Impairment of Collagen and Elastin Crosslinking by an Amine Oxidase Inhibitor[1] (34948)

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Collagen and elastin are crosslinked by a family of compounds derived from α-aminoadipic-Δ-semialdehyde as it exists in peptide linkage (1–4). This aldehyde arises from the enzymically catalyzed oxidation of the epsilon carbon of peptidyl lysine. The enzyme involved is an amine oxidase (5–8) which appears to be both copper and pyridoxal dependent. Similar impairment of crosslink formation results from copper deficiency (7, 9, 10) and pyridoxine deficiency in chicks (11, 12). Additional evidence that a pyridoxal-dependent amine oxidase is involved in crosslink formation is presented here. Iproniazid, an amine oxidase inhibitor (13), decreased intermolecular bonding of tendon collagen when fed to chicks, and reduced lysine oxidation in elastin when added to organ cultures of embryonic aortas. Inhibition of aortic amine oxidase by iproniazid was competitively reversed by pyridoxal phosphate.

Materials and Methods. The control diet was based on nonfat milk solids and was supplemented with adequate copper (9). This diet and a similar diet supplemented with 0.1% iproniazid (IPN) were fed to two groups of day-old broiler strain chicks.[4] Food intake of control chicks was limited to the quantity consumed by the group fed IPN. At 3 weeks, the aortas and Achilles tendons were removed and frozen until analyzed. Tendons were homogenized and extracted successively with 0.15 *M* NaCl, 1.0 *M* NaCl, and 0.5 *M* acetic acid at 3° (7). Each extract and the residue were analyzed for hydroxyproline after hydrolysis in 6 *N* HCl at 110° for 24 hr (14). Aortas were extracted with acetone and ether and dried *in vacuo*. The percentage of insoluble elastin was determined by 0.1 *N* NaOH extraction as previously described (9).

The *in vitro* effect of IPN was observed in tissue culture. Aortas from 14-day-old chick embryos were removed aseptically and cultured in roller tubes containing Puck's medium (15) from which lysine was omitted except for 0.5 μCi [U-^{14}C]-lysine (270 μCi/μmole) per ml of solution. One-half of the tubes contained 5 m*M* IPN. The cultures were aerated (95% O_2; 5% CO_2) and incubated at 41°. After 24 hr, the medium containing [U-^{14}C]-lysine was removed and a complete medium (0.52 m*M* lysine) without radioactive label was added. After an additional 48 hr, the aortas were collected, and the insoluble elastin was isolated by treatment with 0.1 *N* NaOH. The isolated elastin was hydrolyzed with 6 *N* HCl for 24 hr at 110° and the hydrolyzate chromatographed on an amino acid analyzer coupled in series with a scintillation counter equipped with an anthracene flow cell (16). Radioactivity incorporated into the desmosines and other crosslinking compounds was correlated with ninhydrin peaks.

Amine oxidase activity was determined by a spectrophotometric method (17), using benzylamine as the substrate and adapted as previously described (6). Finely homogen-

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TABLE I. Effect of Dietary Iproniazid on the Distribution of Collagen in Chick Tendon.

Fraction or solvent	Collagen in mg/g fresh tissue[b,c] Basal diet	Basal + 0.1% IPN	Stat. sig.[a] $p <$
0.15 *M* NaCl	17.6 ± 2.1	16.9 ± 1.0	NS
1.0 *M* NaCl	20.1 ± 1.2	40.3 ± 1.7	0.001
0.5 *M* HAc	43.1 ± 4.4	70.3 ± 7.1	0.02
Insoluble	146.4 ± 3.7	66.7 ± 5.1	0.001
Total	227.2 ± 4.9	194.2 ± 7.0	0.01

[a] p values as determined by t tests were less than indicated.

[b] Mean of four determinations ± SEM.

[c] Expressed as milligrams hydroxyproline/gram tissue × 7.35, assuming a hydroxyproline content of 13.6% for collagen.

ized suspensions of aorta, liver, and heart were used as sources of the enzyme. In the *in vitro* inhibition assays, IPN was incubated with the other components of the system at least 10 min before the addition of the substrate. When pyridoxal phosphate was added, it was preincubated for 30 min before addition of benzylamine.

Results and Discussion. Although iproniazid slightly decreased the total collagen content of tendon, the most striking effect was upon the solubility of the collagen (Table I). Sixty-six per cent of the tendon collagen of chicks fed IPN was extracted by successive solvent treatments, whereas only 36% was extracted from controls. The increased solubility of collagen in 1 *M* NaCl and 0.5 *M* acetic acid is usually interpreted as decreased intermolecular crosslinking.

The effect of IPN on crosslinking in elastin was not so great as that in collagen, but there was a significant ($p < .05$) decrease in the concentration of elastin in aortas. Insoluble elastin represented 47.4 ± 1.3 % of the dry weight of aortas from IPN-fed chicks compared to 52.2 ± 1.7% for controls. Since the iproniazid-fed and pair-fed control chicks weighed approximately the same, 111 and 115 g, respectively, the effects of IPN on collagen and elastin maturation appear to be true metabolic effects.

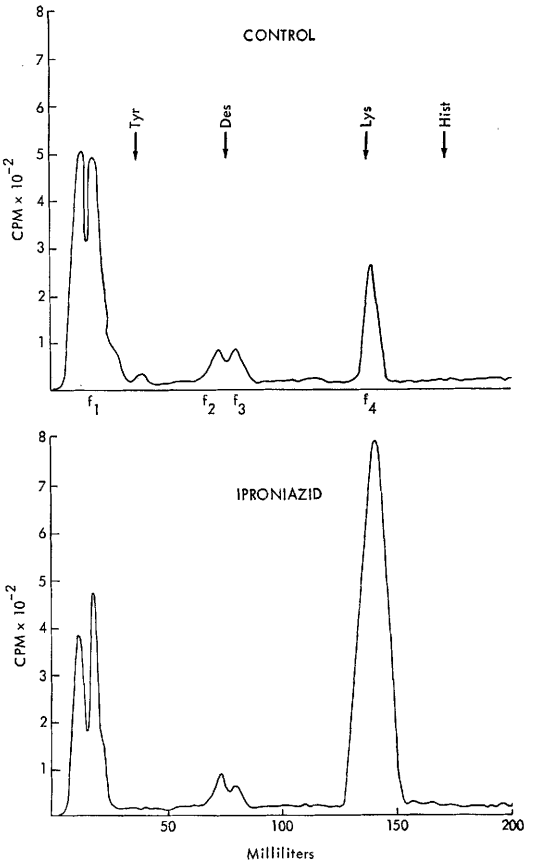

FIG. 1. Distribution of labeled compounds from elastin hydrolyzates. The hydrolyzates were chromatographed on the short column of a Beckman 116 amino acid analyzer. Temperature and buffer (citrate) were maintained at 33° and pH 4.25 for the initial 140 ml of effluent and then increased to 55° and pH 5.35. The flow rate was 50 ml/hr.

The chromatographic distribution of ^{14}C-labeled compounds in the elastic hydrolyzates obtained from aorta cultures shows that iproniazid impairs the formation of elastin crosslinking compounds (Fig. 1). The radioactive counts were divided into four fractions corresponding to neutral–acidic residues (f_1), isodesmosine (f_2), desmosine (f_3), and lysine (f_4). The counts in f_1 were assumed to be due to compounds that arose from oxidation of peptidyl lysine including their condensation and hydrolysis products (1–4).

Seventy-five per cent of the counts in the elastin hydrolyzates from control cultures were present in compounds other than [U-

TABLE II. Effect of Iproniazid on the Incorporation of Radioactivity from [U-^{14}C]-Lysine into Fractions Obtained from the Elastin of Aortas Grown in Culture.

Fraction[a]	Treatment[b]	Proportion of total counts[c] (%)	Radioactivity in fractions f/lysine[c]
f_1 (Neutral–acidic residues)	Control	57.6	2.34
	Iproniazid	24.6	0.38
f_2 (Isodesmosine)	Control	8.5	0.35
	Iproniazid	7.5	0.12
f_3 (Desmosine)	Control	8.5	0.35
	Iproniazid	4.0	0.06
f_4 (Lysine)	Control	25.4	1.00
	Iproniazid	63.9	1.00

[a] Fractions are designated in Fig. 1.
[b] Iproniazid was present in cultures at 5 m*M*.
[c] Representative data from three experiments.

^{14}C]-lysine compared to 36% in hydrolyzates from IPN-treated cultures (Table II). When these data are expressed as counts per minute per milligram of insoluble elastin, 268 and 125 cpm were present in the f_1 fractions of control and IPN-treated aortas, respectively, compared to 115 and 325 cpm in the respective lysine fractions. Iproniazid blocks the initial oxidation step and decreases the proportion of the subsequent condensation products such as the desmosines.

In copper deficiency, benzylamine oxidase activity of connective tissues correlates well with the degree of maturation of the collagen and elastin in these tissues (7). Activity is not affected in tissues such as liver, which contain only small amounts of connective tissue protein and an abundance of an amine oxidase that requires flavin (6, 7). Since IPN inhibits both flavin- and pyridoxal-dependent amine oxidases (18), it is significant that the addition of pyridoxal phosphate *in vitro* greatly enhanced the amine oxidase activity of aorta, but not of liver, in the presence of IPN whether added *in vitro* or as it occurs in tissue from IPN-treated chicks (Table III).

Addition of pyridoxal phosphate *in vitro* increased amine oxidase activity in aorta by a factor of 1.67 in control chicks and 18.5 in IPN-fed chicks. The activity in heart, which contains some connective tissue, was increased 1.1- and 4.5-fold in the control and IPN-fed groups, respectively. However, liver amine oxidase was only slightly affected.

The gross effect of IPN *in vivo* appears to be similar to lathyrogenic agents (19, 20), but it may be unique with respect to site of action. Whereas aorta amine oxidase was inhibited by IPN, the inhibition was time dependent and could be reduced competitively by the addition of pyridoxal phosphate (Fig. 2). These results provide further evidence that a pyridoxal phosphate requiring enzyme with the properties of an amine

TABLE III. Effect of Pyridoxal Phosphate *in Vitro* on Aorta, Heart, and Liver Amine Oxidase Activities.

Tissue	Dietary treatment	Oxidase activity[a] benzaldehyde/hr /mg N moles × 10^8	
		Pyridoxal phosphate	
		None	1 m*M*
Aorta (2)[b]	Control	0.52	0.87
	0.1% IPN	0.02	0.37
Heart (5)	Control	6.21	6.86
	0.1% IPN	0.19	0.86
Liver (4)	Control	1.49	1.81
	0.1% IPN	0.20	0.29

[a] Benzylamine was present in assays at 3.33 m*M*.
[b] Number of determinations shown in parentheses.

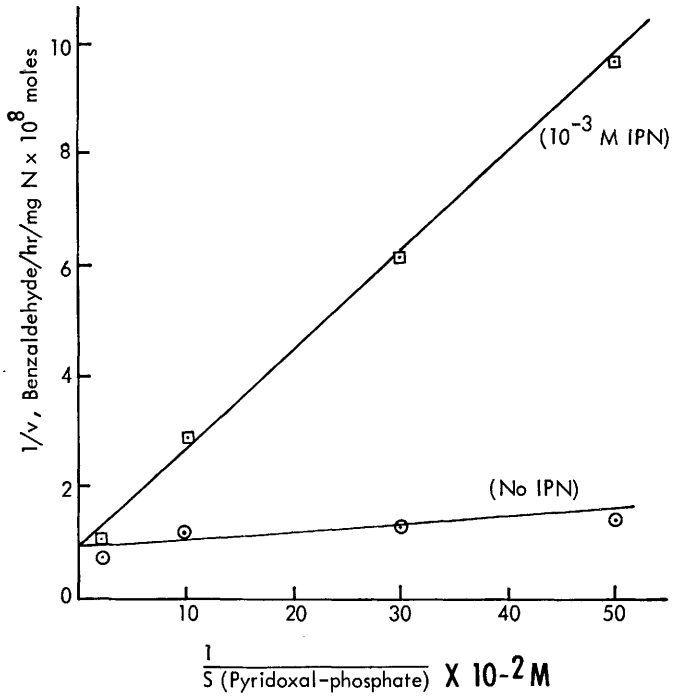

FIG. 2. Competitive effect of pyridoxal phosphate with respect to iproniazid inhibition of aorta amine oxidase. Finely homogenized aortas (20 mg/3 ml) were preincubated at 37° in the presence of iproniazid except for controls. After 10 min, pyridoxal phosphate was added as indicated. The homogenates were further incubated for 30 min before the addition of benzylamine at 3.33 m*M*. Velocity is expressed as moles of benzaldehyde/hr/mg N × 10^8.

oxidase is important in connective tissue crosslinking.

Summary. Tendons of chicks fed 0.1% iproniazid (IPN) contained twice as much 1 *M* NaCl- and 0.5 *M* acetic acid-extractable collagen as tendons from pair-fed controls. The concentration of elastin in aortas was reduced. Iproniazid fed to chicks inhibited the amine oxidase activity in homogenates of liver, heart, and aorta. Addition of pyridoxal phosphate to homogenates from both control and IPN-fed chicks enhanced the activity of amine oxidase in aorta to a greater extent than that of other tissues. Iproniazid decreased the oxidation of peptidyl lysine in elastin isolated from aortas grown in organ culture. *In vitro* inhibition of the aortic enzyme by IPN was competitively reversed by pyridoxal phosphate. These data support the hypothesis that an amine oxidase found in connective tissue and requiring pyridoxal phosphate is an important enzyme in the maturation of collagen and elastin.

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