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Biochemical and immunochemical study of lysyl oxidase in experimental hepatic fibrosis in the rat

(collagen crosslinking/biosynthesis of collagen/carbon tetrachloride-induced liver injury)

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ABSTRACT **Lysyl oxidase catalyzes the crosslinking of collagen and elastin. Lysyl oxidase activity was measured and localized in rat liver during the evolution of hepatic fibrosis induced by CCl_4. Enzyme activity measured with DL-[6-^{3}H]-lysine-labeled collagen substrates in liver and plasma increased sharply after approximately 3 wk of injection, reached a maximum at 6 wk, and then decreased. The increase in activity correlated histologically with early connective tissue septa formation, and the magnitude of increase was significantly greater than that found for the intracellular collagen biosynthetic enzymes protocollagen prolyl hydroxylase and lysyl hydroxylase. Indirect immunofluorescence studies showed that lysyl oxidase was present in association with collagen in the extracellular space. However, it was not possible to correlate the distribution pattern with a particular liver cell type. These observations suggest that serial measurements of lysyl oxidase activity in liver or plasma may be useful for correlating changes in connective tissue formation with histologic connective tissue deposition.**

Lysyl oxidase catalyzes the enzymatic reaction that initiates collagen and elastin crosslinking (1, 2). In this reaction, the ϵ-amino groups of certain lysyl and hydroxylysyl residues are oxidatively deaminated to form the corresponding δ-semialdehydes (3). These then condense either with ϵ-amino groups of other lysyl or hydroxylysyl residues or with other semialdehydes to form Schiff base or aldol crosslinks (4). To date, studies of lysyl oxidase activity in experimental fibrosis have been limited to wounds and granulation tissue forming in implanted sponges (5–7). Investigations comparing the alterations in lysyl oxidase activity during experimental fibrosis to activity of other post-translational collagen biosynthetic enzymes are limited to a single study dealing with the effect of cholesterol on collagen metabolism in the chicken aorta (8). The purposes of the present study are: (*i*) to describe the changes in lysyl oxidase activity that occur during hepatic fibrosis induced by CCl_4 in the rat; (*ii*) to compare these changes with the alterations in activity of other enzymes required for collagen biosynthesis, such as protocollagen prolyl hydroxylase (proline,2-oxoglutarate dioxygenase, EC 1.14.11.2) and lysyl hydroxylase (lysine,2-oxoglutarate dioxygenase, EC 1.14.11.4); and (*iii*) to identify the tissue localization of lysyl oxidase by indirect immunofluorescence. Experimental hepatic fibrosis is a useful model for this type of comparative study because the initial collagen content of liver is quite low and large amounts relative to that initially present may be synthesized during the fibrosis caused by chronic CCl_4 intoxication (9).

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade. DL-[6-^{3}H]Lysine (specific activity 29.3 Ci/mmol), L-[4-^{3}H]proline (specific activity 26.7 Ci/mmol), and L-[4,5-^{3}H]lysine (specific activity 60.3 Ci/mmol) were obtained from New England Nuclear.

Methods. *Preparation of Substrates.* L-[4-^{3}H]Proline-labeled protocollagen for assay of protocollagen prolyl hydroxylase was prepared by the method of Hutton *et al.* (10). L-[4,5-^{3}H]Lysine-labeled protocollagen for assay of protocollagen lysyl hydroxylase was prepared by the method of Peterkofsky and DiBlasio (11). DL-[6-^{3}H]Lysine-labeled collagen substrate for lysyl oxidase assay was prepared as previously described (12).

Induction of Experimental Hepatic Fibrosis. Female Sprague–Dawley rats (100–200 g) were injected with 0.15 ml of CCl_4 mixed with 0.85 ml of mineral oil thrice weekly for the specified time. Control animals were not injected. Animals were bled by cardiac puncture and then decapitated. The liver was rapidly removed by sharp dissection and weighed. Sections were then taken for histologic examination, immunofluorescence studies, and biochemical assays.

Biochemical Studies. Weighed portions of liver were homogenized with a Polytron in 0.1 M NaCl/0.1 M glycine/0.01 M Tris·HCl, pH 8.0, (3 ml/g of tissue) (buffer A) and then centrifuged at 17,000 × *g* for 10 min. The supernatant was dialyzed overnight with two changes against buffer A for prolyl hydroxylase assay (10), 0.4 M NaCl/0.1 M glycine/0.05 mM dithiothreitol/0.02 M Tris·HCl, pH 7.4, for lysyl hydroxylase (11) assay, or 0.15 M NaCl/0.1 M Na_2HPO_4, pH 7.8, for lysyl oxidase assay (12). Aliquots of liver homogenates were taken for DNA (13), hydroxyproline (14), and protein determination (15) prior to the first centrifugation. The pellet was rehomogenized in buffer A and recentrifuged, and aliquots were again taken for dialysis and enzyme assay. After the second centrifugation, the pellet was homogenized twice in 6 M urea/0.05 Tris·HCl, pH 7.5 (2 ml/g of tissue). The supernatant after each homogenization was dialyzed overnight for assay of lysyl oxidase activity. Preliminary experiments indicated that the hydroxylase activity was almost completely extracted in the first two homogenizations and the bulk of lysyl oxidase activity was in the urea extracts. In some cases, liver samples were homogenized directly in 6 M urea without prior extractions. Measured lysyl oxidase activity was similar in both cases.

Assays. Prolyl hydroxylase activity was measured by incubating 100,000 cpm of substrate with 1 ml of dialyzed solution diluted to 2–3 mg/ml from the first two extracts for 90 min at 37° (10). The total volume of the assay mixture was 2 ml. The radioactivity in 1 ml of the product of vacuum distillation was measured with Aquasol in a Beckman liquid scintillation spectrometer (efficiency 30%). The activities measured in the first and second extracts were added together to determine total activity.

The activity of lysyl hydroxylase was measured by incubating 300,000 cpm of L-[4,5-^{3}H]lysine-labeled protocollagen substrate

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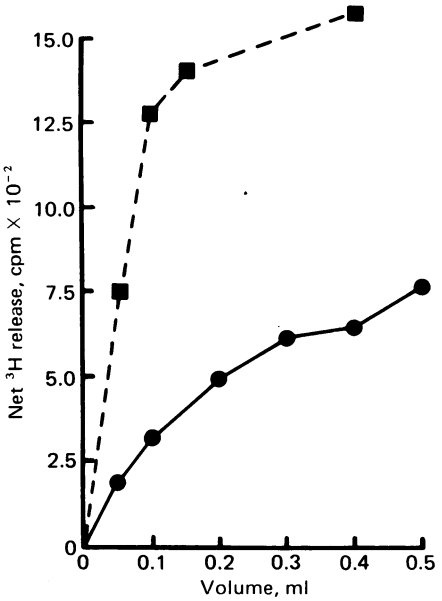

FIG. 1. Tritium release assay for lysyl oxidase activity in rat plasma and liver extracts. Samples of pooled plasma (■ - - ■) or 6 M urea extracts of liver after chronic CCl_4 injection (●—●) were incubated with 909,000 cpm of [6-^{3}H]lysine-labeled collagen fibrils for 2 hr at 37°. Samples were analyzed for activity as described in the text. Protein concentration was approximately 5 mg/ml in the liver extract and 56 mg/ml in the plasma.

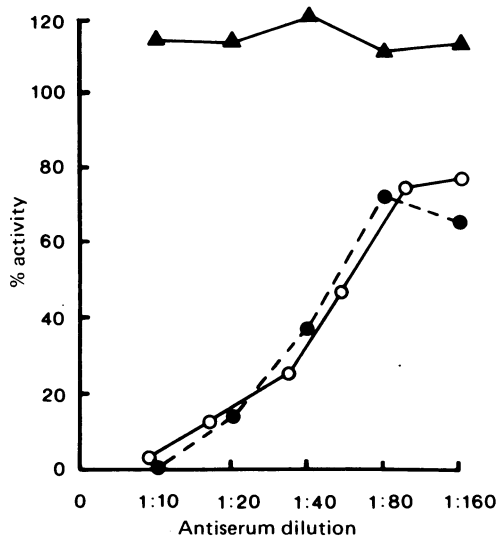

FIG. 2. Effect of rabbit antisera to chick cartilage lysyl oxidase on activity of purified lysyl oxidase from different species. The observed activity was compared to the activity without antisera. Samples of purified rat liver (○—○), chick cartilage (● - - ●), and human skin lysyl oxidase (▲—▲) were incubated with various dilutions of gamma globulin for 15 min with 100 μg of bovine serum albumin and then assayed for lysyl oxidase activity as described in the text.

(11) with 1 ml of dialyzed extract after dilution to 2–3 mg/ml at 37°. The total volume of the assay mixture was 1.5 ml and a 1-ml aliquot of the distillate was measured.

Lysyl oxidase activity was measured by a tritium release assay. For this, 1×10^6 cpm of labeled substrate was incubated with 0.5 ml of dialyzed extract or 0.1 ml of dialyzed plasma for 2 hr at 37° (12). Extracts were assayed either undiluted or after dilution to concentrations that gave values on the linear part of the assay curve (Fig. 1). L-Lysine was added to each tube to a final concentration of 10 mM. The total volume of the assay was 0.9 ml and an aliquot of 0.5 ml was measured after vacuum distillation. The total activity was obtained by combining the activities measured in all four extracts. Usually more than 60% of the total activity was present in the first urea extract. The protein concentration (15) in liver extracts was adjusted to 2–3 mg/ml before assay for lysyl oxidase to minimize artifacts due to high protein concentration. Samples were also assayed undiluted and this value was used when the diluted sample had no measurable activity. In most cases, activity was not detectable in the fractions extracted from control rats in the absence of urea. The activity in similar fractions from rats treated with CCl_4 was 25% or less of that in the urea fractions. The low activity in the fractions isolated without urea was not due to differing concentrations of lysyl oxidase inhibitors. In mixing experiments, combining liver extracts from rats treated with CCl_4 or extracts from untreated rats with chick cartilage lysyl oxidase caused similar degrees of inhibition.

Immunochemical Studies. Rabbit antisera to chick cartilage lysyl oxidase were prepared as described (16). The gamma globulin fraction was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (17). Assays for lysyl oxidase-inhibiting activity were performed by incubating various dilutions of the gamma globulin fraction of the antisera with purified lysyl oxidase preparations from chick cartilage (18), human skin (2), or rat liver‡ or with unpurified extracts from rat liver with 0.1 mg of bovine serum albumin at 37° for 15 min. The mixture was then transferred to tubes containing reconstituted fibrils of DL-[6-^{3}H]lysine-labeled collagen, incubated for 2 hr, and then analyzed for tritium release as a measure of lysyl oxidase activity (12).

Crude or partially purified‡ rat liver lysyl oxidase was studied with antisera raised in rabbits by injecting highly purified chick cartilage lysyl oxidase. Both preparations of rat liver lysyl oxidase were inhibited by rabbit antisera to chick cartilage lysyl oxidase and could be precipitated by incubation with the antisera. The inhibition of the partially purified liver enzyme by rabbit anti-chick antisera (Fig. 2) appeared to be due to formation of a specific enzyme–antibody complex because human skin lysyl oxidase was not inhibited (Fig. 2).

For morphological studies, chick embryo tissues and rat liver were fixed in 4% (wt/vol) formaldehyde in 0.08 M sodium cacodylate buffer at pH 7.2 with 7.5% sucrose and 0.5% $CaCl_2$ at 0°–4° for 24 hr and rinsed in 0.05 M cacodylate buffer at pH 7.2 with 7.5% sucrose at 0°–4° for 24 hr (19). Tissues were quenched in Freon cooled in liquid nitrogen and stored in sealed containers in liquid nitrogen. Cryostat sections were cut at 5 μm and stained by indirect fluorescence for lysyl oxidase. The gamma globulin fraction of the antiserum was used at a 1/10 dilution for 30 min at room temperature as the first layer. The second layer was a 1/10 dilution of a goat antiserum to rabbit gamma globulin (Behring Diagnostics, lot 0102E, fluorescein to protein ratio 4.0) which was also used at room temperature for 30 min. Controls were preimmunization serum as the first layer or the second layer alone.

RESULTS

In initial experiments one set of rats was treated for five consecutive weeks with CCl_4 and a second set served as controls. As shown in Table 1, liver hydroxyproline content increased more than 3-fold; DNA content did not change significantly. Per mg of DNA, prolyl hydroxylase activity rose approximately 3.5-fold and lysyl hydroxylase 1.7-fold. On the other hand, lysyl oxidase activity per mg of DNA increased approximately 30-

‡ K. H. Chen and R. C. Siegel, unpublished data.

Table 1. Collagen biosynthetic activity of liver made fibrotic by CCl_4

	Enzyme activity, cpm*			mg hydroxyproline/	
	Prolyl hydroxylase	Lysyl hydroxylase	Lysyl oxidase	liver	mg DNA/liver
Liver*					
Control (n = 4)	10,000 ± 2800	3010 ± 300	126 ± 97	1.05 ± 0.61	30.2 ± 3.8
5 wk CCl_4 (n = 7)	34,600 ± 9700†	5160 ± 1450†	3670 ± 1320†	3.41 ± 1.13†	37.0 ± 8.2
Plasma*					
Control	640		4,520		
5 wk CCl_4	1920		69,200		

Values are expressed as mean ±1 standard deviation.
* Activities are given per mg of DNA for liver samples and per rat for plasma.
† P <0.001.

fold. On histologic section, broad septa and pseudolobules were present in the CCl_4-treated animals. All of the changes in enzyme activity were statistically significant (P <0.001) by Student's t test (18). The rise in activity of prolyl hydroxylase and lysyl oxidase in pooled plasma samples from control and treated rats corresponded closely to the values in the liver. Prolyl hydroxylase increased approximately 3-fold and lysyl oxidase approximately 15-fold. The time course of the increase in lysyl oxidase activity was then determined by measuring the activity simultaneously in liver and plasma after various times of CCl_4 treatment. As shown in Fig. 3, activity in the liver began to increase sharply after 3 wk of injection, was at a peak at 6 wk, and then decreased again. The curve of activity in the plasma paralleled the liver activity closely. Histologically, fibrosis became apparent after 3 wk and large septa were present after 4 wk of treatment.

Immunofluorescent studies were performed in an attempt to localize the lysyl oxidase antigen. As shown in Fig. 4 *upper*, most intense staining was observed between hepatocytes and in the connective tissue septa. The distribution did not appear to correspond to a single hepatic cell type but was quite similar to the distribution of the reticulin stain (not shown). For comparison, a section of chick embryo tendon was also examined (Fig. 4 *lower*). Staining here also appeared to be predominantly extracellular and localized to the tendon sheaths.

DISCUSSION

Both collagen biosynthesis (9, 20, 21) and the measurable activity of certain enzymes required for collagen biosynthesis (9, 22, 23) increase during experimental hepatic fibrosis (22–25) and human cirrhosis (26, 27). The relative activity of protocollagen prolyl hydroxylase correlated with connective tissue proliferation in several studies of experimental (22–24) or human (27, 28) liver disease. Comparative investigations have shown that lysyl and prolyl hydroxylase activity increase approximately 2-fold (23, 24) while galactosyl and glucosylgalactosyl transferase increase by 1.7-fold or less. In other studies, 4- to 8-fold increases in prolyl hydroxylase activity are found (24, 26–28). However, changes in prolyl hydroxylase activity in either tissue or serum measured by tritium release assay may reflect changes in the proportion of active enzyme tetramer to inactive monomer rather than *de novo* enzyme synthesis (29). Furthermore, collagenous domains with hydroxyproline and hydroxylysine occur in proteins such as C1q (30) and acetylcholinesterase (31). Alterations in apparent activity might therefore result from either a change in the concentration of functional tetramer or an alteration in the synthetic rate of proteins other than collagen. In contrast, lysyl oxidase does not appear to require subunit aggregation for functional activity (2), and the crosslinking reaction it catalyzes is specific for collagen and elastin (4). These findings suggested that alterations in liver lysyl oxidase activity might be more specific for connective tissue deposition than changes in the other enzymes previously studied.

The biochemical data presented document that lysyl oxidase is present in both normal and fibrotic rat liver. Previous work demonstrated that plasma amine oxidase measured with benzylamine as substrate increases during hepatic fibrosis and suggested that this enzyme might catalyze collagen crosslinking in the liver (32–34). It is unlikely that the assay with collagen substrates used here detected amine oxidase rather than lysyl oxidase activity because purified amine oxidase preparations do not utilize collagen substrates (2) and 10 mM L-lysine was added to each assay as a competitive substrate for amine oxidase. In addition, rat liver lysyl oxidase was antigenically similar to chick cartilage lysyl oxidase, but antisera to amine oxidases do not crossreact with purified chick lysyl oxidase (35). The presence of lysyl oxidase activity in the liver implies that collagen crosslinking in the liver is similar to that in organs composed principally of connective tissue, such as skin and tendon.

Comparisons of lysyl oxidase activity with prolyl and lysyl hydroxylase from normal and fibrotic liver suggest that lysyl

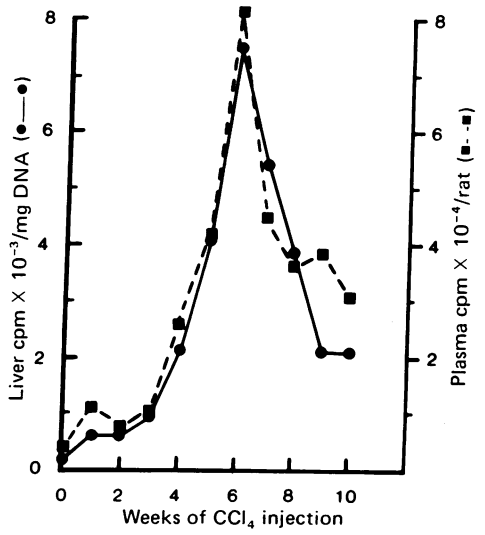

FIG. 3. Activity of lysyl oxidase in rat liver (●—●) and plasma (■ - - ■) during the evolution of CCl_4-induced hepatic fibrosis. Samples of pooled plasma (0.1 ml) and 0.5 ml of liver extract were assayed for lysyl oxidase activity as described in the text. Values obtained in the plasma were converted to total activity per rat by assuming that the total blood volume was 7% of total body weight and that plasma volume was half of blood volume.

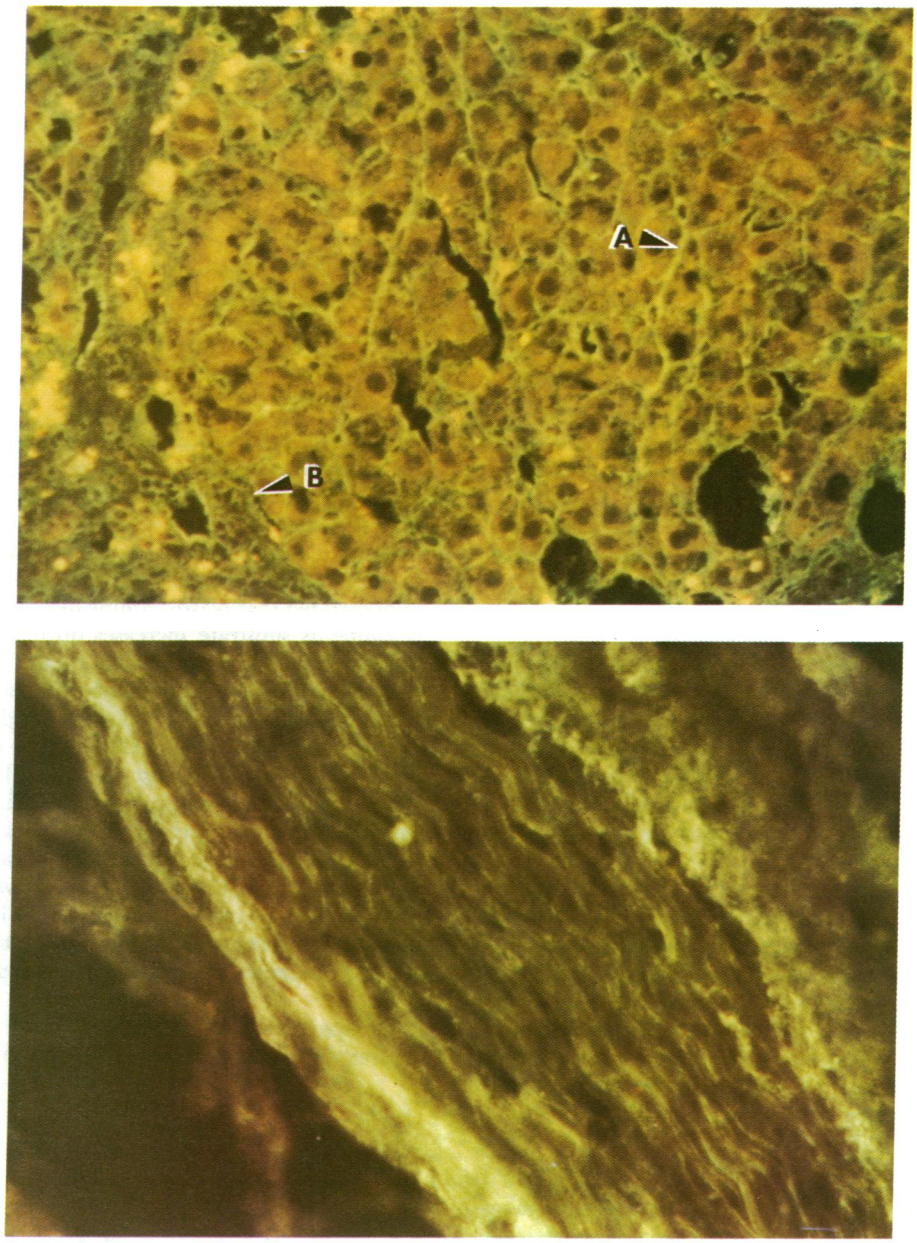

FIG. 4. (*Upper*) Immunofluorescent localization of lysyl oxidase in fibrotic rat liver. Rats were injected with CCl_4 for 6 wk prior to sacrifice. A 1:10 dilution of purified gamma globulin (2 mg/ml) containing rabbit antiserum to chick cartilage lysyl oxidase was incubated with each section. Binding was then localized by adding fluorescein-conjugated goat antiserum to rabbit gamma globulin. Staining for lysyl oxidase was found between hepatocytes (A) and extracellularly in the connective tissue septa (B). (×312.5.) (*Lower*) Immunofluorescent localization of lysyl oxidase in 17-day-old chick embryo patella tendon. Procedure as in *Upper*. Staining in the tendon sheath was also extracellular. (×312.5.)

oxidase may be an accurate and sensitive index of collagen synthesis. The observed increases in prolyl and lysyl hydroxylase activity per mg of DNA of 3.5- and 1.7-fold, respectively, were similar in magnitude to published values (22–24) but were significantly less than the 30-fold change in lysyl oxidase found in the same specimens. Moreover, assay of lysyl oxidase activity in plasma and liver specimens from rats receiving CCl_4 for various times demonstrated that measurable activity increased at similar rates in both. Prior to CCl_4 injection, little activity was detectable in either liver or plasma. With CCl_4 injection, activity reached a maximum in both liver and plasma after 6 wk and then decreased. This suggests that the liver is the principal source of the plasma enzyme under these circumstances; plasma activity is a useful guide to activity in the liver and a means of monitoring liver fibrosis.

The mechanism for the increase in lysyl oxidase activity is unknown. Because both prolyl and lysyl hydroxylase are intracellular membrane-bound enzymes (36), they may be regulated more precisely by changes in soluble cofactor concentration in their microenvironment than lysyl oxidase, which is presumably extracellular (37) and does not have known soluble cofactors (2). Alternatively, the half-life or turnover number of the hydroxylase enzymes may be significantly greater than that of lysyl oxidase. Finally, collagen synthesis and secretion occur in hepatic fibroblasts (9), cloned hepatocytes (38), and perisinusoidal lipocytes (39). If each of these cell types contains the hydroxylase enzymes, selective proliferation of a single cell type might not result in a large relative increase in enzyme activity. On the other hand, if lysyl oxidase is synthesized predominantly by a single cell type, such as the hepatic fibroblast,

that proliferates during fibrosis, relatively large increases in activity might be expected.

Earlier work demonstrated that lysyl oxidase is secreted into the media of fibroblast (37) and endothelial (40) cell cultures, is tightly bound to collagen (12, 16), and has the highest level of activity with ordered aggregates of collagen rather than with collagen monomers (16). These findings suggest that aldehyde synthesis catalyzed by lysyl oxidase occurs on collagen fibrils in the extracellular space. The immunofluorescence results described here also support this hypothesis because immunofluorescent staining was found in chick tendon and fibrotic rat liver in the extracellular space in the same distribution as collagen. On the other hand, it is possible that the localization was similar to that of collagen because the enzyme is tightly bound to collagen *in vivo* and inactivated by binding. In fact, all enzyme protein localized by immunofluorescence may have been functionally inactive. Binding may have occurred either intracellularly or during transport across the membrane after catalysis. Functional histochemical studies, currently unavailable, rather than localization experiments, may be required to define to what extent the identified enzyme antigen is active *in vivo*.

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