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Collagen Crosslinking in Lungs of Rats with Experimental Silicosis

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Abstract

Rats were intratracheally instilled with 50 mg of size-fractionated crystalline quartz to induce silicosis. Lungs were analyzed 1, 4, 6, and 9 months after instillation for their content of the reduced difunctional collagen crosslinks dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL), of the nonreducible trifunctional (mature) crosslink, hydroxypyridinium (OHP), and of hydroxylysine. Ratios of DHLNL : HLNL were elevated in silicotic lung collagen at all times sampled, due both to increased levels of DHLNL and decreased amounts of HLNL. Hydroxylysine content of collagen in the silicotic lungs was also increased as compared with age-matched control rats. Hydroxypyridinium content of silicotic lung collagen was less than control values at 1 month, but was significantly increased to about 120%, 150%, and 175% of the age-matched control values at 4, 6, and 9 months after silica instillation, respectively. The increased levels of OHP in lung collagen were temporally correlated with the appearance of mature silicotic nodules in these lungs. We conclude that the large amounts of excess collagen deposited in silicotic lungs differs biochemically from normal lung collagen despite maintenance of the normal ratio of major collagen types in silicotic lungs.

Key Words: crosslinking, fibrosis, hydroxypyridinium, hydroxylysine, silicosis.

Introduction

In previous studies we have examined several aspects of collagen metabolism in experimental silicosis in rats (Reiser et al., 1982; 1983). We observed an increase in lung collagen synthesis rates, detectable as early as 1 week after a single intratracheal instillation of 50 mg of quartz, that persisted for at least 1 year. Total lung hydroxyproline content steadily increased during the year until it was 3- to 6-times control values. Of particular interest was the observation that collagen type I: type III ratios remained the same as control values in the silicotic rat lungs at all time points (Reiser et al., 1982; 1983). In contrast, we have observed marked increases in the relative amount of type I collagen in other animal models of acute pulmonary fibrosis (Reiser and Last, 1981), as well as in lungs from humans who died of acute respiratory distress syndrome (Last et al., 1983). We thus became interested in futher investigating the

nature of the "fibrosis collagen" in silicosis. Specifically, we wanted to examine the hypothesis that despite the maintenance of apparently normal collagen type ratios, the excess collagen was nevertheless different biochemically than normal lung collagen. In the present study we report on detailed studies of the Schiff base-derived crosslinks of collagen from lungs of silicotic rats and their age-matched controls.

In designing this study, we assumed that abnormalities in silicosis collagen might resemble those found in analogous disease processes. Despite the difference in target organs (skin versus lung), hypertrophic scarring resembles silicosis in certain respects. After a single insult, a sustained pathological response is initiated in which abnormal scar collagen is deposited indefinitely in the target organ. As with silicosis, the underlying mechanisms are not understood. Crosslinking in hypertrophic scar collagen has been studied by several investigators (Bailey et al., 1975; Moriguchi and Fujimoto, 1979). By analogy with these studies, we made several predictions concerning possible changes in crosslinking in silicotic "scar collagen". Based on the previous results with hypertrophic scar collagen, we hypothesized that in silicotic lung collagen we would find the following: 1. an increase in extent of lysine hydroxylation, 2. a relative increase in DHLNL[1], 3. an increase in the trifunctional crosslink hydroxypyridinium (which is derived from DHLNL). We were also interested in determining the time course of any such changes that might occur. We had no basis on which to predict whether any such putative changes would occur rapidly (paralleling the observed increase in collagen synthesis rates and collagen deposition) or if they would gradually appear in parallel with the evolution of silicotic nodules over the course of many months. In addition, we were interested in determining whether any observed changes in collagen crosslinking could be correlated with the histologic progression of the silica nodules. Since frozen lung tissue was available from our previous experiments (Reiser et al., 1982; 1983), we performed these studies on lungs that had been extensively characterized biochemically and histologically. Thus, we hoped to be able to directly correlate the present data with the detailed progression of silicotic lesions from the (early) granulomatous stage to the (late) mature silicotic nodules.

Materials and Methods

Preparation of Tissues

Lung tissue from control (saline-instilled) and silicotic rats was obtained from a previous experiment; the lungs had been stored frozen at –20 °C. Details of the experimental procedures used for inducing silicosis have been described previously (Reiser et al., 1982; 1983). Briefly, Wistar rats weighing 250–300 g were intratracheally instilled with about 50 mg of crystalline silica (quartz) with an average particle size of 0.6 ± 0.6 μm (mean ± SD), with a median particle size of 0.36 μm (range 0.003–4.8). No particles larger than 5 μm were present. The rats were killed at times ranging from 1 week to 1 year after instillation. In our earlier experiments the left lung lobe was used for determination of apparent collagen synthesis rates and of collagen type ratios. The right cranial lobe was used for histological assessment and the right accessory lobe was used for hydroxyproline determination. The right middle lobe, which was frozen immedi-

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[1] Abbreviations used: DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; OHP, hydroxypyridinium (also known as pyridinoline).

ately after the rats were killed, was used in the present experiments for analysis of collagen crosslinks.

Tissue was prepared for crosslink analysis as follows: approximately 40 mg wet weight of lung tissue was minced into fine pieces and washed overnight in 5 mM phosphate buffer containing 0.9% NaCl, pH 7.4. There was no detectable hydroxyproline extracted from the lungs by this washing procedure. We estimate the sensitivity of detection to have been such that less than 2–5% of the total lung hydroxyproline could have been solubilized at this step. The next day, the wash fluid was removed with a Pasteur pipette and the tissue was incubated in 3 ml of 0.1 M sodium phosphate, pH 7.4 for 4 hours at room temperature (about 25 °C). NaB^3H_4 (142 Ci/Mol, Amersham) was then added at a ratio of 1 part per 30 parts (dry weight) of the sample. After one hour the reduction was stopped by the addition of about 1 ml of glacial acetic acid (to pH 3–4). The tissues were then thoroughly rinsed with distilled water, hydrolyzed in 6 N HCl for 18 h at 110 °C, rotary-evaporated to remove HCl, and filtered using a Rainin (Emeryville, CA) microfiltration apparatus. Hydroxyproline content of the hydrolysates was determined by a colorimetric assay (Woessner, 1961).

Chromatography

Crosslinks were analyzed using a modification of an HPLC program described by us in detail previously (Reiser and Last, 1983). The reduced difunctional crosslinks DHLNL and HLNL, and also hydroxylysine, were analyzed by chromatography of aliquots of the lung hydrolysate containing 50 μg of hydroxyproline on a C_{18} reversed-phase column (Ultrasphere 0.4 × 25 cm; Altex, Berkeley, CA). An isocratic elution system was used; the buffer consisted of 22.5% *n*-propanol in 0.1 M phosphate buffer, pH 2.83 containing 0.3% sodium dodecyl sulfate. Flow rate was 0.8 ml/min. Amino acids and crosslinks in the effluent were visualized by their fluorescence (excitation, filter cutoff, 360 nm; emission filter cutoff, 455 nm; Gilson-Spectra-Glo, Gilson, Middleton, WI) after post-column derivatization with *o*-phthalaldehyde (Reiser and Last, 1983). In this system the difunctional crosslinks are eluted well after arginine and thus can be directly visualized even in an unfractionated lung hydrolysate. The difunctional crosslinks are completely separated from each other in this system. Fractions (1.3 ml) were collected from the fluorometer effluent every minute for determination of radioactivity by liquid scintillation counting. Samples were counted in 8 ml of Instagel (Packard, Downers Grove, IL) at an efficiency of about 33%.

For analysis of the trifunctional crosslink hydroxypyridinium (OHP), we used a slightly different solvent system that required a shorter elution time (OHP can also be determined at the same time as the difunctional crosslinks if two fluorometers are used in series; OHP elutes just after HLNL). An aliquot of lung hydrolysate containing about 5 μg of hydroxyproline was chromatographed on a 0.4 × 10 cm C_{18} reversed-phase column (Accupak Short-One, Rainin, Emeryville, CA). The elution solvent was the same as that described above except that it contained 24% *n*-propanol. A Hitachi 2000 adjustable wavelength fluorometer was used (excitation = 295 nm, emission = 395 nm) with a 12 μl flow cell to detect OHP in the eluent by its intrinsic fluorescence. A purified standard was prepared from bovine achilles tendon as described by us previously (Reiser and Last, 1983) and used for calibrating the system. The concentration of the standard was determined using the method of Eyre et al. (1984). Briefly, it was assumed that the molar extinction coefficient of OHP at 295 nm was the same as that of N-ethyl-3-pyridinol (EHP, Aldrich Chemical Co., Milwaukee, WI) in 15 mM

HCl. A standard curve was prepared from EHP. The purified OHP standard was chromatographed by HPLC as described above using UV detection at 295 nm to demonstrate that no other compounds were present that absorbed at 295 nm.

Bio-Gel P-2 Chromatography

Some hydrolysates were rotary-evaporated to remove HCl, then chromatographed on a Bio-Gel P-2 column, 0.8 × 90 cm. The elution buffer consisted of 50 mM Tris hydrochloride, pH 7.2, containing 1 M $CaCl_2$. Flow rate was 18 ml/h. Fractions were desalted on a smaller Bio-Gel P-2 column, 0.5 × 30 cm, with 10 mM acetic acid as the eluant.

Results

Difunctional Crosslinks

Samples of rat lung tissue were reduced with NaB^3H_4, washed, then hydrolyzed. About 5–10% of the hydrolysate was used for determination of hydroxyproline. The remainder of the hydrolysate was prepared for analysis by HPLC. Aliquots containing about 50 μg of hydroxyproline were used for analysis of the difunctional crosslinks. A typical chromatograph of the hydrolysate from a normal rat lung is shown in Figure 1.

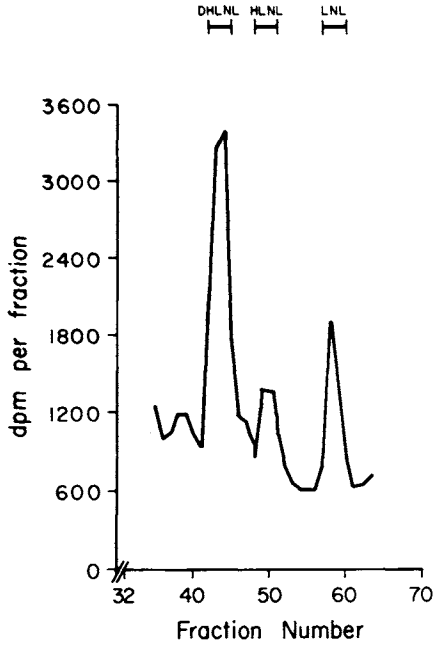

Fig. 1. Analysis of a rat lung hydrolysate by HPLC. Hydrolysate containing 50 μg of hydroxyproline was chromatographed on a C_{18} reversed-phase column using an isocratic buffer system as described in Methods. Radiographic profile of reductively labelled crosslinks. Fractions (0.8 ml of sample + 0.5 ml of OPA solution) were collected from the fluorometer at l-minute intervals and counted by liquid scintillation.

Table I. Reducible Difunctional Crosslinks and Hydroxylysine Content of Normal and Silicotic Rat Lungs. Data are presented as mean values ± 1 SD. Each sample analyzed was from the right middle lobe of an individual animal. DHLNL: dihydroxylysinonorleucine; HLNL: hydroxylysinonorleucine.

Group (n)	Months after silica instillation	Dpm per 50 μg of Hydroxyproline		Ratio, DHLNL: HLNL	Hydroxylysine content, residues per 100 residues of hydroxyproline
		DHLNL	HLNL		
Control (2)	1	5310 ± 310	1550 ± 19	3.4 ± 0.1	11.4 ± 0.4
Silica (4)		10,500 ± 3760[a]	1355 ± 633	7.7 ± 1.6[a]	17.6 ± 1.8
Control (2)	4	5500 ± 151	1761 ± 250	3.1 ± 0.5	10.0 ± 0.6
Silica (4)		7400 ± 2830	937 ± 255	7.9 ± 2.3[a]	13.8 ± 3.4
Control (2)	6	4500 ± 431	1360 ± 257	3.3 ± 0.9	9.2 ± 0.9
Silica (4)		7960 ± 2950[a]	751 ± 90[a]	10.6 ± 4.7[a]	15.9 ± 4.7
Control (2)	9	5570 ± 182	1430 ± 102	3.9 ± 0.1	11.4 ± 0.1
Silica (4)		8660 ± 1330[a]	756 ± 127[a]	11.5 ± 1.4[a]	13.5 ± 1.7

[a] Significantly different ($P < 0.05$) from control values by analysis of variance.

Figure 1 shows the radiographic profile of the reduced difunctional crosslinks. The three difunctional crosslinks, DHLNL, HLNL, and LNL, are well resolved. LNL was not quantified in this experiment as it is primarily derived (in the lung) from elastin. Accurate quantification of collagen-derived LNL requires prior fractionation of lung tissue using such techniques as extraction of collagen with hot alkali (data not shown).

We examined control rat lungs at all time points so as to allow us to correct for any age-associated changes that might potentially complicate interpretation of the results in the silicotic lungs. As shown in Table I, we found no significant differences in DHLNL or HLNL content of normal rat lungs at any of the times examined in this experiment. Control lungs contained 5220 ± 510 dpm of DHLNL per 50 μg of hydroxyproline, 1520 ± 215 dpm of HLNL per 50 μg of hydroxyproline, and a ratio of 3.4 : 1 of DHLNL : HLNL when data were pooled for all of the samples of lungs studied (n = 8 control lungs).

The silicotic lungs show an increased amount of DHLNL and decreased HLNL at all time points relative to control values. There also seems to be a trend towards increased ratios of DHLNL to HLNL at longer times after instillation of silica, due to progressively greater decreases in the apparent levels of HLNL per collagen (hydroxyproline) molecule. The variability in difunctional crosslink content among different samples (note the relatively larger coefficient of variance for silicotic samples as compared to control samples) may be in part a reflection of the proportion of silicotic nodules to normal lung tissue obtained in any given sample or of biological variability in the response of different animals to silica.

Hydroxylysine was also separated from other amino acids by HPLC and quantified by its fluorescence after post-column derivatization with OPA. Data were expressed relative to lung hydroxyproline content to normalize for the large increases in total lung collagen content between 1 and 9 months. Hydroxyproline content was determined by a colorimetric assay (Woessner, 1961). As shown in Table I, we found increased relative amounts of hydroxylysine in the silicotic lungs at all times as compared with control lungs. Hydroxylysine levels per collagen molecule do not seem to

change between one and six months in the silicotic lungs. Control lung values were constant between 1 and 9 months and averaged 0.81 ± 0.08 μmol per mg of hydroxyproline (n = 8 control lungs).

Hydroxypyridinium

Hydroxypyridinium (OHP) is a nonreducible trifunctional crosslink first identified in tendon (Fujimoto et al., 1977). We prepared standards from monkey articular cartilage, obtained from adult rhesus monkeys at necropsy (these monkeys had died of natural causes and excess tissue was available) and from bovine achilles tendon (Sigma Chemical Co., St. Louis, MO). The tissue was hydrolyzed in 6 N HCl for 24 h without prior reduction; OHP was separated from the other amino acids by Bio-Gel P-2 chromatography as described in Methods. The fractions containing OHP, as confirmed by examining the sample with a scanning fluorometer, were pooled and desalted. OHP has characteristic fluorescent properties: at acid pH the excitation maximum is 297 nm and the emission maximum is 395 nm. In neutral or alkaline solution, however, the excitation maximum increases to 325 nm, while the emission maximum remains at 395 nm (Fujimoto et al., 1977). Our purified OHP standards exhibited these properties. Fluorescence was not appreciably quenched by the buffers used in our HPLC system (data not shown). We also examined unfractionated articular cartilage hydrolysate and observed a fluorescent peak that eluted in the same position as authentic OHP, thus confirming that the OHP peak was not an artifact of the isolation procedure. In addition, the mobility of the standard OHP on Bio-Gel P-2 (Fig. 2) was consistent with the size expected of a trifunctional crosslink.

OHP from lung eluted at the same position upon HPLC as did the standard material, and had the same excitation and emission maxima as did authentic OHP. The OHP standard and OHP from lung were both destroyed by ultraviolet photolysis overnight at room temperature, determined essentially as described by Wu and Eyre (1984).

We quantified the nonreducible crosslink hydroxypyridinium by its natural fluorescence. Data, shown in Table II, are expressed as pmol of OHP per 5 μg of hydroxyproline. We measured OHP content of control rat lungs at each time point to determine

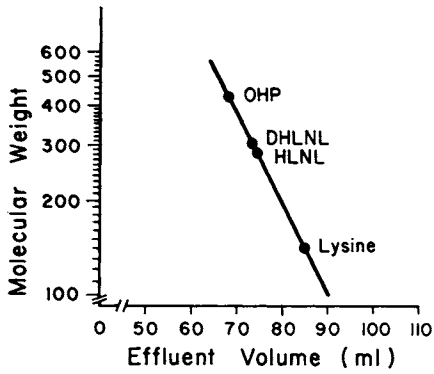

Fig. 2. Chromatography on Bio-Gel P-2 of crosslinks isolated from various tissues reduced with NaB^3H_4. Chromtography conditions are described in Methods. HLNL was isolated from adult rat tail tendon and DHLNL was isolated from rat tibia. The column was calibrated with lysine.

Table II. Hydroxypyridinium Content of Normal and Silicotic Rat Lungs. Data are presented as mean values ± 1 SD. Each sample analyzed was from the right middle lobe of an individual animal. Analysis by HPLC as illustrated in Figure 3.

Months after silica instillation	Group (n)	Hydroxypyridinium, pmol per 5 μg of hydroxyproline
1	control (4)	40 ± 3
	silica (4)	29 ± 5[a]
4	control (3)	39 ± 5
	silica (5)	47 ± 5[a]
6	control (3)	53 ± 2
	silica (5)	79 ± 8[a]
9	control (2)	53 ± 3
	silica (4)	92 ± 17[a]

[a] Significantly different ($P < 0.05$) from age-matched control values by ANOVA.

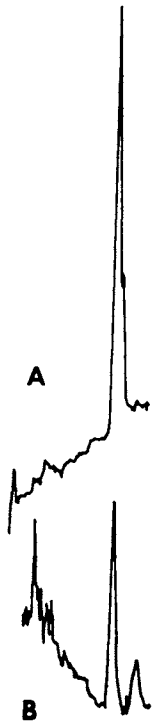

Fig. 3. Analysis of OHP content of rat lung hydrolysate. Hydrolysate containing 5 μg of hydroxyproline was chromatographed on a 0.4 × 10 cm C_{18} RP column as described in Methods. OHP was visualized by its intrinsic fluorescence (excitation = 295 nm; emission = 390 nm). A = Standard OHP; B = Lung tissue hydrolysate.

whether there were any age-associated changes. OHP values appeared to increase as the rats aged; however, more animals would have to be examined at each time point to determine whether this trend was real. We observed a biphasic response in OHP content of the collagen in silicotic lungs. At 1 month after silica instillation, OHP values (expressed per 5 μg of hydroxyproline) were significantly less than control values. At 4, 6, and 9 months after instillation OHP values were greater than controls. The highest values were observed at 9 months.

Histological Changes

As noted in our earlier studies (Reiser and Last, 1982; 1983), silica-containing granulomas could be observed as early as 1–2 weeks after silica administration. At 1 month, granulomas were scattered throughout the lung lobes; they were especially apparent around airways. Collagen deposition could be noted at the periphery of some of the granules. By 2 months distinct collagenous bands could be seen in some of the granulomas. By 4 months mature silicotic nodules could be seen; their central areas were acellular and hyalinized. The collagenous bands were thick and haphazardly woven. Over the next 5 months the central hyalinized areas gradually increased in size. At all time points, however, granulomas in varying stages of development were present, indicating a continuous process.

Discussion

In this study we have analyzed collagen crosslinks in lungs of rats that had been intratracheally instilled with silica. Lungs were analyzed at time points between 1 month and 9 months after instillation. Matched control animals were also examined at each time point to rule out the possibility that any observed changes in crosslinking were associated with normal aging of these rats. Silicotic lungs differed from control lungs in a number of respects. Lungs were larger, had a decidedly different texture, and contained more hydroxyproline (Reiser and Last, 1983). In addition, we found in the present study that the amount of the difunctional reducible crosslink DHLNL was increased at all time points. Direct visualization of the reduced crosslinks by OPA derivatization allowed us to determine specific activities of the crosslinks; we found no obvious differences in specific activity of either DHLNL or HLNL between control and silicotic lungs (data not shown) at any time point. Thus, the changes in difunctional crosslinks in silicotic lungs seen in Table I cannot be attributed to alteration in reducibility of the crosslinks, but rather must be due to changes in amounts of crosslink per collagen molecule.

Hydroxylysine content was also increased in the silicotic lungs. OHP, a nonreducible crosslink generally accepted as an example of a "mature" collagen crosslink, was also quantified. We found an initial decrease in this crosslink at 1 month, while by 4 months OHP content was greater than control values. OHP content continued to increase with time after instillation; highest values were seen at 9 months. Studies *in vitro* have suggested that biosynthesis of this crosslink is substantially slower than that of the difunctional crosslinks, occurring over weeks to months (Siegel et al., 1982). Thus, one interpretation of our data is that during the first weeks to months after silica instillation the pool of newly synthesized collagen is relatively poor in OHP content. As OHP is slowly synthesized, OHP content in the collagen begins to attain control values.

However, the silicotic collagen does not stabilize at a "normal" OHP level. Instead, "silicotic" collagen may have a larger than normal number of OHP crosslinks per molecule. Thus, as increasing amounts of "silicotic" collagen are deposited in the lungs, OHP content rises above control values. The values for OHP content shown in Table II are thus probably not values for pure "silicotic" collagen; instead, they represent the average OHP content of up to three pools of lung collagen: normal lung collagen, newly synthesized "silicotic" collagen (relatively low in OHP), and mature "silicotic" collagen (relatively high in OHP). The normal lung collagen pool may result from two pools: both the collagen present before instillation of silica, and collagen synthesized by parts of the parenchyma unaffected by the silica. Certainly histological evidence (Reiser et al., 1983) suggests that much of the lung remains relatively normal.

An important question posed at the beginning of the study was whether silicotic lung collagen could be distinguished from normal lung collagen. It appears that silicotic lung collagen has an increased level of lysine hydroxylation, an increase in DHLNL, and ultimately an increase in OHP. Since DHLNL is believed to be a precursor of OHP, it seems reasonable that some of the rapidly synthesized DHLNL is slowly converted into OHP. We examined within the limitations of tissue availability the time course of these changes. As can be seen in Table II, at least 6 to 9 months are necessary to appreciate the full spectrum of changes in crosslinking occurring in silicotic collagen. Of particular interest is the relationship between the rapid increase in DHLNL and the biphasic change in OHP. Our data, while not conclusive, are compatible with the interpretation that DHLNL is a precursor of OHP. More precise calculations of the kinetics of DHLNL conversion to OHP in lung would require techniques such as *in vivo* labelling. Such experiments, analogous to those previously reported by us in bone, tendon and skin (Reiser and Last, 1986), are presently underway; preliminary data suggest that turnover of lung collagen crosslinks is very slow (data not shown).

Our final question involved correlating the biochemical changes with histological changes. Up to 4 months after instillation of silica, the lesions in the silicotic rats consisted primarily of cellular granulomas with just the beginnings of collagenous bands. The increased content of OHP in the lung collagen correlates with the evolution of these granulomas to mature silicotic nodules containing hyalinized acellular cores.

In the only other study of which we are aware that examined collagen crosslinking in pulmonary fibrotic disorders, Seyer et al. (1981) measured DHLNL and HLNL by reductive labelling in the lungs of patients with idiopathic pulmonary fibrosis and scleroderma. The single patient with idiopathic fibrosis had essentially normal values for both crosslinks. In contrast, lungs from two patients with scleroderma showed marked decreases in both crosslinks, with a relatively greater decrease in DHLNL. OHP was not quantified in this study.

Crosslinking in tissues other than lung has been investigated in various disorders associated with derangements in collagen metabolism. Bailey et al. (1975) reported a sustained increase in the DHLNL:HLNL ratio in hypertrophic scars as compared with normal scars. More recently, Moriguchi and Fujimoto (1979) reported increased amounts of OHP in hypertrophic scar as compared with normal scars, which had barely detectable levels of OHP. Normal skin had no detectable OHP. An increased ratio of DHLNL:HLNL in other conditions involving pathological fibrosis has also been reported by Barnes et al. (1976), Brickley-Parsons et al. (1981), and Skirving et al. (1984).

In summary, we have presented new data on biochemical abnormalities in collagen metabolism in experimental silicosis. Although intratracheally instilled silica produces

detectable biochemical changes in lungs within a week, the present study suggests that the evolution of mature nodules is a multistep process with biochemically definable stages. Of particular interest is the observation that in the rat model used in this study, greater than normal amounts of the nonreducible crosslink OHP did not occur until 4–6 months after silica instillation. This observation may have clinical relevance.

We have prepared and analyzed lungs from rats instilled 1 and 2 weeks earlier with silica (cf. Table 1). There were no significant differences in any of the listed parameters at 1 week, although a trend (155% of control value) towards increased DHLNL : HLNL ratio was already apparent. The DHLNL : HLNL ratio was significantly higher (185% of control values) 2 weeks after silica administration, as was hydroxylisine content (increased by about 36%).

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