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Glycosaminoglycan Synthesis in Endotoxin-Induced Lung Injury (41746)

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Abstract. Endotoxin-induced lung injury has previously been shown to produce lesions that resemble emphysema morphologically and biochemically as demonstrated by the reduction in the content of lung elastin. The purpose of this study was to define the changes in one other connective tissue component, glycosaminoglycans, during the acute phase of the lung injury. Intravenous administration of a single dose of endotoxin in rats resulted in an increase in the total synthesis of glycosaminoglycans by the pulmonary parenchyma. There was a significant increase in the proportion of dermatan sulfate synthesized during the first 48 hr and a concomitant decrease in heparin/heparan sulfate synthesis. At 48 hr the increased synthesis of dermatan sulfate had reached 7.3 times control values and began to decline, whereas the synthesis of chondroitin-4-sulfate rose from 4.1 to 10.7 times control values between 48 and 72 hr. Analysis of the rates of synthesis revealed that the total amount of heparin/heparan sulfate remained constant while the synthesis of chondroitin-6-sulfate increased proportionally to the overall synthesis of glycosaminoglycans. These findings indicate that dramatic changes in glycosaminoglycan synthesis are an integral part of endotoxin lung injury.

Various alterations have been observed in the glycosaminoglycan (GAG) composition in different organs in response to tissue damage (1–6). In a recent paper we have shown that repeated injection of endotoxin into rats with galactosamine-induced reduction in α_1-antitrypsin results in alveolar injury that biochemically, morphologically, and physiologically resembles pulmonary emphysema (7). In these animals there was a loss of connective tissue elastic fibers and a decrease in alveolar septa. To increase our understanding of the consequences in pulmonary parenchyma of an increment in neutrophils and macrophages and an associated increase in endogenous proteases, the synthesis of sulfated GAGs following intravenous administration of *E. coli* endotoxin has been studied.

Endotoxin or septic shock causes increases in pulmonary vascular resistance (8, 9), ventilation, alveolar surface tension, and pulmonary airway resistance with a decrease in lung compliance (9–11). The effects of intravenous administration of endotoxin can be visualized ultrastructurally by the resulting agglutination of platelets and the sequestration of polymorphonuclear leukocytes (PMNs) in the pulmonary capillaries (11–14). The sequestration of PMNs results in their degranulation, fragmentation, and ultimate release of proteolytic enzymes that are capable of degrading proteoglycans (15).

All seven of the recognized glycosaminoglycans are present in the lung (1, 16) but keratan sulfate has not been found in the parenchyma. Although the exact function of the GAGs has not been elucidated, their association with other elements of connective tissue has been demonstrated. They are associated with collagen fibers, forming an incompressible barrier that both retards and directs the flow of water (17, 18) as well as inhibiting the diffusion rate of small macromolecules (19).

Thus, GAGs may play an important role in the initial sequence of events following endotoxin administration which ultimately leads to breakdown of connective tissue fibers with eventual rupture of the alveolar septa. Chemically defining these changes in GAGs may provide insight into their relationship to collagen and elastin during lung injury. In addition results obtained in this model may be compared with changes observed in other types of lung injury such as interstitital pneumonitis and elastase-induced emphysema (20).

Materials and Methods. To elucidate the effect of endotoxin on pulmonary GAG synthesis, female Sprague–Dawley rats weighing 190–200 g and fed an unrestricted commercial diet were injected iv with 0.5 ml of an endotoxin suspension consisting of 1 mg *E. coli* lipopolysaccharide (Sigma Chemical Co., St. Louis, Mo.)/1 ml of sterile 0.9% NaCl. The GAGs were then labeled by intraperitoneal

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injections with a total dose of 400 μCi ^{35}S in the form of carrier-free sodium sulfate (New England Nuclear, Boston, Mass.). Each animal received 200 μCi at 0, 12, 24, 48, or 72 hr following endotoxin administration and a second dose of 200 μCi 12 hr later. This procedure has previously been used to label GAGs and measure their turnover rates both *in vivo* and *in vitro* (1, 6, 21).

Tissue preparation. Animals were killed 12 hr after the last injections of ^{35}S by intraperitoneal administration of 1 ml of a 2.75% solution of chloral hydrate in 0.9% NaCl per 100 g body wt. The lungs were resected from the thorax, and the trachea, mainstem bronchi, and major pulmonary vasculature were dissected free and discarded. The lung parenchyma was then cut into small fragments, washed three times in normal saline to remove free blood, and homogenized. The homogenates were dialyzed free of salts, lyophilized, weighed, and analyzed for labeled GAGs.

Analysis of labeled glycosaminoglycans. The lyophilized lung homogenates were delipidated by extraction with three changes of acetone and allowed to air dry. The tissues were then suspended in 0.1 *N* sodium acetate buffer, pH 5.5, and digested with papain (Sigma Chemical Co., St. Louis, Mo.) activated with 5 m*M* cysteine hydrochloride and 5 m*M* EDTA, at 57°C for 48 hr. The undigested proteins were precipitated with 10% TCA at 4°C and centrifuged at 10,000*g* for 30 min and the precipitates discarded. The supernatants were dialyzed free of TCA and small polypeptides and 3 mg of cold chondroitin sulfate added. They were then lyophilized and resuspended in 2 ml of distilled water. To precipitate the GAGs 6 ml absolute ethanol was added and the samples maintained at 4°C for 48 hr. This method has previously been shown to recover approximately 90% of the isolated GAGs (1). The precipitates were resuspended in 1 ml of distilled water and aliquots were analyzed for total ^{35}S incorporation.

Additional aliquots of the isolated GAGs were digested with chondroitinase ABC or chondroitinase AC-II (Miles Laboratories, Elkhart, Ind.) according to the methods of Saito and co-workers (22). The digests were chromatographed on Whatman 3MM filter paper in butanol, acetic acid, and 1 *N* ammonia (2:3:1) for 18 hr. Digestion with chondroitinase ABC yielded three labeled constituent pools: (i) chondroitinase-resistant material, predominantly heparin and/or heparan sulfate since keratan sulfate has not been detected in lung parenchyma; (ii) disaccharides of chondroitin-6-sulfate, and (iii) disaccharides of chondroitin-4-sulfate and dermatan sulfate. After digestion with chondroitinase AC-II, dermatan sulfate remains undigested and chromatographs in pool (i) rather than (iii). The strips were cut into 1-cm sections, immersed in scintillation fluid and analyzed for radioactivity in a liquid scintillation spectrometer. Tissues were analyzed for total ^{35}S incorporation, relative distribution of ^{35}S among specific GAGs, and relative rates of ^{35}S incorporation into specific GAGs. Results were expressed as means ± standard deviations as shown by the horizontal bars and the rectangles on Figs. 3–7. Statistical significance was determined by Student's *t* test.

Results. Histological examination of slide sections of the lungs 24 hr following intravenous administration of *E. coli* endotoxin revealed marked hypercellularity and engorgement of the capillary beds. Numerous red cells could be seen in the alveolar spaces, and the interstitium was infiltrated with PMNs and mononuclear cells (Figs. 1 and 2).

Uptake of ^{35}S into lung glycosaminoglycans. Total incorporation of ^{35}S into lung GAGs at 0, 12, 14, 48, and 72 hr following the intravenous administration of endotoxin was compared. As shown in Fig. 3 there was an increase in the total incorporation of ^{35}S into pulmonary GAGs as early as 12 hr post-endotoxin injection (401 ± 172 cpm/mg lung as compared to 279 ± 141 cpm, $P < 0.05$). The rate of incorporation steadily increased over the 72-hr period studied and attained a threefold increase in total synthesis by 72 hr (Fig. 3).

Analysis of lung parenchyma from control animals not injected with endotoxin revealed a distribution of GAG synthesis of: 66.0% heparin/heparan sulfate; 9.9% chondroitin-6-sulfate; 10.3% dermatan sulfate; 14.0% chondroitin-4-sulfate. Twelve hours following the administration of endotoxin only 55% of the GAGs synthesized were heparin/heparan sulfate and by 48 hr they comprised only 23% of the total GAG synthesized (Fig. 4). In contrast, analysis of the rate of heparin/heparan sulfate synthesis revealed that it remained rel-

FIG. 1. Section through the lung of a normal rat. H & E, ×40.

atively constant over the 72-hr period studied (cf. Fig. 8). The relative proportion of chondroitin-6-sulfate remained constant at approximately 10% of the total glycosaminoglycan synthesized (Fig. 5); its rate of synthesis thus paralleled the increase in the rate of total GAG synthesis (Fig. 8).

By 12 hr 15.5% of the GAGs synthesized were dermatan sulfate (Fig. 6). The relative amount of ^{35}S incorporated into dermatan sulfate continued to increase over the first 48 hr reaching a maximum of 39.7%, after which time it began to revert toward control levels. The rate of dermatan sulfate synthesis also increased over the first 48-hr period (Fig. 8) attaining a maximum of 7.3 times control values and decreasing toward control levels from 48 to 72 hr.

For the first 24 hr the relative distribution of chondroitin-4-sulfate remained slightly above control values (Fig. 7); however, it rose to 27% of the total GAGs synthesized by 48 hr and 46.5% by 72 hr. The rate of synthesis of condroitin-4-sulfate increased steadily over the first 48 hr to 4.1 times control values (Fig. 8). Between 48 and 72 hr there was a dramatic increase in the rate of synthesis of chondroitin-4-sulfate from 4.1 to 10.7 times control values.

Discussion. Sequestration of PMNs in the pulmonary parenchyma following intravenous administration of endotoxin is well documented (11–14). The presence of PMNs not

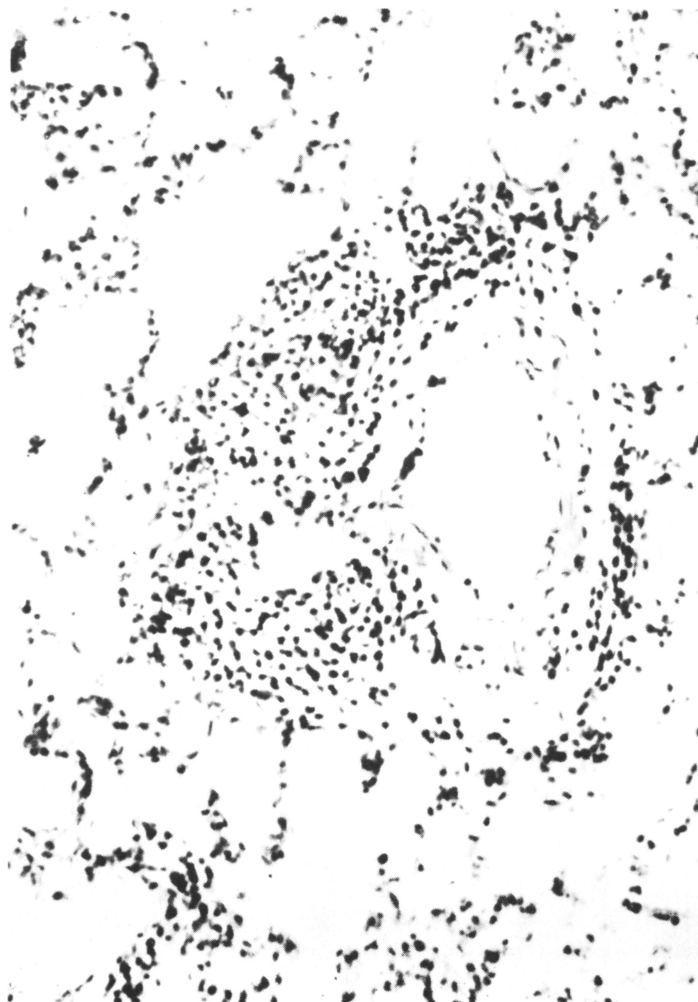

FIG. 2. Section of a rat lung 24 hr post-endotoxin. Note the hypercellularity and numerous PMNs within the interstitium. H & E, ×40.

only results in an increase of proteolytic enzymes capable of digesting proteoglycans (15) but also causes the release of factors capable of stimulating DNA and GAG synthesis by fibroblasts (23). In addition, the accumulation of PMNs is associated with hemodynamic changes within the pulmonary parenchyma which include alterations in capillary wall permeability, platelet agglutination, and fibrin deposition (8, 9, 12, 14), although the mechanisms by which these changes are effected are not clearly understood. Nonetheless the intimate association of proteoglycans with the other elements of the connective tissue may influence these functions. The observed changes in GAG synthesis may be a response to structural alterations induced by the influx of neutrophils which may also have functional effects.

Alteration in endothelial cell basement membrane connective tissue secondary to the release of proteolytic enzymes from PMNs may be responsible for platelet agglutination and fibrin deposition. Although heparan sulfate is most closely associated with the maintenance of an antithrombogenic surface, dermatan sulfate has also been shown to increase clotting time (24), whereas chondroitin-4-sulfate is known to prevent the adhesion of RBCs to endothelial cells (3). Thus, the early

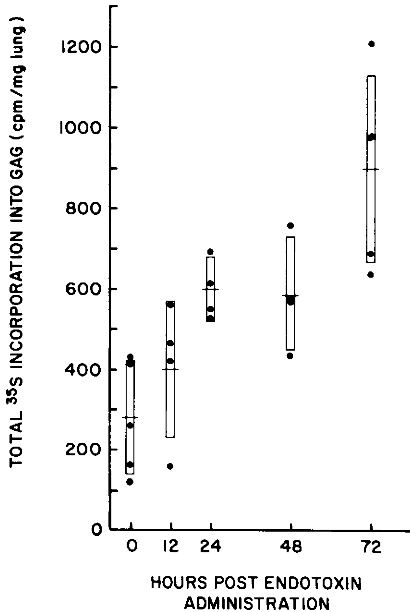

FIG. 3. Total ^{35}S incorporation into GAGs, showing an increase as early as 12 hr post-endotoxin administration.

rise in dermatan sulfate and the late increase in chondroitin-4-sulfate may function to reduce platelet agglutination and maintain pulmonary capillary blood flow.

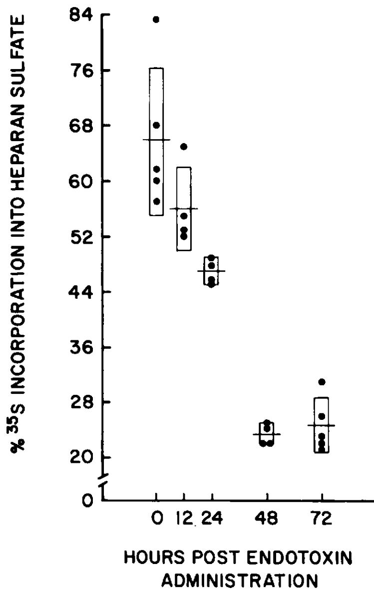

FIG. 4. Percentage ^{35}S incorporation into heparin/heparan sulfate showing a relative decrease beginning 12 hr after endotoxin administration.

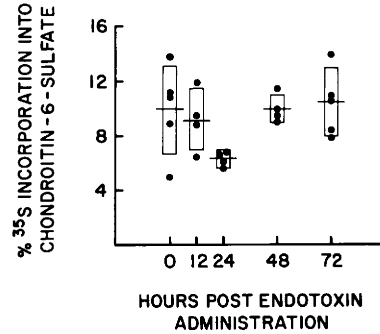

FIG. 5. Percentage incorporation of ^{35}S into chondroitin-6-sulfate which remains constant throughout the experiment.

Following different tissue injuries, there appears to be an early increase in the synthesis of hyaluronic acid (2, 3, 25). It is proposed that hyaluronic acid forms a matrix which supports the migration of cells into the affected area and also facilitates the regeneration of collagen fibers. Following the early increase in hyaluronic acid synthesis there is an increase in sulfated GAG synthesis. The only consistent finding is that the synthesis of dermatan sulfate closely parallels the synthesis of collagen.

While the changes in total uptake of ^{35}S-sulfate into the various GAGs most probably reflect actual differences in rates of synthesis between lungs of animals with and without endotoxin induced lung injury, other factors

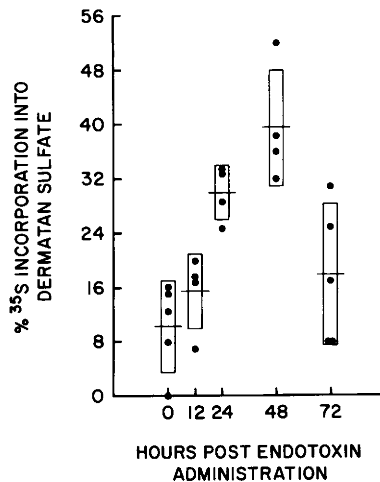

FIG. 6. Percentage incorporation of ^{35}S into dermatan sulfate showing an increase over the first 48 hr followed by a relative decrease.

which conceivably influence label incorporation should also be considered. Among these is an alteration in the pool size, i.e., the specific radioactivity of the labeled sulfate in the lung following endotoxin administration. While differences in pool sizes of ^{35}S cannot be completely ruled out, it is noteworthy that correlative *in vivo* and explant studies of ^{35}S labeling of GAGs in bleomycin-induced lung injury indicated that there was no significant pool size effect on the observed changes in GAG synthesis (6). Other factors which might influence uptake data include changes in degree of sulfation of individual GAGs and/or changes in their chain length. The relative percentages of ^{35}S-sulfate incorporation into GAGs should not be influenced by pool size changes.

In a previous study (7) we demonstrated that 10 weeks after endotoxin administration there was a loss of parenchyma, an increase in compliance and a decrease in the elastin content of rat lungs, but the early pulmonary consequences were not explored. In the present study one of the early consequences of endotoxin induced pulmonary injury has been shown to be an increase in the synthesis of GAG associated with a change in the relative concentration of the subtypes of this connective tissue component.

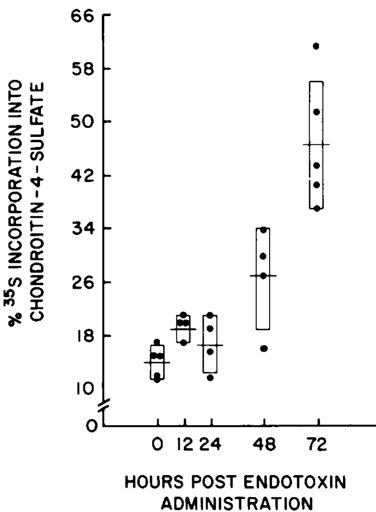

FIG. 7. Percentage incorporation of ^{35}S into chondroitin-4-sulfate which remains relatively constant over the first 48 hr followed by a dramatic increase.

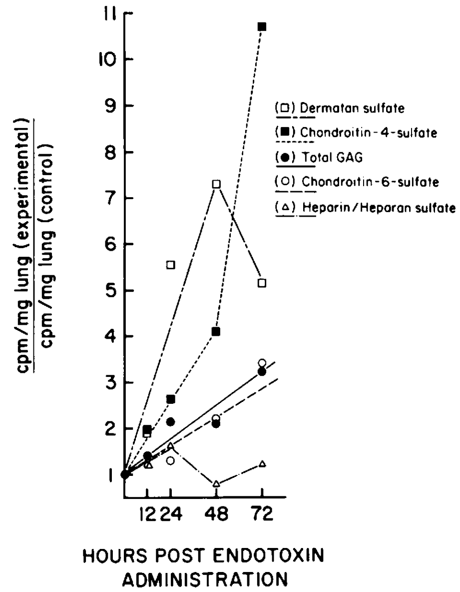

FIG. 8. Comparison of rates of synthesis of different GAGs. Note that the rate of chondroitin-6-sulfate synthesis parallels that of total GAG incorporation, whereas the rate of synthesis of heparin/heparan sulfate remains constant and that of dermatan sulfate increases during the first 48 hr and then decreases. Chondroitin-4-sulfate synthesis increases at a rate similar to that observed for total GAG synthesis for the first 48 hr and then rises dramatically.

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1. Cantor JO, Bray BA, Ryan SF, Mandl I, Turino GM. Glycosaminoglycan and collagen synthesis in *N*-nitroso-*N*-methylurethane induced pulmonary fibrosis. Proc Soc Exp Biol **164**:1-8, 1978.
2. Suzuki S, Suzuki S, Nakamura N, Koizumi T. The heterogeneity of dermatan sulfate and heparan sulfate in rat liver and a shift in the glycosaminoglycan content in carbon tetrachloride-damaged liver. Biochim Biophys Acta **428**:166-181, 1976.
3. Gillard GC, Birnbaum P, Reilly HC, Merrilees MJ, Glint MH. The effect of charged synthetic polymers on proteoglycan synthesis and sequestration in chick embryo fibroblast cultures. Biochim Biophys Acta **584**:520-528, 1979.
4. Niada R, Mantovani M, Pescador R. Antithrombotic activity of glycosaminoglycans of mammalian origin. In vivo experiments in the rabbit. Pharmacol Res Commun **11**:349-356, 1979.
5. Edward M, Long WF, Watson HH, Williamson FB. Sulphated glycosaminoglycans in regenerating rat liver. Biochem J **188**:769-773, 1980.
6. Cantor JO, Osman M, Cerreta JM, Mandl I, Turino

GM. Glycosaminoglycan synthesis in explants derived from bleomycin-treated fibrotic hamster lungs. Proc Soc Exp Biol Med **173**:362–366, 1983.

7. Blackwood RA, Moret J, Mandl I, Turino GM. Emphysema induced by intravenous endotoxin in an alpha-1-antitrypsin deficient rat model. Amer Rev Respir Dis, in press.
8. Kuida H, Hinshaw LB, Gilbert RP, Visscher MB. Effect of gram-negative endotoxin on pulmonary circulation. Amer J Physiol **192**:335–347, 1958.
9. Halmagyi DFJ, Starzechi B, Horner GJ. Mechanism and pharmacology of endotoxin shock in sheep. J Appl Physiol **18**:544–553, 1963.
10. Guenter CA, Fiorica V, Hinshaw LB. Cardiorespiratory and metabolic responses to live E. coli and endotoxin in the monkey. J Appl Physiol **26**:780–787, 1969.
11. Harrison LH, Beller JJ, Hinshaw LB, Coalson JJ, Greenfield LJ. Effect of endotoxin on pulmonary capillary permeability, ultrastructure and surfactant. Surg Gynecol Obstet **129**:723–737, 1969.
12. Stetson CA. Studies on mechanism of Schwartzman phenomenon; certain factors involved in producing a local hemorrhagic necrosis. J Exp Med **93**:489–504, 1951.
13. Coalson JJ, Hinshaw LB, Guenter CA. The pulmonary ultrastructure in septic shock. Exp Mol Pathol **12**:84–103, 1970.
14. McKay G, Margarethen W, Csavassy I. An electron microscope study of the effect of bacterial endotoxin on the blood vascular system. Lab Invest **15**:18–28, 1966.
15. Malemud CJ, Janoff A. Human polymorphonuclear leukocytes, elastase and cathepsin G mediate the degradation of lapine articular cartilage proteoglycans. Ann NY Acad Sci **256**:233–254, 1975.
16. Sjoberg I, Fransson LA. Synthesis of glycosaminoglycans by human embryonic lung fibroblasts. Different distribution of heparan sulfate, chondroitin sulfate and dermatan sulfate in various fractions of cell culture. Biochem J **167**:383–392, 1977.
17. Day TD. Connective tissue permeability and the mode of action of hyaluronidase. Nature (London) **166**:785–786, 1950.
18. Day TD. The permeability of the interstitial connective tissue and the nature of the interfibrillary substance. J Physiol (London) **117**:1–8, 1952.
19. Kantor TG, Schubert M. The difference in permeability of cartilage to cationic and anionic dyes. J Histochem Cytochem **5**:28–32, 1957.
20. Karlinsky JB. Glycosaminoglycans in emphysematous and fibrotic hamster lungs. Amer Rev Respir Dis **125**:85–88, 1982.
21. Ehrlich KO. Proteoglycan synthesis by rat lung cells cultured in vitro. J Biol Chem **256**:73–80, 1981.
22. Saito H, Yamagata T, Suzuki S. Enzymatic methods for the determination of isomeric chondroitin sulfates. J Biol Chem **243**:1536–1542, 1968.
23. Myers SL, Castor CW. Connective tissue activation. XV. Stimulation of glycosaminoglycan and DNA synthesis by a polymorphonuclear leukocyte factor. Arthritis Rheum **23**:556–563, 1980.
24. Kindness G, Long WF, Williamson FB. The anticoagulant activity of dermatan sulfates: Evidence against the involvement of antithrombin III. Brit J Pharmacol **72**:81–88, 1981.
25. Koizumi T, Nakamura N, Abe H. Changes in acid mucopolysaccharides in the liver in hepatic fibrosis. Biochim Biophys Acta **148**:749–756, 1967.

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