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Cross-Linking of Collagen and Elastin. Properties of Lysyl Oxidase*

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ABSTRACT: Lysyl oxidase catalyzes the formation of the lysine-derived aldehyde, allysine. This is the first step in the cross-linking reaction of collagen and elastin. The present study describes the partial purification and some of the properties of this enzyme.

The specific activity of the enzyme extracted from chick embryo cartilage was increased 440-fold by $(NH_4)_2SO_4$ precipitation, acid precipitation, and gel filtration. Its molecular weight was approximately 170,000 as judged by gel filtration. Elevated oxygen tension was found to stimulate activity. A metal cofactor is required for activity since dialysis against solutions with α,α-dipyridyl abolished activity. Cupric ion restored full enzymatic activity and cobaltous and ferrous ions restored some activity. It is likely that copper is the naturally occurring cofactor since enzyme activity was absent in extracts from copper-deficient chicks. The lathyrogen β-aminopropionitrile acts as an irreversible inhibitor of lysyl oxidase *in vitro* and *in vivo*.

Lysyl oxidase converts specific lysyl residues in collagen and elastin into the corresponding δ-semialdehyde, allysine[1] (Pinnell and Martin, 1968; Martin *et al.*, 1970; Siegel and Martin, 1970a). These aldehyde residues then condense, probably by a spontaneous reaction, to form covalent cross-links (Gross, 1969; Deshmukh and Nimni, 1969; Schiffmann and Martin, 1970). When this normal biosynthetic process is impaired in either experimental lathyrism (Martin *et al.*, 1961) or copper deficiency (Miller *et al.*, 1965; Shields *et al.*, 1962; O'Dell *et al.*, 1961; Kimball *et al.*, 1964), the collagen and elastin from these animals have increased numbers of lysyl residues and decreased amounts of allysine (Piez, 1968; Chou *et al.*, 1969).

Lysyl oxidase activity was initially detected in extracts of embryonic chick bone (Pinnell and Martin, 1968). In one assay elastin biosynthetically labeled with [6-^3H]lysine was prepared as a substrate. As allysine was formed during the incubation with the lysyl oxidase preparation, tritium was released from the substrate, isolated by distillation, and used as a measure of the reaction. In a second assay, elastin biosynthetically labeled with [^{14}C]lysine was used as a substrate. The allysine formed during incubation with lysyl oxidase was subsequently converted by oxidation into α-aminoadipic acid. The radioactivity in this compound served as a measure of the enzymatic reaction. With these assays it was possible to demonstrate that lysyl oxidase activity was inhibited by low levels of the lathyrogen (BAPN)[2] (Pinnell and Martin, 1968). This compound inhibits the cross-linking of collagen and elastin *in vivo*.

In a separate study (Siegel and Martin, 1970a), the action of lysyl oxidase on collagen was examined. Enzyme catalyzed formation of allysine was observed in both the α1 and α2 chains. In addition, the formation of the β_{12} component of collagen, a dimer composed of an α1 and α2 chain covalently linked, was shown to occur. The formation of allysine and of the cross-linked dimer was inhibited by BAPN.

The purpose of the present paper is to report on the partial purification and further characterization of lysyl oxidase. The enzyme has been purified 440-fold and found to have a tightly bound metal cofactor. The relationship between experimental lathyrism or copper deficiency and lysyl oxidase activity has also been studied.

Materials and Methods

Preparation of Enzyme from Chick Embryos. The 105,000*g* supernatant fluid obtained by homogenizing the cartilaginous ends of the tibiae and femora from 6 dozen 17-day-old chick embryos (approximately 25 g of tissue) in 50 ml of 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, was prepared as described previously (Pinnell and Martin, 1968). A saturated solution of $(NH_4)_2SO_4$ adjusted to pH 7.5 at 4° with concentrated NH_4OH was added to the fluid to give a final concentration of $(NH_4)_2SO_4$ of 277 g/l. or 45% saturation. The precipitate that formed was collected by centrifugation at 17,300*g* for 10 min. This pellet was suspended in 8–10 ml of 0.01 M NaH_2PO_4–0.015 M NaCl which had been adjusted to pH 7.7 with NaOH and

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[1] The trivial name used is: allysine, α-aminoadipic acid δ-semialdehyde.

[2] The abbreviation used is: BAPN, β-aminopropionitrile fumarate.

dialyzed against the same solution for 4–6 hr with two solution changes. After dialysis the precipitate was removed by centrifugation at 27,000*g* for 10 min and discarded. The supernatant fluid was used for the experiments described below except as noted. Before each experiment, the salt concentration of this and all other enzyme preparations was adjusted to 0.35 M by addition of 1.75 M NaCl. Lysyl oxidase activity was maximal at this salt concentration.

In some experiments, the precipitate resulting from the initial $(NH_4)_2SO_4$ precipitation was resuspended in 8–10 ml of 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, containing 176 g/l. of $(NH_4)_2SO_4$ (30% saturation). This suspension was stirred for 10 min in the cold in order to allow proteins soluble at this $(NH_4)_2SO_4$ concentration to dissolve. The suspension was then centrifuged at 17,300*g* for 10 min and the pellet was discarded. The supernatant fluid was dialyzed against 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, for 4–6 hr with two solution changes.

After dialysis, the pH of the solution was adjusted to 5.0 with 1 N HCl and the resulting precipitate was collected by centrifugation at 17,300*g* for 10 min. The precipitate was then suspended in 1 ml of 0.15 M NaCl–0.1 M NaH_2PO_4 adjusted with NaOH to pH 7.7 and dialyzed overnight against 1 l. of this same solution.

Molecular Sieve Chromatography. In some cases, the enzyme preparation obtained after the acid precipitation step described above was purified further by molecular sieve chromatography. The acid precipitate dissolved in 1 ml was applied to a 112 × 1.5 cm column of 10% agarose (Bio-Gel A 0.5) equilibrated with 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, and maintained at 4°. The column was eluted with the same buffer at 12 ml/hr flow rate. The effluent was monitored continuously at 280 mμ and the absorbance recorded with a Sargent linear-log recorder. The column effluent was collected in either 3- or 6-ml fractions. Aliquots (1 ml) from each tube were assayed for lysyl oxidase activity with the [^3H]elastin substrate described below.

Preparation of Enzyme Extract from Lathyritic Chick Embryos. BAPN (20 mg) in 0.5 ml of sterile water was injected into the yolk sac of embryonated eggs 15.5 days old. The eggs were incubated for an additional 36 hr. The embryos were removed, the cartilaginous portion of the tibiae and femora were dissected, and the initial extract was prepared as described above. The 105,000*g* supernatant obtained was dialyzed extensively against 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, in order to remove any free BAPN. After the second solution change, aliquots of bone extracts prepared from normal 17-day chick embryos were dialyzed in the same beaker but in a separate dialysis sac from the extract prepared from the lathyritic chicks. This was done as an additional check to determine whether BAPN in the dialysate was present at a level that would not inhibit enzyme activity. The $(NH_4)_2SO_4$ precipitation step and dialysis of the precipitate against 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, was performed as described above in the purification of the enzyme from normal chick tissue.

Preparation of Enzyme from Copper-Deficient Chicks. For the study of enzyme activity in copper-deficient chicks, 1-day-old chicks were raised for periods of up to 3 weeks on a copper-deficient diet as previously described (Miller *et al.*, 1965). In the case of the controls this diet was supplemented with copper. The chicks were sacrificed at various times and the cartilaginous ends of the tibial and femoral bones were dissected free from surrounding tissue. Extracts of these bones were prepared by a procedure identical with that described above for the extract from normal embryonic bones.

Removal and Readdition of Copper and Other Cations. To determine the effect of copper and other cations on enzyme activity, the enzyme preparation from normal embryonic chick bone that had been purified by $(NH_4)_2SO_4$ precipitation and dialysis against 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, was dialyzed overnight in the cold against 1 l. of 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, containing 2.5×10^{-3} M α,α-dipyridyl, a metal chelator. The α,α-dipyridyl was removed by a subsequent overnight dialysis against 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, with several solution changes. The pH of the enzyme preparation was adjusted to 5.0 in order to decrease the formation of sparingly soluble metal hydroxides upon addition of cations. $CuCl_2$ was then added to final concentrations of 10^{-3} M, 5×10^{-4} M, or 10^{-4} M. The pH was readjusted to 7.7 prior to assay. All of the other cations, Fe^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Zi^{2+}, Ni^{2+}, Ca^{2+}, and Mg^{2+}, were added as their chloride salts to a final concentration of 5×10^{-4} M. They were also added at pH 5.0 as described for the addition of $CuCl_2$ and the pH of the preparation was readjusted to 7.7 prior to assay.

Preparation of Labeled Elastin Substrates. Eighteen aortas obtained from 17-day-old chick embryos were incubated for 24 hr in 10 ml of Eagle's minimal essential medium minus lysine and glutamine. BAPN (50 μg/ml), ascorbic acid (50 μg/ml), and penicillin G (2000 units/ml) were added as supplements to the media. In addition, at the start of the incubation, the media was supplemented with 200 μCi of either [6-^3H]-D,L-lysine or [4,5-^3H]-D,L-lysine. After the incubation, the aortas were rinsed with distilled water and lyophilized. The lyophilized aortas were stored at 4° until used.

Incubation Mixtures and Assays. The lyophilized aortas were homogenized at 4° in a ground glass homogenizer with 1 ml of 0.15 M NaCl per aorta. The insoluble material was collected by centrifugation at 17,300*g* for 10 min. The pellet was resuspended in 0.15 M NaCl by homogenization and reisolated by centrifugation. This pellet was then suspended in sufficient 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, buffer to ensure that each 0.5-ml aliquot contained approximately 600,000 cpm. These aliquots were then pipetted directly into each incubation tube. The enzyme preparation was then added to the incubation tubes. The final volume of each tube was adjusted to 1.5 ml by addition of 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, buffer. Toluene (0.05 ml) was added to each tube to inhibit bacterial growth. BAPN was added to certain tubes either before or during the incubation to a final concentration of 50 μg/ml. Incubations were performed at 37° for varying times. Certain samples were gassed for 10 min with either N_2 or O_2 at 2 l./min before incubation. To determine the effect of pH on enzyme activity, the pH of some incubation mixtures was adjusted with either 1 N HCl or NaOH to a pH other than 7.7. All tubes were tightly closed with rubber stoppers. At the end of the incubation, the reaction was terminated either by freezing or by adding BAPN to a final concentration of 50 μg/ml. Both of these procedures inhibited enzyme-dependent tritium release. The tritium released from the protein was isolated by distillation as described previously (Pinnell and Martin, 1968). The tritium was then measured as tritium water by standard counting procedures with toluene counting fluid with 8 g/l. of 2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-

TABLE I: Tritium Release from Elastin Substrate Labeled with [4,5-^3H]Lysine or [6-^3H]Lysine.[a]

	[4,5-^3H]-Lysine (cpm)	[6-^3H]Lysine (cpm)
Buffer	260	200
Buffer + BAPN (50 μg/ml)	158	107
Enzyme (1 ml)	1021	1150
Enzyme (1 ml) + BAPN	110	90

[a] Samples were incubated for 4 hr at 37° with elastin labeled with either [4,5-^3H]lysine or [6-^3H]lysine. Each incubation tube contained aliquots of substrate with approximately 600,000 cpm of ^3H. Release of tritiated water from the substrate was measured.

TABLE II: Purification of Lysyl Oxidase.[a]

	Total Protein (mg)	Specific Activity[b]	Total Units[c]	% Recovery
Initial extract	540	144.8	783.0	100
45% $(NH_4)_2SO_4$ precipitate	57	762.7	434.0	55.4
30% $(NH_4)_2SO_4$ supernatant	23.0	1,356.0	312.0	49.8
Acid precipitate	3.0	8,160	245.0	31.3
Gel filtration peak tube	0.28	64,500	181.0	23.1

[a] Incubations were performed for 4 hr at 37° with 600,000 cpm of ^3H per incubation tube present as [6-^3H]lysine-labeled elastin. Aliquots following each purification procedure were assayed to determine the total enzyme activity at that stage of purification. [b] The specific activity was defined as the cpm of tritium water formed per mg of protein in 4 hr. [c] One unit of enzyme activity is defined as that amount of enzyme that catalyzes the formation of 100 cpm of tritiated water per 4-hr incubation.

1,3,4-oxadiazole and 0.5 g/l. of 2-(4′-biphenyl)-6-phenylbenzoxazole with 15% by volume of Beckman Bio-Solv BBS-3. A Packard Model 3375 liquid scintillation spectrometer was used to measure the tritium.

The protein content of the various enzyme preparations was estimated by a spectrophotometric method (Warburg and Christian, 1941).

Materials. BAPN fumarate was obtained from Aldrich Chemicals. [6-^3H]-D,L-lysine, specific activity 7900 mCi/mmole, and [4,5-^3H]-D,L-lysine, specific activity 11,600 mCi/mmole, were obtained from New England Nuclear Corp.

Results

The tritium-release assay for lysyl oxidase depends upon the release of tritium ions from lysine-labeled substrate as lysyl residues are converted into allysine. In previous studies [6-^3H]lysine was used to label the substrate. If aldehyde formation were the principal reaction contributing to tritium release, then elastin with lysine labeled with tritium at the 5 position should be as good a substrate as the [6-^3H]lysine-labeled elastin. As soon as aldehyde forms at position 6, tritium at position 5 should become readily exchangeable (Bell and Smith, 1958). Indeed, the lysyl oxidase preparation catalyzed similar increases in tritiated water from each of these substrates (Table I). In both cases enzyme activity was inhibited by BAPN. Since [4-5-^3H]-D,L-lysine can be prepared with higher specific activity than [6-^3H]-D,L-lysine, this compound may be used when a more sensitive assay is required.

A previous study (Pinnell and Martin, 1968) demonstrated that the formation of tritiated water is linear for approximately 6 hr when the lysyl oxidase preparation is incubated with labeled elastin substrate. In addition, the formation of tritium water is proportional to the amount of lysyl oxidase present (Martin *et al.*, 1970). (Similar results are shown as part of Figures 3 and 4.) These observations permit the definition of a unit of enzyme activity. A unit of enzyme activity is defined as the amount of enzyme activity causing the formation of 100 cpm of tritium water during 4 hr in the standard incubation mixture. Because of variations among different preparations of labeled aortas from which the substrate is prepared, comparisons can be best made using the same preparation of substrate.

Table II illustrates the recovery of activity after various steps in the purification of lysyl oxidase. In these experiments, a single preparation of substrate was used. $(NH_4)_2SO_4$ (45% vol/vol) precipitated enzyme activity. This precipitate was collected by centrifugation, resuspended in a dilute phosphate buffer, and dialyzed. This step was of use in purification because of the large amount of collagen in the original preparation. Under these conditions any collagen in the preparation remained insoluble and was removed by centrifugation. Enzyme prepared through this stage retained full activity for periods up to 1 week.

A significant purification was achieved by the subsequent steps listed in Table II. A single peak of enzyme activity was obtained by molecular sieve chromatography (Figure 1). The peak of enzyme activity coincided with the trailing edge of the major absorbing peak. The molecular weight for a globular protein eluting at the position of maximum enzyme activity was approximately 170,000. This was estimated by the usual techniques of molecular weight determination by gel filtration (Andrews, 1967) with proteins of known molecular weight as standards. Enzyme purified by acid precipitation and molecular sieve chromatography was unstable. Approximately half of the activity was lost upon standing at 4° for 24 hr. For this reason, the studies reported below were carried out with enzyme that had been purified only through the first $(NH_4)_2SO_4$ step. Attempts to preserve enzyme activity by the use of sulfhydryl compounds such as dithiothreitol and mercaptoethanol were unsuccessful. These compounds and sulfhydryl reagents such as *p*-mercuribenzoate and 1-fluoro-2,4-dinitrobenzene inhibited lysyl oxidase activity when added to incubations at 10^{-4} to 10^{-5} M.

Figure 2 illustrates the influence of pH on enzyme activity.

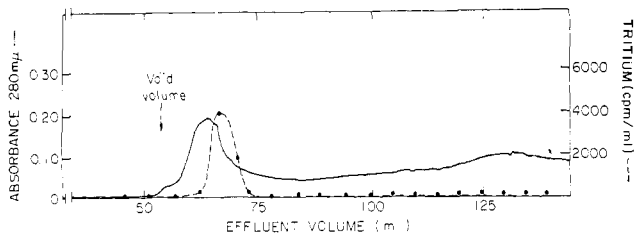

FIGURE 1: Molecular sieve chromatography (Bio-Gel A 0.5, 112 × 1.5 cm column) of a lysyl oxidase preparation that had been purified by fractional $(NH_4)_2SO_4$ precipitation and acid precipitation. Protein concentration is represented by the solid line and enzymatic activity by the dashed line. Enzyme activity was measured by incubation of 1-ml aliquots from each fraction with elastin labeled with [6-^3H]lysine and measuring the production of tritiated water.

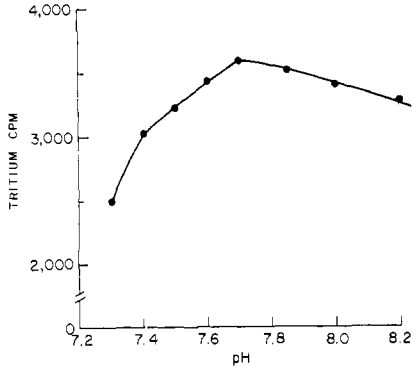

FIGURE 2: Effect of pH on lysyl oxidase activity. Samples of substrate plus a sixfold-purified lysyl oxidase preparation were incubated for 4 hr at 37°. The pH of the samples was adjusted to the values indicated in the figure just prior to incubation.

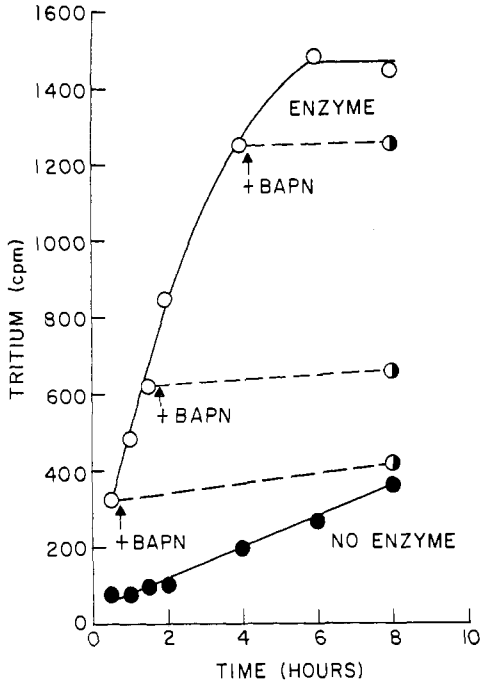

FIGURE 3: Activity of sixfold-purified lysyl oxidase preparation with increasing incubation time. Enzyme activity was measured by incubation of the lysyl oxidase preparation with elastin labeled with [6-^3H]lysine and measuring the production of tritiated H_2O. The closed circles represent samples of substrate plus buffer incubated for the times indicated. The open circles indicate the tritium release resulting from incubation of substrate plus a lysyl oxidase preparation. At the arrows BAPN (50 μg/ml) was added to one set of samples which were then incubated for the remainder of the 8 hr (half-closed circles connected by dotted line to the open circles). The open circle next to the arrow represents the ^3H released from a set of samples that were frozen at the time BAPN was added to the other set.

Maximum activity was observed between pH 7.6 and 7.8. Little activity was observed outside the range pH 7.2–8.4. For these reasons, the incubations were routinely performed at pH 7.7.

In previous experiments (Pinnell and Martin, 1968) the reaction between lysyl oxidase and substrate was terminated by freezing the incubation mixture, since little or no reaction occurs at low temperature. However, a more specific method of terminating the tritium release due to allysine formation seemed desirable. Since BAPN inhibits lysyl oxidase (Pinnell and Martin, 1968; Martin *et al.*, 1970), BAPN was added at various times during the course of the reaction to determine whether it would stop the reaction. Figure 3 demonstrates that it was possible to inhibit almost all further formation of tritiated water by adding BAPN (50 μg/ml) to the incubation tubes.

Since lysyl oxidase is inhibited by low levels of BAPN *in vitro* (Pinnell and Martin, 1968), it has been postulated that the basic defect in experimental lathyrism is the inhibition of lysyl oxidase by BAPN *in vivo*. To test this hypothesis, the level of lysyl oxidase activity in enzyme preparations from lathyritic and normal chick embryos was compared. The embryos that received BAPN were grossly abnormal with marked tissue fragility, soft bones, flexion contractures, and joint hemorrhages. No lysyl oxidase activity could be demonstrated in the extracts from lathyritic chicks in either the initial preparation after extensive dialysis to remove free BAPN or after partial purification by $(NH_4)_2SO_4$ precipitation (Figure 4; only the partially purified preparation is illustrated). When either the initial or the partially purified preparation from the lathyritic chick embryos was added to a similar preparation from control embryos (Figure 4), enzyme activity decreased only by that amount obtained by dilution with an equal proportion of buffer.

When animals are raised on a copper-deficient diet, they develop many lesions similar to those found in lathyritic animals (O'Dell *et al.*, 1961; Kimball *et al.*, 1964; Shields *et al.*, 1962) and they have an increased amount of soluble collagen as compared with normal animals (Chou *et al.*, 1969). To determine whether copper was a cofactor necessary for lysyl oxidase activity, epiphyseal cartilage extracts from chicks raised on either a copper-deficient or normal diet were prepared. There was no lysyl oxidase activity in the extracts from the copper-deficient chicks (Figure 5) although there was significant activity in the control preparation (Figure 5). There was no change in the observed activity in the control preparation when it was incubated in the presence of the copper-deficient chick preparation beyond that expected from the dilution (Figure 5). The absolute levels of lysyl oxidase

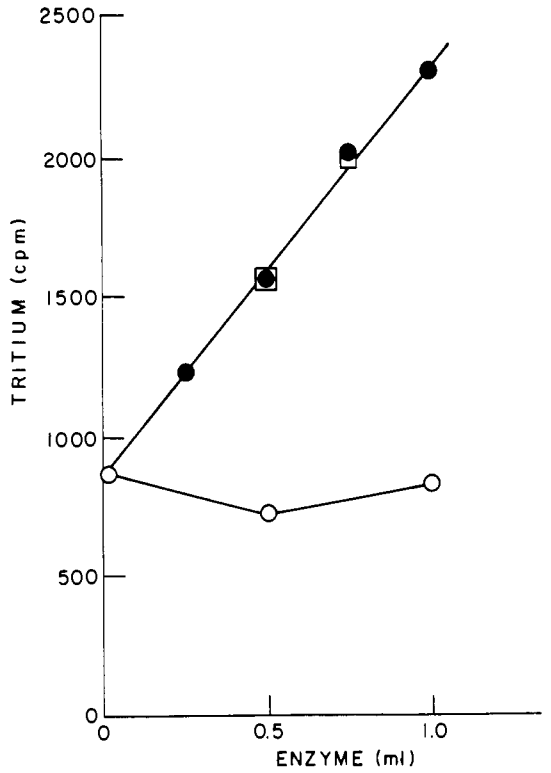

FIGURE 4: Lysyl oxidase activity in an enzyme preparation from lathyritic chick embryos. Various amounts of the enzyme preparation after $(NH_4)_2SO_4$ precipitation from lathyritic chick embryos (open circles) and normal chick embryos (closed circles) were incubated with substrate for 4 hr at 37°. Normal enzyme (0.5 ml) was mixed with 0.5 ml of lathyritic enzyme (open square above 0.5 ml) and 0.75 ml of normal enzyme was mixed with 0.25 ml of lathyritic enzyme preparation (open square above 0.75 ml) and assayed.

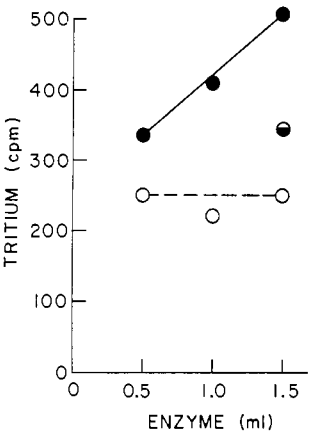

FIGURE 5: Comparison of lysyl oxidase activity in the original enzyme preparation from normal and copper-deficient, three-week-old chicks. The open circles indicate lysyl oxidase activity in copper-deficient chick epiphyseal cartilage as measured by the elastin tritium release assay. The closed circles indicate lysyl oxidase activity in normal chick epiphyseal cartilage. The half-closed circle represents lysyl oxidase activity observed when equal amounts of normal and copper-deficient enzyme preparations were mixed.

activity were less in this experiment since 3-week-old chicks rather than 17-day-old chick embryos were used.[3]

The effect of copper on lysyl oxidase activity was further evaluated by dialyzing the sixfold purified enzyme preparation from normal chick embryos against the metal chelator α,α-dipyridyl. After dialysis no lysyl oxidase activity was observed (Table III). However, addition of copper in increasing amounts to the apoenzyme resulted in restoration of full activity (Table III). Ferrous and cobaltous ions were also able to restore some lysyl oxidase activity (Table III). However, activity was not as great at an equivalent concentration of these cations as it was with cupric ion.

Little is known about the mechanism of action of lysyl oxidase. One possibility is that lysyl oxidase is an oxygenase (Hayaishi, 1963). Determining the source of oxygen in allysine would establish this point (Hayaishi, 1963, 1969). However, it has not yet been possible to obtain sufficient allysine formation to allow the required ^{18}O experiment to be done. To investigate in a preliminary way the role of oxygen in the reaction, incubation tubes were gassed briefly (10 min) with oxygen or nitrogen. The effect of these treatments is shown in Table IV. The yield of tritiated water was considerably greater in those samples gassed with oxygen than in those gassed with nitrogen. BAPN prevented the increased production of tritiated water. Gassing with nitrogen reduced but did not abolish enzyme activity. However, it is doubtful that oxygen would be completely removed by this treatment. These experiments indicate that molecular oxygen is involved in allysine formation.

[3] We have observed that lysyl oxidase activity in chick cartilage reaches a peak in the 17-day embryo. In the three-week-old chick, enzyme activity was approximately 10% of this level.

Discussion

The present study is an extension of the description of the enzymatic properties of lysyl oxidase orginally reported from this laboratory (Pinnell and Martin, 1968). The purification outlined above resulted in approximately a 440-fold increase in specific activity over the original preparation. Highly purified lysyl oxidase preparations were unstable and attempts to prevent rapid loss of enzymatic activity were unsuccessful. For this reason, the sixfold-purified preparation was used in the majority of these studies as well as when collagen rather than elastin was used as a substrate (Siegel and Martin, 1970a). Assessing the relative specific activity of the various enzyme preparations toward collagen or elastin substrates is difficult since much of the allysine in collagen is consumed by the formation of covalent cross-links. All of the more highly purified preparations utilized collagen as a substrate as well. However, even the most highly purified preparations contained more than one protein since the final purification step only separated proteins by size. Maximum enzyme activity was found in the trailing edge of the major protein peak. Therefore the question of whether a single enzyme catalyzes allysine formation in both collagen and elastin is unanswered.

The $(NH_4)_2SO_4$ precipitation step and subsequent dialysis against a low phosphate buffer used to prepare the stable enzyme resulted in removal of most of the collagen present in the 105S preparation. This step also proved useful for concentrating crude enzyme preparations of low specific activity. Although the chick cartilage preparation was used exclusively

TABLE III: Addition of Cu^{2+} and Other Cations to Lysyl Oxidase Apoenzyme.[a]

Substrate +	Net Tritium Release (cpm)	Activity (%)
Control enzyme	1210.0	100
Apoenzyme	28.0	2.3
Apoenzyme + 10^{-4} M $CuCl_2$	905	74.5
Apoenzyme + 5×10^{-4} M $CuCl_2$	1060	87.5
Apoenzyme + 10^{-3} M $CuCl_2$	1200	99.0
Substrate + apoenzyme +		
$FeCl_2$[b]	813	67.2
$FeCl_3$	0	0
$CoCl_2$	692	57.2
$MnCl_2$	50.0	4.0
$CdCl_2$	41.5	3.4
$ZnCl_2$	11.8	1.0
$NiCl_2$	0	0
$CaCl_2$	0	0
$MgCl_2$	0	0

[a] The apoenzyme preparation was prepared by dialyzing the sixfold-purified enzyme against a solution of 0.01 M NaH_2PO_4–0.015 M NaCl, pH 7.7, containing 2.5×10^{-3} M α,α-dipyridyl. All incubations were performed for 4 hr at 37° with 600,000 cpm of elastin labeled with [6-^3H]lysine. [b] All metal salts were added to a final concentration of 5×10^{-4} M.

in this study, lysyl oxidase activity has been detected in extracts of bone from rats, guinea pigs, fetal calves, and fetal pigs (Siegel and Martin, 1970a,b). In several of these studies, the initial extract contained very low levels of enzyme activity. However, adequate levels of lysyl oxidase activity were obtained after the $(NH_4)_2SO_4$ step.

Several investigators have suggested that there is a similarity between the connective tissue disorders observed in experimental lathyrism and copper deficiency (Shields *et al.*, 1962; Miller *et al.*, 1965; Piez, 1968). The present study provides additional evidence that the connective tissue lesions arise by the same mechanism, namely, inhibition of the lysine to aldehyde step that initiates cross-linking. In neither case was there demonstrable lysyl oxidase activity in epiphyseal cartilage extracts. Previous studies have shown that lysyl oxidase is inactivated following incubation with BAPN *in vitro* (Pinnell and Martin, 1968). In the lathyritic extracts, the absence of enzymatic activity was probably also due to irreversible inhibition by BAPN. Preliminary studies with radioactive BAPN indicates that it binds to lysyl oxidase and remains bound despite extensive dialysis (A. S. Narayanan, R. C. Siegel, and G. R. Martin, unpublished data).

The lack of enzymatic activity in the copper-deficient preparations was probably due to the fact that lysyl oxidase is a metalloenzyme that requires copper for activity. The experiments utilizing the metal chelator α,α-dipyridyl are additional evidence that a metal ion is essential for lysyl oxidase activity. After dialysis of lysyl oxidase against α,α-dipyridyl, full activity was recovered by addition of copper. Two other cations in the first transition period, ferrous and cobaltous ion, also restored some lysyl oxidase activity. Substitution of other cations with restoration of enzymatic activity is a well recognized phenomenon with other metalloenzymes such as carboxypeptidase A (Folk and Gladner, 1960; Vallee *et al.*, 1960; Coleman and Vallee, 1960). However, it is uncertain whether metalloenzymes with these other cations bound to the apoenzyme occur naturally (Vallee and Riordan, 1968). In the case of lysyl oxidase, the experiments with copper-deficient chicks and with α,α-dipyridyl indicate that the copper form of the metalloenzyme is the one that exists *in vivo*.

TABLE IV: Effect of Gas Phase on Lysyl Oxidase Activity.[a]

Substrate +	Air (cpm)	Nitrogen (cpm)	Oxygen (cpm)
Buffer	478	402	803
Buffer + BAPN[b]	240	235	250
Enzyme	2350	2052	3128
Enzyme + BAPN	220	210	230

[a] All samples were incubated for 4 hr at 37°. Each incubation contained 600,000 cpm [^3H]elastin labeled with [6-^3H]lysine. Samples were gassed with either 100% oxygen, nitrogen, or air for 10 min just prior to incubation. [b] BAPN at a concentration of 50 μg/ml.

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Inhibition of Ribonucleoside Diphosphate Reductase by 1-Formylisoquinoline Thiosemicarbazone and Related Compounds*

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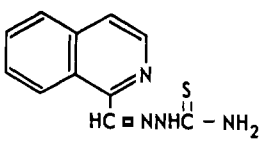
ABSTRACT: Interference with the biosynthesis of DNA by 1-formylisoquinoline thiosemicarbazone (IQ-1) was shown to be due to inhibition of ribonucleoside diphosphate reductase. The molecular mechanism of the inhibition by IQ-1 was investigated with an enzyme from a rat tumor, purified approximately 20-fold over the 100,000*g* supernatant fraction. The concentration of the nucleoside diphosphate substrate, the allosteric activator ATP, or magnesium ion did not influence the inhibition by IQ-1. Dithioerythritol or dithiothreitol, model dithiols used in place of the natural substrate thioredoxin (dithiol form), were partially competitive with the inhibitor. Although IQ-1 has a great affinity for ferrous ion, the inhibition of the enzyme was not reversed, and in certain conditions was enhanced, by increasing the concentration of this cation. The findings are compatible with a model in which either IQ-1 binds to an iron-charged enzyme or the iron chelate of IQ-1 interacts with the enzyme at the site occupied by the dithiol substrate.

A number of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones that form coordination compounds with iron, cobalt, nickel, copper, zinc, and manganese have been shown to be potent inhibitors of the growth of a variety of transplanted rodent neoplasms (Sartorelli and Creasey, 1969). Isoquinoline derivatives are among the most effective tumor inhibitors in this class, one of the most potent agents being 1-formylisoquinoline thiosemicarbazone (IQ-1)[1] (structure I) (Sartorelli *et al.*, 1968). The precise biochemical site

I



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[1] The following abbreviations are used: [^{32}P]CDP, [α-^{32}P]cytidine diphosphate; DTE, dithioerythritol; DTT, dithiothreitol; IQ-1, 1-formylisoquinoline thiosemicarbazone.