PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 172–181 (1983)

Glycosaminoglycan Synthesis in Bleomycin-Induced Pulmonary Fibrosis: Biochemistry and Autoradiography[1] (41721)

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Abstract. At 5, 15, and 45 days following induction of interstitial pulmonary fibrosis by intratracheal administration of bleomycin in hamsters, glycosaminoglycan synthesis was measured, using [^{35}S]sulfate. Total labeled sulfate incorporation into lung glycosaminoglycans was maximally increased over that of saline-instilled controls at 5 days ($P \leq 0.05$), declined markedly at 15 days, and returned to control values at 45 days. Separation of the various labeled glycosaminoglycans by chondroitinase digestion and chromatography revealed a transient rise from controls ($P \leq 0.05$) in the proportion of labeled chondroitin 4-sulfate at 5 days, followed by an increase from controls ($P \leq 0.05$) in proportionate labeling of dermatan sulfate at 15 and 45 days postbleomycin. Autoradiography, using [^{35}S]sulfate, performed at 21 days postbleomycin, revealed an increase from controls in film grain formation in areas of interstitial reaction. Grain formation was greatly reduced by pretreatment of the slide sections with hyaluronidase and chondroitinase, demonstrating the specificity of the label for glycosaminoglycans. The results indicate that glycosaminoglycan synthesis is significantly altered from normal in this model of interstitial lung disease and that dermatan sulfate is preferentially synthesized during the fibrotic phase of the lung reaction.

Interstitial pulmonary fibrosis is a disease process usually marked by an inflammatory cell reaction, an increase in fibroblasts in the interstitium, and subsequent tissue fibrosis with derangement of lung architecture. The progression of anatomic changes is associated with significant alteration in the content and distribution of connective tissue in the lung parenchyma (1–11). Whereas collagen and elastin content and synthesis in interstitial lung reactions have been studied in some detail, the nature of the changes in glycosaminoglycans are less well understood. Although this connective tissue component comprises only a very small percentage of the tissue mass of the lung, it may nevertheless play a strategic role in the pathogenesis of pulmonary fibrosis by virtue of its ubiquitous presence on cell surfaces and in the extracellular matrix. Glycosaminoglycans are known to be structurally associated with collagen, to influence cell growth and differentiation, and may play a significant role in the regeneration of injured tissue of other organs (12–18).

The purpose of the present study was to define the temporal sequence of changes in pulmonary glycosaminoglycans in relation to the morphological alterations in experimental interstitial lung fibrosis induced by intratracheal instillation of bleomycin (19, 20). The investigation is an outgrowth of earlier studies of glycosaminoglycans in this laboratory on another model of pulmonary fibrosis induced by *N*-nitroso-*N*-methylurethane (10) which indicated that significant changes from normal occur in the pattern of synthesis of this connective tissue component. In addition to permitting comparison of glycosaminoglycan alterations in two distinct experimental models of pulmonary fibrosis, the present study on the well-characterized bleomycin model provides information regarding the temporal relationships between glycosaminoglycan synthesis and other manifestations of interstitial injury, including synthesis of collagen and elastin and derangements in lung morphology.

Materials and Methods. *General experimental plan.* Glycosaminoglycan synthesis in bleomycin-treated and control hamster lungs was studied by injecting the animals with [^{35}S]sulfate at 5, 15, and 45 days post-intratracheal instillation of bleomycin. Differences between the experimental and control groups were determined with regard to total incor-

[1] This study was supported by an American Lung Association Grant, USPHS HL 26496 and USPHS Program Project Grant HL15832.

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0037-9727/83 $1.50

poration of ^{35}S into lung glycosaminoglycans and the distribution of label among the various subgroups of those molecules, i.e., heparan sulfate, dermatan sulfate, and chondroitin sulfate. In addition, animals were labeled with [^{35}S]sulfate at 21 days postinstillation of bleomycin for subsequent autoradiography to determine the anatomic distribution of the newly synthesized glycosaminoglycans in both diseased and control lungs.

Analysis of glycosaminoglycan synthesis. (1) Induction of interstitial lung disease. Forty-eight Syrian hamsters, outbred females weighing 130–150 g each, were divided into experimental and control groups (24 experimental, 24 control). Fifteen of the 24 experimental animals received a single intratracheal injection of 1.0 unit bleomycin sulfate (Bristol Laboratories, Syracuse, N.Y.) in 0.2 ml normal saline. The remaining 9 animals were given 0.5 unit of the agent in 0.2 ml saline. Controls received a single intratracheal injection of 0.2 ml normal saline. All insufflations were performed following tracheostomy while the animals were under light anesthesia induced with 0.05 ml Ketaset (Bristol Laboratories, Syracuse, N.Y.) injected im.

(2) Labeling of the glycosaminoglycans. At 5, 15, and 45 days postinstillation of bleomycin, both experimental and control animals were given ip injections of [^{35}S]sulfate (New England Nuclear, Boston, Mass.) to label the glycosaminoglycans. [^{35}S]Sulfate has been shown to be incorporated almost exclusively into sulfated polysaccharides and to reflect *de novo* synthesis of these molecules (21). The isotope was administered daily for 4 consecutive days at a dose of 1 mCi/kg/day.

(3) Preparation of tissue for analysis of glycosaminoglycans. The animals were sacrificed on the day following the final injection of ^{35}S by ip administration of a lethal dose of barbiturate. The lungs were removed from the thorax; extraparenchymal structures, i.e., trachea, mainstem bronchi, and major blood vessels, were discarded. Multiple biopsies were taken from each specimen for histology. The lungs were then cut into small fragments, washed in normal saline, homogenized, dialyzed, and lyophilized.

(4) Measurement of labeled lung glycosaminoglycans. Weighed samples were prepared as previously described (10, 22). Briefly, the method is as follows: After delipidation in acetone, the glycosaminoglycans were isolated by papain digestion, TCA precipitation of undigested proteins, dialysis of the supernatants, and ethanol precipitation of the glycosaminoglycans in the presence of carrier chondroitin sulfate. After measurement of total labeling of the glycosaminoglycan fraction, aliquots of the material were degraded with either chondroitinase ABC or AC-II (Seikagaku Kogyo Co., Tokyo, Japan), according to the method of Saito *et al.* (23). The characteristics of these enzymes have been described previously (23–25), as well as their usefulness in measuring microamounts of glycosaminoglycans (23, 25). The resultant digests were chromatographed on Whatman 3MM filter paper in a system of butanol:acetic acid:1 *N* ammonia (2:3:1). This yielded three constituent pools: (i) chondroitinase-resistant heparin and/or heparan sulfate, which remains at the origin; (ii) disaccharides of chondroitin 6-sulfate; (iii) disaccharides of chondroitin 4-sulfate and dermatan sulfate (with chondroitinase AC-II, dermatan sulfate remains undigested and chromatographs with heparin/heparan sulfate). The developed chromatograms were cut into strips, each of which was immersed in 10 ml scintillation fluid and measured for radioactivity in a liquid scintillation spectrometer. Incorporation of label into each of the products of enzyme digestion was expressed as a percentage of the total labeled material in the three pools obtained by chromatography.

Histology. Biopsies taken from the lungs of experimental and control animals were inflated and fixed in 10% neutral-buffered Formalin under a negative pressure of approximately 20 cm H_2O. The fixed tissues were embedded in paraffin, sectioned at 5 μm thickness, mounted on glass slides, and stained with hematoxylin and eosin. The slide sections were examined with the light microscope for determination of the presence of disease.

Autoradiography. Syrian hamsters (three experimental, two control) were injected intratracheally with either 0.2 ml bleomycin (1.0 unit) or 0.2 ml saline. At 21 days postintratracheal instillation of these agents, each animal was injected ip with 1 mCi [^{35}S]sulfate in 1.0 ml normal saline. The animals were sacrificed 24 hr later by ip injection of a lethal

dose of barbiturate. Thoracotomies were performed and the lungs were inflated *in situ* via the trachea with 10% neutral-buffered Formalin at a pressure of 20 cm H_2O. Two hours later, the lungs were removed from the chest cavity after ligating the trachea and additionally fixed in 10% Formalin for several days. Tissue blocks were embedded in either paraffin or methacrylate and sectioned at 5 and 2 μm, respectively. Prior to coating the slide sections with film emulsion, some were treated consecutively with ovine hyaluronidase (Miles-Servac, Ltd., Berkshire, England) and chondroitinase ABC to demonstrate that ^{35}S was incorporated into the glycosaminoglycans. Digestion with hyaluronidase was performed by overlaying the slide section with the enzyme dissolved in 0.1 *M* phosphate buffer at a concentration of 1 mg/ml. Treatment was for 1 hr at 37°C. After removal of the hyaluronidase solution, the slides were treated with chondroitinase ABC for a similar period, using Tris-enriched buffer (23), at an enzyme concentration of 1.0 unit/ml. These slide sections were then washed extensively in water. Both enzyme-treated and untreated slides were coated with NTB-2 emulsion (Eastman Kodak, Rochester, N.Y.) and exposed for 2 to 4 weeks prior to development. The slide sections were then stained with hematoxylin and eosin and examined with the light microscope.

Results. *Histology.* The histological features of bleomycin-induced lung injury have been described previously in detail (19, 20). Lung biopsies from bleomycin-treated animals sacrificed after labeling from Days 5 to 9 showed patchy interstitial thickening. The interstitial reaction was composed of a mixed inflammatory cell infiltrate, i.e., neutrophils and mononuclear cells, fibrinous exudates, and alveolar epithelial hypertrophy and hyperplasia. By 19 days, the mononuclear cell infiltrates were still prominent in the interstitium as was the hyperplastic alveolar epithelium. Scattered fibroblast proliferation was also seen in the thickened septa and fibrosis was apparent. Many of the terminal bronchioles and alveolar spaces were dilated and lined by cuboidal epithelium and were surrounded by areas of alveolar collapse and consolidation. The interstitial reaction was patchy with abundant skip zones. The severity of the disease varied among the animals, but all showed at least some evidence of this interstitial reaction. Lungs from animals sacrificed at 49 days showed morphologic changes similar to those at 19 days postbleomycin. However, the mononuclear cell infiltrates had receded somewhat and the amount of fibrosis in the interstitium was greater than at 19 days.

Biochemistry. As seen in Fig. 1 (and Table I), total incorporation of [^{35}S]sulfate into the bleomycin-treated lungs was greatest at the 5-day interval. There was a marked decrease in total label incorporation at 15 days, but it was still significantly greater than in controls ($P \leq 0.05$). Between 15 and 45 days, there was a continued decrease in total [^{35}S]sulfate incorporation in the experimental lungs and the values at the latter time interval were similar to controls. Label incorporation in control lungs was greater at 5 days than at either 15 or 45 days, probably as a result of an initial inflammation caused by the saline infusion, as seen in biopsies of the 5-day lungs. Analysis of total ^{35}S incorporation according to the dose of bleomycin (1.0 or 0.5 unit) showed that, at 15 days, differences in the amount of the agent instilled produced a significant difference in total labeling of the glycosaminoglycans (Fig. 2). However, at 45 days, varying the dose of the agent did not result in an overall difference in label incorporation.

The apportionment of the ^{35}S label among the various subgroups of glycosaminoglycans differed between the experimental and control groups at 5, 15, and 45 days postbleomycin instillation. At 5 days, the experimental lungs had a small, but significant ($P \leq 0.05$), increase in the percentage of labeled chondroitin 4-sulfate compared to controls (Table II). Conversely, at this time, the percentage of labeled heparin/heparan sulfate was lower in the bleomycin-treated lungs than in controls. The percentage of ^{35}S associated with dermatan sulfate and chondroitin 6-sulfate was similar in both groups at the 5-day interval (Table II).

At 15 days postbleomycin, the proportionate distribution of [^{35}S]sulfate among the various glycosaminoglycans in the experimental lungs differed from that at 5 days. In contrast to the earlier time interval, there was no difference in the proportion of label in chondroitin 4-sulfate between treated and control lungs. Rather, the bleomycin-instilled lungs had a significant increase from control tissues

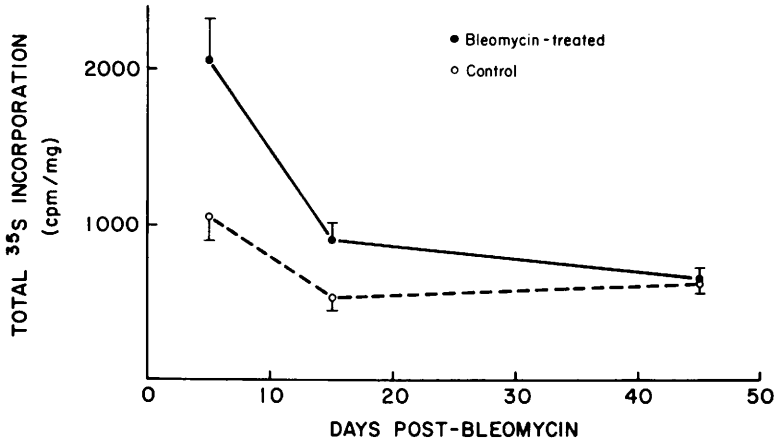

FIG. 1. Graph comparing total [^{35}S]sulfate incorporation into lung glycosaminoglycans of bleomycin-treated and control hamsters. Differences between the groups were greatest at 5 days and became less pronounced with time. (From Table I.)

($P \leq 0.05$) in the percentage of labeled dermatan sulfate (26.3 vs 19.5%). The proportion of labeled heparin/heparan sulfate was again lower in the experimental group (Table II) while no difference between the groups was seen with regard to chondroitin 6-sulfate.

At 45 days postbleomycin, the percentage of labeled dermatan sulfate in the experimental lungs was again significantly greater ($P \leq 0.05$) than in controls (30.4 vs 23.5%). As at 5 and 15 days, the percentage of labeled heparin/heparan sulfate was lower in the bleomycin-treated group as compared to controls (37.6 vs 43.3%).

The most marked change in label distribution over the course of the study occurred with regard to dermatan sulfate. Between 5 and 45 days, there was a 40% increase in the percentage of [^{35}S]sulfate associated with this glycosaminoglycan in the experimental lungs (Fig. 3). No other glycosaminoglycan changed to this extent, though smaller changes were observed with respect to heparin/heparan sulfate and chondroitin 4-sulfate.

TABLE I. TOTAL [^{35}S]SULFATE INCORPORATION INTO LUNG GLYCOSAMINOGLYCANS

Group	No. animals	Day postbleo	cpm/mg dry lung wt
Expt	5	5	2059 ± 264*
Cont	5	5	1031 ± 132*
Expt	10	15	904 ± 110*
Cont	10	15	528 ± 69*
Expt	9	45	657 ± 81
Cont	9	45	621 ± 55

Note. Values are means ± SE.
* $P \leq 0.05$, Mann–Whitney–Wilcoxon rank sum test, 2-tailed.

Autoradiography. Examination of the autoradiographs of 21-day bleomycin-treated lungs revealed an increase from controls in film grain concentration in areas exhibiting an interstitial cellular reaction (Fig. 4). These grains were associated with newly synthesized glycosaminoglycans as indicated by the very marked reduction in grains in slide sections treated consecutively with hyaluronidase and chondroitinase (Fig. 5). Even with such digestion, some grains would be expected to form since hyaluronidase does not digest heparan sulfate or dermatan sulfate while chondroitinase ABC leaves heparan sulfate intact.

The grain pattern was not quantitated because even the thinner, 2-μm sections did not permit localization of grains to particular cells. However, this was not the case for mast cells, which were overlaid with grain densities and were generally localized in the bronchial stroma. High concentrations of grains were also associated with reactive areas of the pleural surface and were possibly due to increased secretion of glycosaminoglycans by

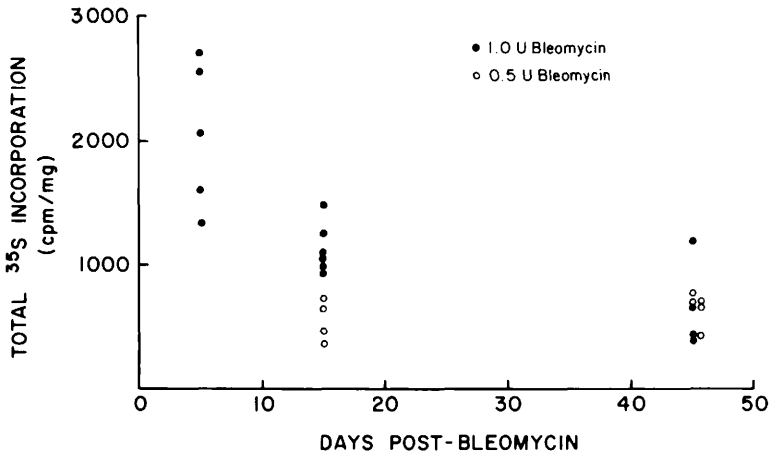

FIG. 2. Graph comparing total [^{35}S]sulfate incorporation into lung glycosaminoglycans of hamsters receiving either 1.0 or 0.5 unit of bleomycin intratracheally. At 15 days postbleomycin, animals receiving 1.0 unit of the agent had significantly greater label incorporation than those given 0.5 unit ($P \leq 0.05$; Mann–Whitney–Wilcoxon rank sum test). At 45 days, varying the dose of bleomycin did not result in labeling differences.

mesothelial cells at this 21-day interval (26, 27).

Discussion. This study demonstrates that changes in the pattern of synthesis of glycosaminoglycans in lung parenchyma occur shortly after administration of bleomycin and continue to evolve over a 7-week period. Most notably, there is a twofold increase compared to control lungs in total incorporation of [^{35}S]sulfate into glycosaminoglycans at the 5- to 9-day interval followed by a decline in such incorporation to control values at 45 days. With regard to apportionment of the sulfate label among the various subgroups of glycosaminoglycans, there is a transient rise in the proportion of labeled chondroitin 4-sulfate during the first week after bleomycin instillation which returns to control values by 15 days. In contrast, there is a steady increase in the proportionate labeling of dermatan sulfate which is 40% greater at 45 days than at 5 days postbleomycin. None of the other glycosaminoglycans demonstrated such preferential synthesis during the fibrotic phase of the interstitial lung reaction.

When compared to studies of collagen and elastin synthesis in this experimental model (6, 28), the changes in glycosaminoglycan synthesis occur somewhat earlier. With regard to collagen, studies indicate that the earliest increase over control values is seen at about 6 days post-intratracheal instillation of bleo-

TABLE II. RELATIVE INCORPORATION OF [^{35}S]SULFATE INTO GLYCOSAMINOGLYCANS

Group	No. animals	Day	Hep/Hep S (%)	Derm S (%)	Ch-4S (%)	Ch-6S (%)
Expt	5	5	43.9 ± 1.3*	21.6 ± 0.8	21.6 ± 1.0*	12.8 ± 0.4
Cont	5	5	48.7 ± 1.0*	21.5 ± 0.8	16.7 ± 0.9*	13.1 ± 0.4
Expt	10	15	40.4 ± 2.7*	26.3 ± 2.4*	19.0 ± 0.5	14.3 ± 0.8
Cont	10	15	47.2 ± 1.9*	19.5 ± 1.9*	19.4 ± 0.8	13.9 ± 0.7
Expt	9	45	37.6 ± 1.8*	30.4 ± 2.0*	16.3 ± 0.9	15.6 ± 0.6
Cont	9	45	43.3 ± 1.0*	23.5 ± 1.9*	17.6 ± 1.3	15.6 ± 1.2

Note. Values are means ± SE.
* $P \leq 0.05$, Mann–Whitney–Wilcoxon rank sum test, 2-tailed.

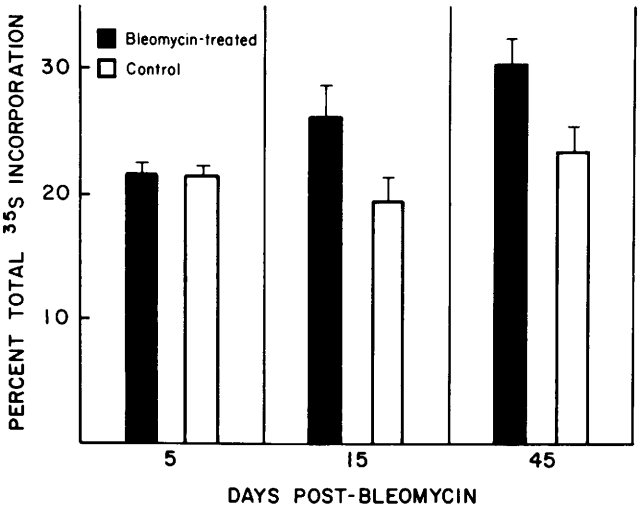

FIG. 3. Bar graph depicting proportionate labeling of dermatan sulfate in bleomycin-treated and control animals over time. Between 5 and 45 days, there was a 40% increase in the percentage of [^{35}S]sulfate associated with this glycosaminoglycan in the bleomycin-treated lungs. (From Table II.)

mycin and the maximum increase occurs between 8 and 14 days (6). Studies of crosslinked elastin synthesis in this model (28) show that it is maximal 2 to 3 weeks postinitiation of lung injury. Thus, the changes in glycosaminoglycan synthesis do not simply reflect participation in a general increase in connective tissue synthesis but may comprise an independent response to interstitial injury, preceding changes from normal in collagen and elastin and possibly influencing their deposition.

In this regard, there is evidence that glycosaminoglycans can influence formation of collagen fibers *in vitro* (29) and may stimulate proliferation of fibroblasts as well as regulate collagen synthesis by these cells (16, 17). Glycosaminoglycans may also provide the support and cohesion needed in the restoration of damaged connective tissue matrix and cells (14).

Dermatan sulfate has been shown to be elevated in other tissues undergoing repair (30, 31), either selectively or in conjunction with other glycosaminoglycans, e.g., heparan sulfate. This component may therefore play an important role in the reorganization of the connective tissue in the parenchyma following bleomycin-induced injury. While the relationship of dermatan sulfate to either collagen or elastin remains speculative, Longas and Meyer (32) recently reported that dermatan sulfate isolated from ligamentum nuchae is attached to proteins by a lysine linkage, rather than via serine or threonine. This finding may be important to the understanding of the role of dermatan sulfate in connective tissue synthesis in view of the ubiquity of lysine in both collagen and elastin.

The etiology of the observed changes in glycosaminoglycan synthesis is poorly understood. Little is known about glycosaminoglycan synthesis by individual lung cell types, both in the normal state and following cell damage. Changes in the cells populating the lung after instillation of bleomycin may be responsible for at least some of the findings in this study. There is an initial, variable, acute inflammatory response lasting several days, followed by mononuclear cell infiltration of the interstitium, rapid regeneration of injured alveolar epithelium, fibroblast proliferation, and fibrosis of the parenchyma by 2 to 3 weeks, as manifested both histologically and biochemically (6, 20). Thus, the initial rise in total [^{35}S]sulfate incorporation in lung parenchyma is seen at a time when there is morphological evidence of inflammatory cell infiltrates in the interstitium and alveolar epithelial hyperplasia. It is probable that the influx of inflammatory cells into the lung as well as the regeneration of alveolar epithelium ac-

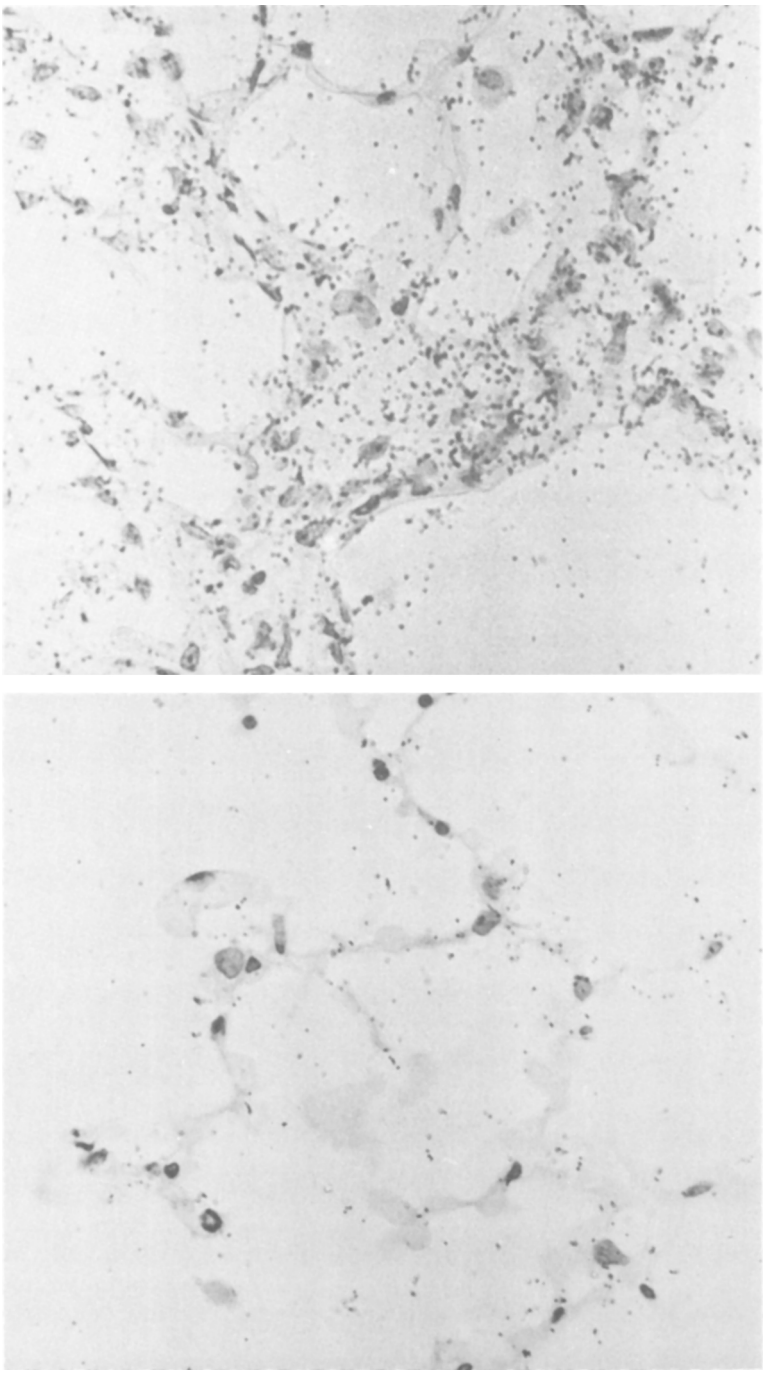

FIG. 4. Autoradiographs of [^{35}S]sulfate incorporation into lung parenchyma of control (left-side) and bleomycin-treated (right-side) animals. Film grain concentration is greater in an area of interstitial reaction in the bleomycin-treated lung. Hematoxylin and eosin. ×660.

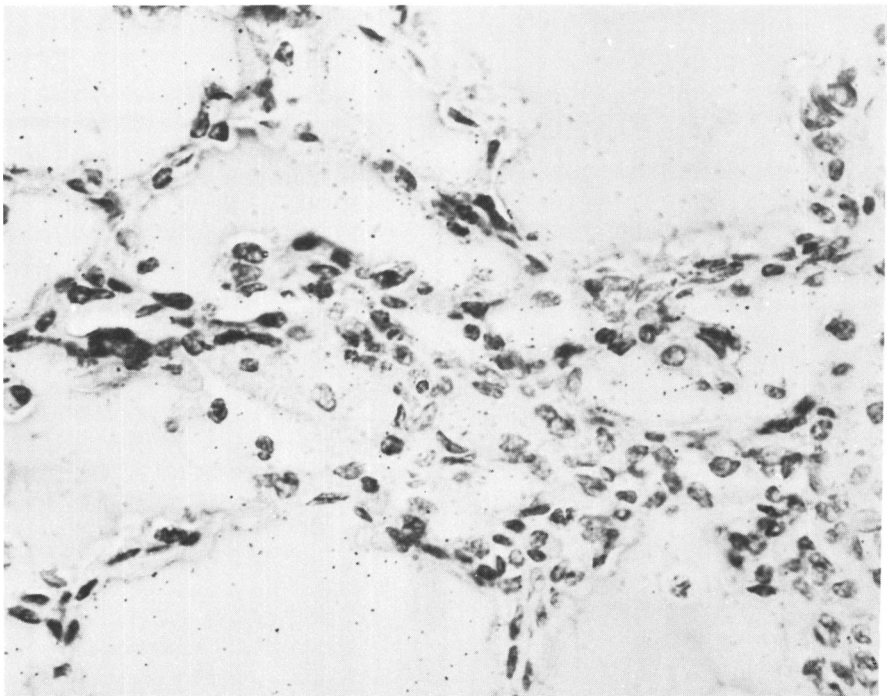

FIG. 5. Autoradiograph of lung parenchyma from a bleomycin-treated animal, pretreated with hyaluronidase and chondroitinase. In comparison with untreated slide sections, there is a marked reduction in grain formation following enzyme digestion. Hematoxylin and eosin, × 660.

count for a significant portion of the labeling increase at this early phase of injury. The relatively late shift in labeling toward dermatan sulfate suggests that the initial cellular changes are not responsible for the preferential synthesis of this glycosaminoglycan. This may instead be due to the increase in number of another cell type, i.e., the fibroblast, occurring in the later phases of the injury.

Although relatively few studies of glycosaminoglycan synthesis in lung injury have been undertaken, the present findings are consistent with previous reports of glycosaminoglycan alterations in interstitial fibrosis. Previous work in this laboratory, using the *N*-nitroso-*N*-methylurethane model of lung fibrosis, also demonstrated a shift in proportionate [^{35}S]sulfate labeling toward dermatan sulfate at a later phase of the lung injury (10). Furthermore, recently completed explant studies (33) of glycosaminoglycan synthesis in the bleomycin model, performed at 15 and 45 days postinstillation of the agent, also showed preferential labeling of dermatan sulfate in the treated explants compared to controls at both time intervals as well as substantially increased total [^{35}S]sulfate labeling of glycosaminoglycans at 15 days. The explant studies are particularly relevant to the present *in vivo* work because they address the problem of radioactive pool-size differences between experimental and control animals by fixing the extracellular pool of [^{35}S]sulfate.

In addition, a recent report by Karlinsky (11), regarding total content of glycosaminoglycans in bleomycin-induced pulmonary fibrosis of hamsters, demonstrated a significant increase in glycosaminoglycans 30 days post-intratracheal instillation of the agent. In that study, however, all measured glycosaminoglycans, including dermatan sulfate, were increased in similar proportions which may reflect differences in the methodological approach to studying the glycosaminoglycans or differences in their individual rates of degradation.

It may be concluded from the present study that glycosaminoglycan synthesis is altered from normal in bleomycin-induced fibrotic lung disease. In contrast to collagen and elastin, maximal changes in total synthesis occur at a very early phase of the lung injury. Dermatan sulfate, which has a proportionately greater amount of the sulfate label as the disease progresses, may play a significant role in the interstitial fibrotic reaction. Further studies are required to delineate the role of dermatan sulfate and other glycosaminoglycans in the increases in collagen and elastin in this model.

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Received April 20, 1983. P.S.E.B.M. 1983, Vol. 174.