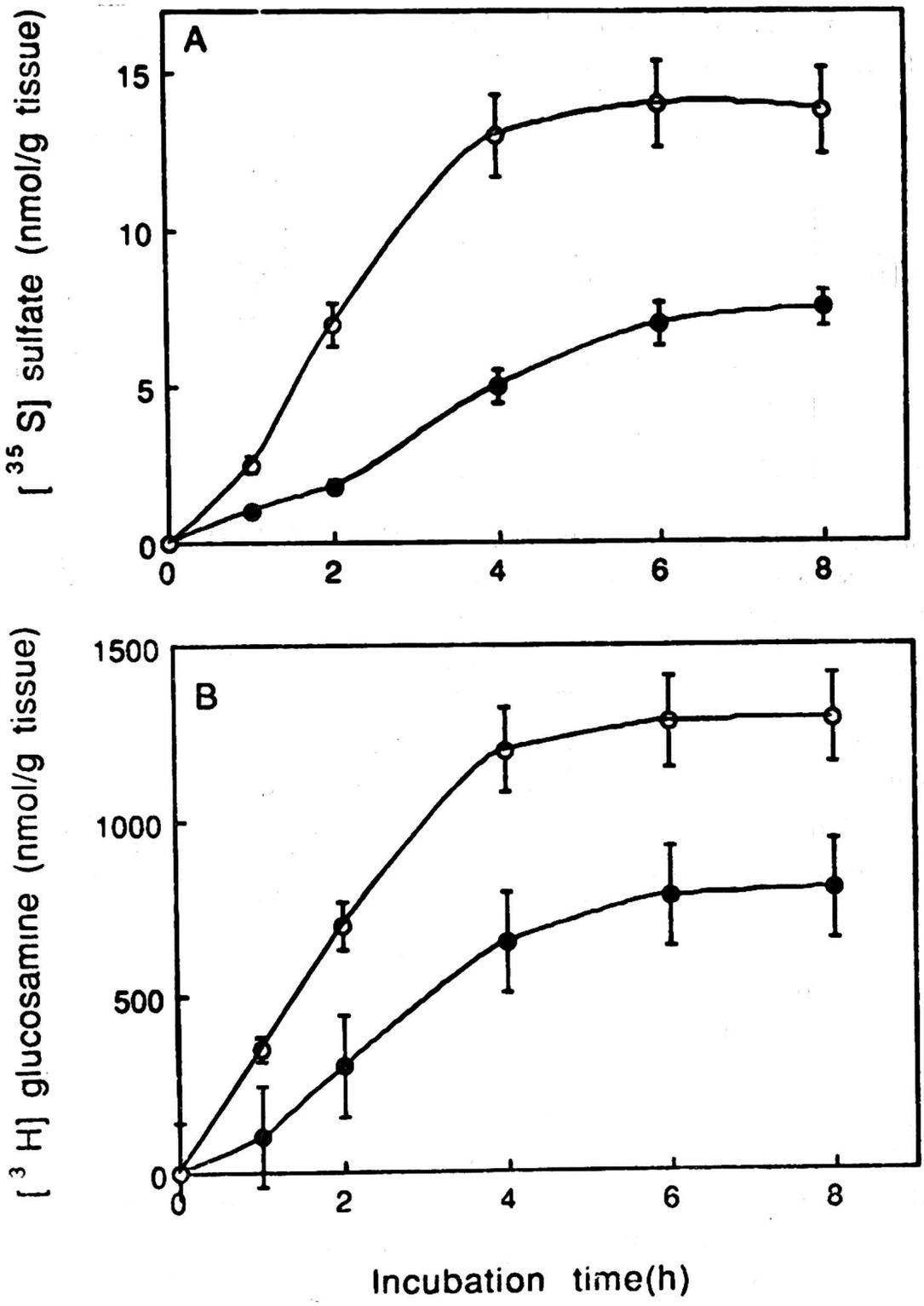

Fig. 3. Incorporation of [^{35}S]Na_2SO_4 (A) and [^{3}H] glucosamine (B) into the intracellular (○) and extracellular (●) mucus glycoprotein fractions. Incubation time 0—8 h, medium sulfate concentration 300 μM. The data show the means ±SD of five separate experiments performed in duplicate.

Table 2 shows the degree of sulfation of the high and low molecular weight mucin forms with the increasing concentration of medium sulfate, without and in the presence of chlorate. The results demonstrated that higher sulfate concentrations in the incubation medium caused higher degree of sulfation

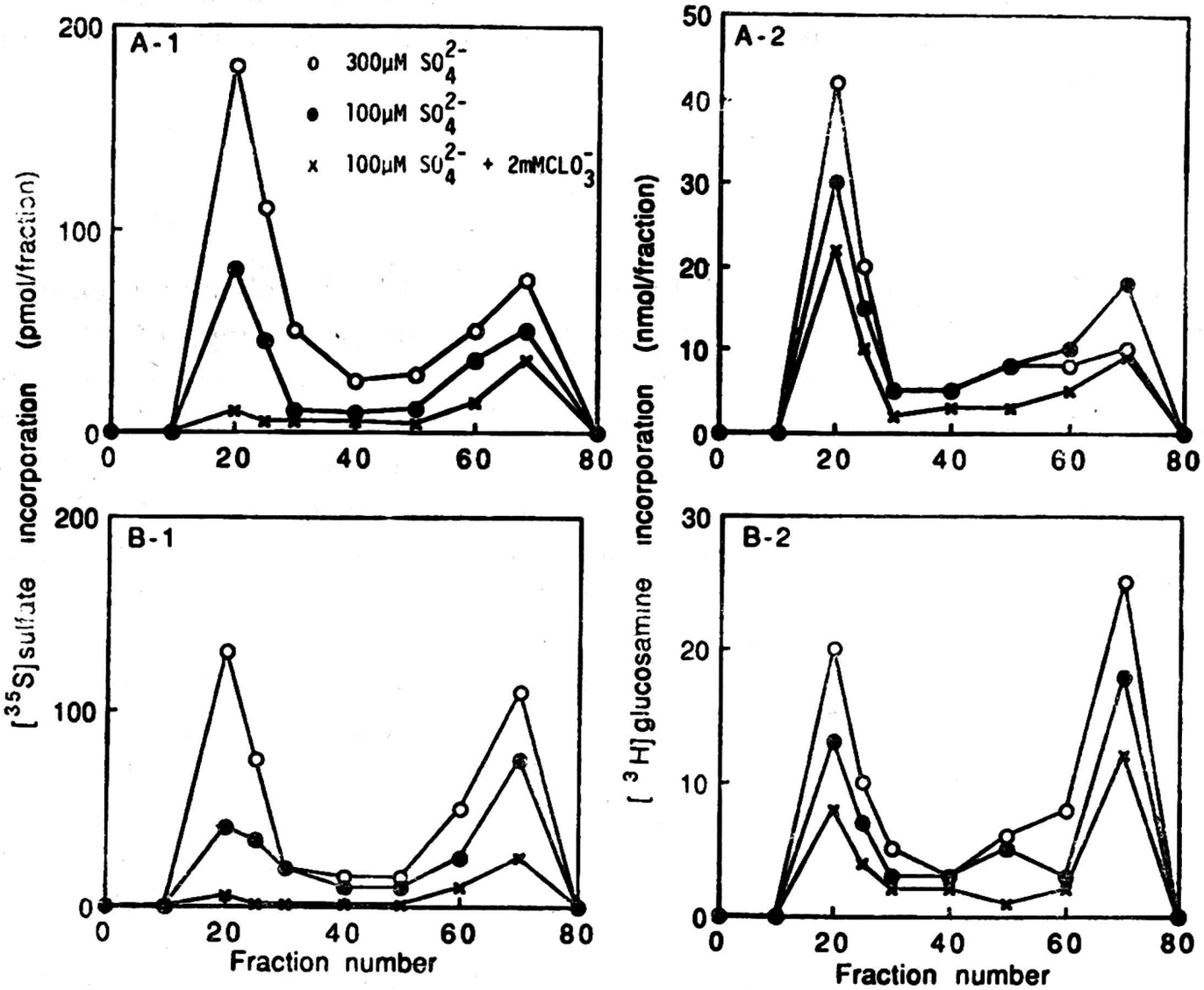

Fig. 4. Bio-Gel A-50 column chromatography in 6 M urea -0.05 M phosphate buffer, pH 7.0 of intracellular (A) and extracellular (B) mucin fractions excluded from Bio-Gel A-1.5. The profiles show superimposed patterns for [^{35}S] sulfate distribution (A and B) and that for [^{3}H]glucosamine (A_2 and B_2) obtained following 4h incubation with the radiolabeled markers at different medium sulfate concentrations, and in the presence of 2 mM chlorate.

Table 2. Distribution of sulfate incorporation among the lower and higher mucin molecular forms elaborated at different medium sulfate concentration in the absence and in the presence of chlorate.

Medium sulfate conc. (μM)	Specific activity (pmol SO_4^{-2}/mg protein)	
	Higher mucin molecular form	Lower mucin molecular form
800	920±80	180±12
300	890±75	170±13
100	310±26	60± 5
100+$C10_3^-$ (2 mM)	35±31	10± 2

The data show the means ±SD of five separate experiments performed in duplicate.

of the high molecular weight mucin form relative to that of the low molecular weight mucin, and that this effect of sulfate was inhibited in the presence of chlorate.

DISCUSSION

In this study, [^{35}S]Na_2SO_4, [^{3}H] glucosamine and [^{3}H] proline were employed as markers for mucus glycoprotein synthesis and processing in the intracellular compartment and following secretion. In both cases, the labels distribution among the high and the low molecular forms of mucin. The [^{35}S] radioactivity was found to be much higher in the intracellular fraction, especially when sulfate concentration of the medium was increased over 300 μM. The specific [^{35}S] activity and the time course studies thus suggest that the sulfation occurred first in the intracellular compartment. At higher medium sulfate concentrations ($>300\,\mu$M), the gastric cells in culture clearly produced more high molecular weight mucin form in the extracellular fraction, and as the medium sulfate concentration decreased, the amount of the low molecular weight mucin form became predominant. The effect was even more evident in the presence of chlorate. These results are in keeping with the findings of Van Beurden-Lamers et al. (19) who reported that chlorate causes both inhibition in the degree of sulfation and reduction in molecular size of the glycoprotein.

Our data demonstrated that the increase of medium sulfate concentration produced the increase in both mucin sulfation and its glycosylation. The inhibition of PAPS formation by chlorate not only affected the extent of sulfation but also caused the inhibition of glycosylation. From the viewpoint of metabolic pathway, the increase in sulfate concentration or inhibition of ATP sulfurylase by chlorate should affect only the PAPS formation and consequently the process of sulfation, and should not have an effect on the apomucin glycosylation. In the light of the obtained data showing that chlorate did not inhibit and even caused a slight increase in glycosylation, it is apparent that sulfation occurs at the stage of carbohydrate chains elongation and seems to be mandatory for the mucin carbohydrate chains growth. Our results indicate that the effect of medium sulfate concentration on the process of mucin glycosylation is somewhat different from that on the sulfation. The incorporation of glucosamine seemed to be less affected than that of sulfate with medium sulfate concentration, and when chlorate was added to the culture medium, the incorporation of glucosamine was less inhibited than that of sulfate. These data, therefore, suggest that some stage of mucin glycosylation may be independent of the process of sulfation. As in glycoprotein synthesis, the process of initiation of glycosylation is followed by the elongation of carbohy-

drate chains, it is apparent that sulfation plays a role only in the event of carbohydrate chain elongation.

The results of the studies presented herein show that the accessibility to active donor sulfate not only affects the degree of mucin carbohydrate chain sulfation, but also its macromolecular assembly. Easy availability of sulfate favors the large molecular weight mucin form formation, whereas in low sulfate environment lower molecular weight mucin form predominates. As a decrease in the synthesis of sulfated mucus glycoprotein and increase in the low molecular weight mucin form have been implicated in the etiology of peptic ulcer (20—22), the obtained results provide further insight as to the origin of this disturbance. From our data, it is apparent that the processes of mucus glycoprotein polymer assembly and the decoration of carbohydrate chains with sulfate are intimately related. Hence, in peptic ulcer disease, the increase in the low molecular weight mucin form may not be due exclusively to excessive peptic erosion as suggested (22), but rather results from the disturbances in mucin sulfation process.

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