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# Activity and Extractability of Lysyl Oxidase and Collagen Proteins in Developing Granuloma Tissue[1,2] (38173)

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In order to control scar-tissue formation efficiently in various organs and tissues, the most promising method now known is prevention of collagenous structure polymerization (1, 2). Several studies on the effect of lathyrogens, mainly of $\beta$-aminopropionitrile (3–5) administered systemically or locally (6) and of *d*-penicillamine (7, 8), present the best evidence that controlling the synthesis of cross links is the primary approach to interference with physical characteristics of collagen.

Lysyl oxidase is the enzyme that synthesizes through oxidative deamination of some $\epsilon NH_2$ groups of lysyl or hydroxylysyl residues the aldehyde necessary to form either stable covalent cross links of an aldol-condensate type or Shiff base with reaction with the other free $\epsilon NH_2$ group in collagen polypeptides. Although several studies have been presented on the characterization of some basic properties of this enzyme in *in vitro* conditions, we present the first report on the function of lysyl oxidase in intact animals during the development of fibrotic granuloma tissue and the relation of these changes to the degree of collagen cross linking. We will show that the extractability and total activity of the enzyme significantly decreases with the age of the reactive granuloma tissue while the total amount of collagen and its fraction extractable in acid medium increases.

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[2] According to personal communications by Dr. G. Martin, Bethesda, Maryland.

*Material and Methods. Biological material.* Sixteen Sprague–Dawley male rats (200 g body weight) were each implanted with 2 polyvinylalcohol sponges, 0.7 × 0.7 × 5 cm in size, (Ivalon, Unipoint Laboratories, North Carolina) subcutaneously, paravertebrally in the back region of the neck. At different time intervals (Tables I and II), the sponges were harvested, pooled, and analyzed for the total activity of lysyl oxidase; the activity of the enzyme in extracts of phosphate, 4 *M* urea, and 8 *M* urea; the total collagen content; the fractions of collagen extractable into neutral salt and acid; and the total glycosaminoglycans content. There were 4 rats for each time period (after 10, 18, 26, and 34 days). A representative sample was studied by histological techniques. Fixed tissue was imbedded in hard paraffin. Sections that were 5 $\mu$ thick were stained by hemotoxylin and eosin, trichrome and PAS.

*Determination of extractability of lysyl oxidase.* The reactive granuloma with the polyvinylalcohol sponge was dissected and homogenized by a Polytron (Brinkmann Instruments, Westburg, New York) in a 0.1 *M* phosphate–0.16 *M* NaCl buffer, pH 7.7, in a ratio of 1:2.5 (w/v). The homogenate was centrifuged for 20 min at 12,000*g* at 4° and the supernatant filtered through glass wool to remove insoluble lipids. An equal volume of saturated ammonium sulfate was added to the filtrate and it was stirred for 60 min at 4°. The pellet obtained after centrifugation (20,000*g* for 20 min at 4°) was resuspended in the original buffer to an original volume, mixed, and





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dialyzed for 3–4 hr, first against 0.01 *M* $NaH_2PO_4$–0.016 *M* NaCl, pH 7.7, and then against a ten-fold higher molarity of the same buffer for another 16 hr at 4°. The protein content (9) and lysyl oxidase (phosphate extractable) was determined in aliquots.

The pellet left after phosphate-NaCl extraction was similarly treated with 4 *M* urea and finally with 8 *M* urea. The pellet was rehomogenized every time with urea solutions in a Polytron and was stirred for 3–4 hr at 4°. Further steps were similar to those described above for the phosphate-extractable fractions of lysyl oxidase were obtained. The residual pellet was also assayed for the activity of lysyl oxidase.

*Preparation of the substrate for lysyl oxidase.* We followed the procedure by Pinnell and Martin (10) with several modifications. Aortas from 17-day-old chick embryos were incubated with (6-$H^3$) DL-lysine (spec. act. 30.3 Ci/mmole, New England Nuclear Corp.) in the presence of β-aminopropionitrile (Aldrich Chemicals) for 24 hr at 37°. Samples (2.5 mg) were weighed and homogenized by hand in small, conical glass centrifuge tubes in 1 ml of 0.5 *N* hydrochloric acid. After being spun at 12,000*g* for 15 min, the pellet was washed twice with a 0.1 *M* phosphate–0.16 *M* NaCl buffer, pH 7.7, and finally resuspended in 0.5 ml of the same buffer.

*Assay of lysyl oxidase activity.* One ml of enzyme extract (see above) with 1 drop of toluene was incubated in stoppered test tubes with 0.5 ml of substrate for 4 hr at 37° under slight shaking. The reaction was stopped by freezing the sample. Determination of the released tritium by distillation was carried out as described by Pinnell and Martin (10).

The total activity of lysyl oxidase represents the sum of activities present in individual extracts, whether related to weight unit of the total protein in extracts or to the mg of collagen in the sponge. The activities in either extract are presented as percent of total activity.

*Determination of total collagen content and neutral- and acid-extractable fractions.* An aliquot of sponge granuloma tissue was extracted repeatedly with 0.3 *M* hot trichloroacetic acid (11). In the dialyzed and hydrolyzed sample (6NHCl at 105° for 16 hr), the content of collagen hydroxyproline was measured by an automated Technicon Autoanalyzer procedure. Another aliquot was homogenized in liquid nitrogen and extracted three times for a total of 48 hr at 4° under continuous shaking in 0.45 *M* NaCl and then in 0.5 *M* acetic acid. Neutral-salt and acetic-acid extracts were extracted with hot trichloroacetic acid and further processed for hydroxyproline determination as indicated above.

*Total glycosaminoglycans content.* This was determined by a uronic acid automated procedure in a Technicon Autoanalyzer (12).

*Statistics.* All data are presented by mean ± S.E. The Student's *t*-test was used to evaluate the statistical significance.

*Results. Morphologic characteristics* of granuloma tissue formed as a result of inflammatory reaction to subcutaneously implanted porous Ivalon sponges and the resulting deposition of collagenous structures within the sponge are shown in Fig. 1–4. These sponges were isolated 10, 18, 26, and 34 days after implantation. The 10 day sponge (Fig. 1) shows the formation of a thin collagenous fibrous capsule and the infiltration of the inflammatory cells which permeate only the surface layer of the sponge. As time progressed, 18 days, Fig. 2, the deeper invasion of cells into the porous sponge increased and collagen fibers and glycosoaminoglycans filled the interstices of the sponge. At 26 days (Fig. 3), the density of cells in the capsule decreased and more collagen was deposited both around and inside the sponge. Finally, at 34 days (Fig. 4), the capsule contained solid collagen bundles as a result of the fibrogenesis. Over this period of time, the highly cellular granuloma tissue within the sponge, was gradually replaced by collagenous structures.

*The lysyl oxidase total activity* fell progressively from 19,000 DPM/mg of protein in 10 day-old tissues to 10,000 DPM/mg of protein in 34 day-old granuloma (Table I) with significant differences between individual following sampling periods. The

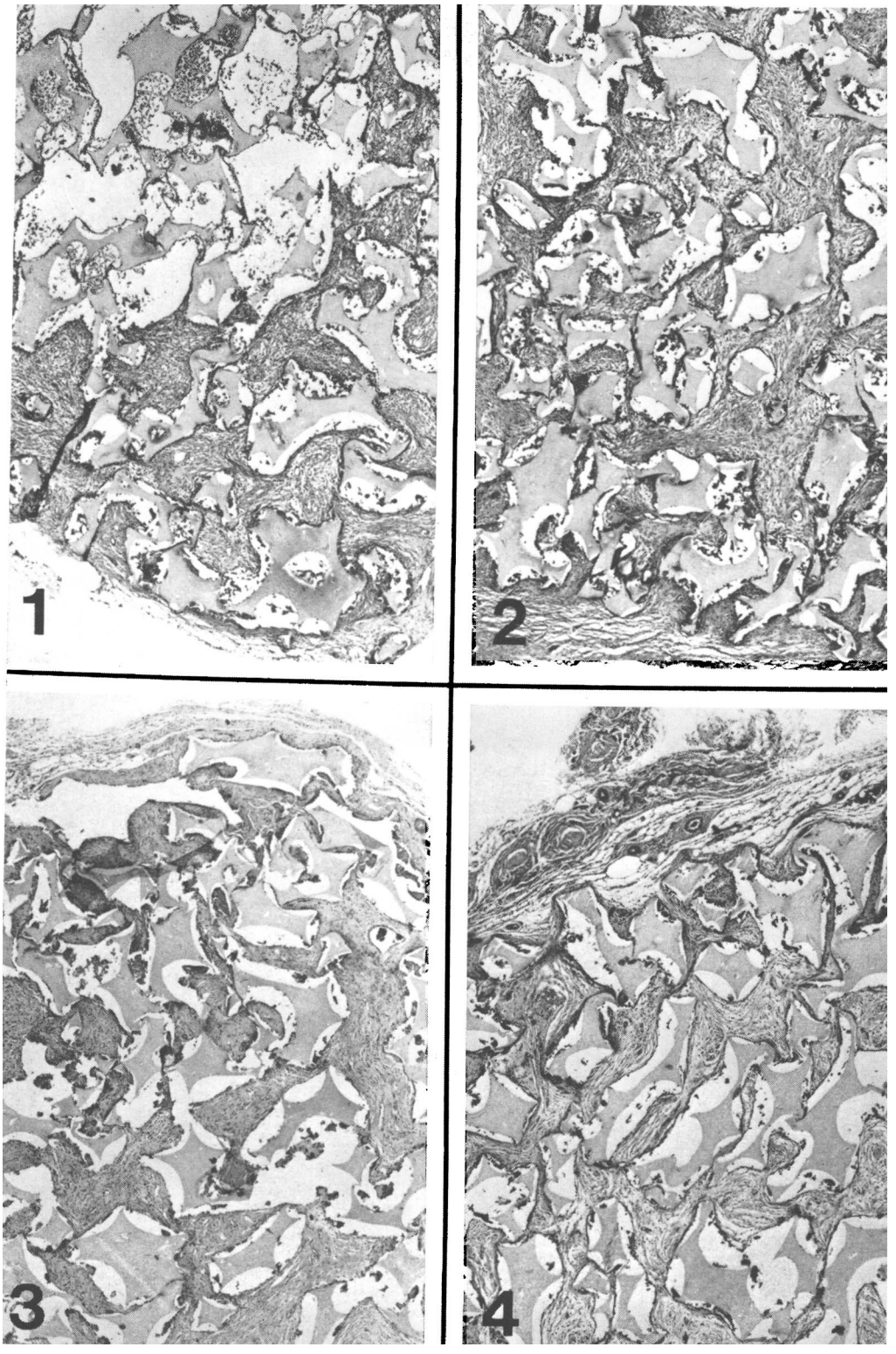
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TABLE I. Activity and Extractability of Lysyl Oxidase in Developing Granuloma Tissue.

| Group[a] (days) | Lysyl oxidase—total[b] $10^2 \times$ DPM/ mg protein | Lysyl oxidase—total[b] $10^2 \times$ DPM/ mg collagen | Fraction of lysyl oxidase[b] (in % of total activity) Phosphate | 4 *M* urea | 8 *M* urea |
|---|---|---|---|---|---|
| 10 | 186 ± 4 | 242 ± 12 | 40.3 ± 1.6 | 38.6 ± 2.2 | 20.9 ± 2.5 |
| 18 | 158 ± 5*** | 125 ± 5*** | 46.4 ± 1.7*** | 32.6 ± 0.9* | 20.5 ± 2.3 |
| 26 | 127 ± 7** | 84 ± 5*** | 45.5 ± 2.7 | 32.7 ± 1.7 | 21.7 ± 1.4 |
| 34 | 104 ± 8* | 66 ± 5** | 60.8 ± 2.0*** | 8.7 ± 4.4*** | 28.6 ± 3.2 |

[a] Four rats in each group.

[b] Number of asterisks refer to significance between individual subsequent groups, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Variability is given by mean ± SE.

continuous decrease of the activity with the age of the granuloma tissue was even more prominent when the activity of lysyl oxidase was compared to the content of collagen present in the sample (Table I, column 2).

Concerning *extractability of lysyl oxidase,* there was significantly less lysyl oxidase extractable into phosphate buffer in 10 day-old granuloma than in the tissue from the following sampling period. Also, significantly more enzyme was released into the phosphate medium from the oldest granuloma tissue analyzed (34 days). The extractability into 4 *M* urea followed the reverse pattern: more active enzyme was extracted from young granuloma tissue than from the older samples (Table I). The portion of enzyme activity extracted into 8 *M* urea was the same in tissue from all sampling periods, averaging 22% of the total lysyl oxidase activity. No detectable activity of lysyl oxidase was found in the pellet left after the extraction with 8 *M* urea.

*Collagen amount and collagen extractability data* are presented in Table II. A significant increase in total collagen was found between 10–18-day-old tissue and 18–26-day-old tissue. Collagen extractable into neutral salt medium (NSC) formed 2.5% of total collagen in 10-day-old tissue and decreased to 1.2% in 34-day-old granuloma. There was only 7.4% collagen extractable into acid medium (ASC) in 10-day-old tissue, the content increasing to 20.5% of the total collagen in 34-day-old samples.

The content of uronic acid of glycosaminoglycans was the same in 10–26-day-old tissue and was significantly higher only in the 34 day-old sample (Table II).

*Discussion.* The dynamics of developing reactive granuloma tissue formed around the implanted porous Ivalon sponges represents a model of fibroproliferative inflammation. The early cellular stages are characterized with a higher activity of a cross-linking enzyme which is firmly associated with the insoluble pellet, possibly with its collagenous substrate, and therefore it is not dissociated by a phosphate buffer. The extraction with 4 *M* urea is needed to separate most of the enzyme from the pellet; still almost 20%

FIGS. 1–4. Represent 20-fold magnifications of trichrome stains of reactive granuloma tissue formed around and in a subcutaneously implanted polyvinyl-alcohol porous sponge. Figure 1 shows a highly cellular reaction 10 days after implantation with still incomplete permeation of sponge interior. The amorphous homogenous structures represent the septa of the sponge. A thin cellular layer has formed around the implant. In Fig. 2, cells permeate the whole sponge at 18 days. Figure 3 shows that fine collagenous structures have become confluent, and a solid fibrous capsule formed around the sponge 26 days after the implantation. In Fig. 4, at 34 days, continuous dense collagenous structures occupy most of the span of the sponge with a thick fibrous capsule formed around the implant. At this stage, the relative paucity of cells was obvious.

TABLE II. Collagen Protein and Glycosaminoglycan Content in Developing Granuloma Tissue.

| Group[a] (days) | Total collagen (Hyp μg/g) | NSC[b] (% total) | ASC[c] (% total) | Uronic acid (μmoles/g) |
|---|---|---|---|---|
| 10 | 770 ± 59 | 2.5 ± 0.27 | 7.4 ± 0.24 | 0.67 ± 0.087 |
| 18 | 1262 ± 46[d]*** | 2.1 ± 0.03 | 14.3 ± 0.70*** | 0.56 ± 0.052 |
| 26 | 1510 ± 79* | 1.8 ± 0.12* | 20.1 ± 1.00*** | 0.66 ± 0.024 |
| 34 | 1581 ± 76 | 1.2 ± 0.11** | 20.5 ± 0.76 | 0.92 ± 0.055*** |

[a] Four rats in each group.
[b] Collagen extractable into 0.45 *M* NaCl.
[c] Collagen extractable into 0.5 *M* acetic acid.
[d] Number of asterisks refers to significant differences with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ between 2 sampling-period groups.

of enzyme activity is not extractable into 4 *M* urea medium. At early stages of the development of granuloma tissue, less collagen is present; but this collagen is more soluble in neutral salt extraction (2.5%). Surprisingly, the content of collagens extractable into acid medium is at this time period significantly less than in samples from older tissue. The meaning of this finding is difficult to interpret. It is assumed that into neutral salt medium, only collagen subunits, $\alpha,\beta$ chains, as well as tropocollagen or procollagen molecules will be extracted. The acid medium should also contain collagens aggregated by unreduced Schiff-base cross links which dissociate under a mild acid environment. The high activity of lysyl oxidase less dissociable from the pellet on one hand and the higher content of NSC with significantly less ASC indicates that the enzyme is bound to the tissue components, possibly forming the complex with the substrate. But not enough cross links have been formed yet at this time period (10 days). It may well be that in young loose connective tissue (10 days), the enzyme is inactive and is activated only by experimental processing (homogenization, extraction procedures). This is shown by a higher content of NSC fraction and paucity of non-reducable cross links. A small portion of collagen extractable into 0.45 NaCl in this young, newly formed tissue indicates that there are obviously other mechanisms which make the collagen structure not dissociate in this milieu.

The only existing information about lysyl oxidase activity in animal tissues is an abstract of a lecture by Riley and Martin (13). They studied the activity of lysyl oxidase in skin-wound granulation tissue and found that lysyl oxidase levels were low in the early postwounding period, but from 8 to 28 days activity in wounds exceeded activity in unwounded skin.

The results of this paper point for the

TABLE III. Extractibility of Lysyl Oxidase in Various Tissues.

| Tissue characteristics | Activity in % of total | | |
|---|---|---|---|
| | 0.1 *M* phosphate[b] | 8 *M* urea[b] | Pellet |
| Sternum, 17 days chicken embryo | 37 | 53 | 9 |
| Calvaria, 17 days chicken embryo[c] | 5–15 | — | — |
| Skin, 1 day old rat | 29 | 23 | 48 |
| Skin, 5 month old rat | 25 | 60 | 15 |
| Granuloma tissue,[a] 10 days old | 40.3 ± 1.6 | 59.7 ± 2.3 | 0 |
| 34 days old | 60.8 ± 2.0 | 37.3 ± 3.9 | 1.8 ± 0.2 |

[a] See Table I for details, variability is given by mean ± S.E.
[b] See Methods.
[c] Refers to analysis of three independent samples.

first time to the fact that lysyl oxidase in granuloma tissue is not easily extractable into neutral salt medium. In our studies on physical properties of lysyl oxidase and its substrate (14), we isolated this enzyme from various tissues. As shown in Table III, the extractability varies not only with the kind of tissue, but mainly with the age of the biological structure. Embryonal tissues, such as calvaria, contain a minimal activity of the enzyme extractable into phosphate; in tissues from older animals, the extractability increases.

The comparison of the histology of the granuloma tissue with the total activity of lysyl oxidase at different sampling periods would suggest that the high activity of the enzyme goes together with the size of the population of fibroblasts in the sample.

In the assay system, we used the biosynthetically labeled substrate with the assumption that only lysyl oxidase and not other monoaminooxidases can deaminate it. The substrate is, however, poorly defined and the specificity of this assay may be questionable. The only evidence that we assayed the real lysyl oxidase is our finding (6) that local injection of $\beta$-aminopropionitrile into the implanted sponge inhibits almost all the activity of the enzyme. It has been shown by Pinnell and Martin (10) that $\beta$-aminopropionitrile inhibits only lysyl oxidase and not other monoaminooxidases.

*Summary.* The total activity of lysyl oxidase and its extractability into phosphate and 4 *M* and 8 *M* urea solutions were studied in granuloma tissue formed around subcutaneously implanted polyvinylalcohol sponges in rats and harvested on days 10, 18, 26, and 34. Furthermore, total collagen content and fractions of collagen extractable into 0.45 *M* NaCl (NSC) and 0.5 *M* acetic acid (ASC) were studied. Lysyl oxidase activity was assayed by measuring the release of tritium from a substrate biosynthetically labeled with $H^3$-6-Lysine. Biochemical data were compared to the histology of the tissue.

With the maturation of the granuloma tissue, the total activity of lysyl oxidase significantly decreases, and its extractability into phosphate significantly increases. Extractability into 4 *M* urea was the highest in 10-day-old granuloma and the lowest in 34-day-old tissue. Only 80% of the total activity was extracted into 4 *M* urea medium. Total collagen content and ASC fraction increased continuously, and NSC decreased from 2.5% to 1.2% in 34-day-old tissue.

We conclude that the highest activity of lysyl oxidase in young granuloma is related to a high proportion of fibroblasts and that the enzyme at this time is tightly bound to the pellet, possibly to the collagen substrate.

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