The inhibition of lysyl oxidase *in vivo* by isoniazid and its reversal by pyridoxal

Effect on collagen cross-linking in the chick embryo

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Isonicotinic acid hydrazide (isoniazid) causes a large increase in the salt-solubility of collagen when injected into chick embryos; this change is accompanied by the inactivation of lysyl oxidase (EC 1.4.3.13), the enzyme responsible for initiating cross-link formation in collagen and elastin. In addition, isoniazid markedly decreases the liver content of pyridoxal phosphate. The depletion of pyridoxal phosphate takes approx. 6h, whereas the inhibition of lysyl oxidase and the increase in collagen solubility occur more slowly. A reversal of these effects of isoniazid can be produced by the subsequent injection of a stoichiometric amount of pyridoxal, supporting the role of pyridoxal as a cofactor for lysyl oxidase. Treatment of chick embryos with β-aminopropionitrile, an irreversible inhibitor of lysyl oxidase, causes an inhibition of the enzyme, which begins to recover within 24h but which is not affected by the administration of pyridoxal; with isoniazid inhibition, however, lysyl oxidase activity does not show any sign of recovery by 48h. It is proposed that isoniazid may cause the inhibition of lysyl oxidase by competing for its obligatory cofactor, pyridoxal phosphate. The potential clinical implications in the therapeutic control of fibrosis are briefly discussed.

Collagen must be cross-linked in order to achieve the mechanical strength necessary for it to perform its major physiological function. Covalent cross-links have been shown to occur between lysine residues on adjacent polypeptide chains (Bailey *et al.*, 1974). The reaction that initiates cross-link formation is catalysed by lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13), first demonstrated by Pinnell & Martin (1968), and shown to be an extracellular, copper-requiring, enzyme (Siegel *et al.*, 1970). This enzyme converts ε-amino groups of lysine or hydroxylysine residues in collagen into aldehydes, producing allysine or hydroxyallysine respectively (for review, see Siegel, 1979). These products can react spontaneously with neighbouring allysine or lysine residues to yield a variety of covalent cross-links [for reviews, see Tanzer (1976) and Light & Bailey (1980)]. Oxygen has also been shown to be necessary for enzyme activity (Siegel *et al.*, 1970), by assaying

the enzyme in the presence of decreased oxygen concentrations.

Strong evidence exists that pyridoxal phosphate is also necessary as a cofactor of lysyl oxidase (Levene, 1961a). Reagents which react with carbonyl groups [e.g. phenylhydrazine, hydroxylamine (Harris *et al.*, 1974), isoniazid (Arem & Misiorowski, 1976)] all inactivate the enzyme. Experiments *in vivo* with chicks have shown that inhibition of lysyl oxidase by a pyridoxine-deficient diet (Murray *et al.*, 1978) or by lathyrogens (Levene, 1961a) can be reversed by pyridoxal or pyridoxine. Other aldehydes were less effective in reversing the effects of lysyl oxidase inhibition (Levene, 1961a). In addition, the studies by Murray & Levene (1977) and Bird & Levene (1982) have produced physical data that are consistent with pyridoxal being tightly bound to the enzyme. A model system containing Cu^{2+} and pyridoxal has been shown to catalyse the conversion of lysine into allysine in collagen (Fowler *et al.*, 1970).

The ability to regulate collagen deposition may be of great value in a variety of pathological conditions, such as liver cirrhosis and silicosis, or in wound healing and scar formation (Levene *et*

Abbreviations used: PBS, phosphate-buffered saline (0.1 M-sodium phosphate/0.15 M-NaCl, pH 7.6); BAPN, β-aminopropionitrile.

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al., 1968). Probably the most specific way of achieving this would be to regulate one of the stages in the post-translational modification of collagen. Subsequent studies on the therapeutic inhibition of fibrosis have been hampered by the toxicity of the drugs used (Chvapil, 1982). Isoniazid may represent a very useful tool, since it is already used extensively in the treatment of tuberculosis and its side effects are well known. One side effect of this drug is the production of a peripheral neuropathy which can be reversed in man by the administration of pyridoxine. The ability to control lysyl oxidase activity both negatively with isoniazid, and positively with pyridoxal, could be of clinical significance; we have therefore attempted to further define the role of vitamin B-6 in lysyl oxidase catalysis by studying the inhibition of the enzyme by isoniazid.

Experimental

Incubation of chick embryos

Fertile eggs were incubated at 37°C in a humid atmosphere with periodic turning, until they were used for experiments; embryos were generally incubated for 14–17 days before being used.

Injection of eggs

Drugs were dissolved in distilled water, and 0.1 ml of solution was injected through a pin-hole in the surface of the shell. Most injections were administered after 14 and 15 days of incubation, and the embryos harvested on day 16. Unless otherwise indicated, 7.4 mg of isoniazid and 11.0 mg of pyridoxal were injected into each egg; these are stoichiometric amounts of the two compounds.

Assays

Leg bones and cartilages from the embryos were homogenized either in PBS or in 4M-urea/0.1M-Tris/HCl (pH 7.6 at 4°C). After overnight shaking at 4°C, the homogenate was centrifuged at 30000 g for 30 min and the supernatant collected.

Lysyl oxidase assay. PBS extracts were used immediately, whereas urea extracts were dialysed exhaustively against PBS before assay, since urea interferes with the assay. Lysyl oxidase activity was determined by the release of ^3H_2O from an aortic elastin substrate which had been radioactively labelled with [4,5-^{3}H]lysine by a published variation (Kagan et al., 1974) of the method of Pinnell & Martin (1968). The activity in PBS and urea extracts were compared as described in the Results section (Table 3). As the differences between control and isoniazid-treated samples were greater in PBS extracts than in urea extracts, the PBS extraction procedure was gener-

ally employed. It has been proposed that the difference is caused by the extraction of both newly synthesized and old enzyme by urea, whereas PBS extracts only the new enzyme, which has not become trapped within the collagen matrix. In addition to enhancing changes in enzyme activity, the extraction into PBS is simpler and quicker and, by eliminating extraction into a denaturant, removes a step that may lead to a variable proportion of the enzyme being inactivated. However, since lysyl oxidase is not as stable in PBS as in 4M-urea (Narayanan et al., 1974) the assays were always carried out as soon as possible after extraction.

The results of lysyl oxidase assays are expressed as percentages of control values, because there was significant variation between batches of aortic elastin substrate; in addition, background radioactivity increased with storage in both enzyme-free blanks and blanks in which enzyme had been pretreated with BAPN. We therefore only stored substrate for 2–3 months. Control assays were usually 1000–3000 d.p.m. above the BAPN-pretreated blanks.

Hydroxyproline assay. The PBS extracts were also used in the measurement of extractable hydroxyproline. Samples were hydrolysed at 110°C overnight in 6M-HCl. The acid was removed by repeated vacuum distillation and washing with distilled water. The free hydroxyproline was determined by the method of Bergman & Loxley (1965), and the result was used as a measure of un-cross-linked collagen, since hydroxyproline is a specific marker for collagen in this system and only collagen that has not been cross-linked can be extracted with PBS. The fact that cross-link synthesis is initiated by lysyl oxidase means that hydroxyproline also gives an indirect measure of lysyl oxidase activity, as long as the rate of collagen synthesis remains constant.

Pyridoxal phosphate assay. Pyridoxal phosphate was measured in liver homogenates by the fluorimetric method of Adams (1979). Livers were frozen in liquid N_2 immediately after removal from the animal, and were thawed and assayed within 3 days.

Results

Effect of isoniazid on collagen solubility and liver pyridoxal phosphate concentration; reversal of effect by pyridoxal

The increase in collagen solubility caused by isoniazid, and its reversal by pyridoxal (Levene, 1961a), were confirmed by assaying PBS-extractable collagen from leg bones of 16-day chick embryos which had been treated with isoniazid at day 14 (7.4 mg/egg) and subsequently with pyri-

doxal at day 15 (11.0 mg/egg) (Table 1). To give a clearer picture of the changes occurring after injection of isoniazid and pyridoxal, liver pyridoxal phosphate, the active form of vitamin B-6 and cartilage lysyl oxidase were also measured. The results are presented in Table 2. Lysyl oxidase activity was first measured in urea extracts, the classical method of extracting the enzyme (Narayanan *et al.*, 1974); however, when we compared the extraction of enzyme activity into urea and PBS, the results of a typical experiment demonstrate that the differences between control and isoniazid-treated embryos are amplified in the PBS-extracted samples (Table 3).

Time course of the effects of isoniazid and pyridoxal

We have shown that over a period of 2 days the amount of pyridoxal phosphate in the liver and the activity of lysyl oxidase in leg bones were depressed by isoniazid and that the effects could be reversed by the injection of a stoichiometric amount of pyridoxal. These results do not give any indication of the rate at which the changes were occurring. A series of time-course studies were performed, and these showed that the decreases in pyridoxal phosphate and lysyl oxidase both occurred very rapidly (Fig. 1). However, no recovery of either parameter was detected within 4.5 h of injection of either pyridoxal or pyridoxine. Since this suggested that re-activation was much slower than inactivation, a much longer time course was

Table 1. *Effect of isoniazid on hydroxyproline extracted with PBS from 16-day chick-embryo leg bones: reversal of the effect by pyridoxal*

Isoniazid (7.4 mg/egg) was injected into eggs after 14 days incubation. Pyridoxal (11.0 mg/egg) was injected 24 h later and the embryos were harvested a further 24 h later, at 16 days incubation. Hydroxyproline is given as μg extracted into 4 vol. (v/w) of PBS per g wet wt. of bone. Values are means $\pm$ half the difference between duplicates. 'I' and 'II' represent separate experiments.

Group		Hydroxyproline extracted (μg/g wet wt. of bone)	(% of control value)
Control	I	149 ± 15	100
	II	175 ± 3	100
Isoniazid	I	999 ± 30	669
	II	734 ± 14	419
Isoniazid + pyridoxal	I	247 ± 5	165
	II	293 ± 1	167
Pyridoxal	I	87 ± 3	58
	II	102 ± 6	58

Table 2. *Effect of isoniazid on liver pyridoxal phosphate content and cartilage lysyl oxidase activity in 16-day chick embryos*

Isoniazid and pyridoxal were injected as in Table 1. All values are given as percentages of control values, as quite large variations occurred between experiments. The control values for liver pyridoxal phosphate were 54 nmol/g wet wt. of liver in Expt. I and 26 nmol/g wet wt. in Expt. II. Lysyl oxidase was extracted into 4 vol. of 4 M-urea buffer. All assays were performed as detailed in the Experimental section.

Group		Liver pyridoxal phosphate (% of control)	Cartilage lysyl oxidase (% of control)
Control	I	100	100
	II	100	
Isoniazid	I	57	59
	II	56	
Isoniazid + pyridoxal	I	86	120
	II	101	
Pyridoxal	I	177	108
	II	157	

Table 3. *Comparison of the method of extraction of lysyl oxidase from 16-day chick-embryo leg bones*

Injection of isoniazid was as described in Table 1. Leg bones were homogenized in 4 vol. of PBS or 4 M-urea (details are given in the Experimental section). Urea extracts were shaken for 2 h at 4°C, and the supernatant was then removed by centrifugation. The extract was dialysed twice against PBS before assay. PBS extracts were shaken overnight at 4°C and were centrifuged just before assay. Extracts were of separate samples and were not sequential. Lysyl oxidase was assayed in 0.5 ml samples in a total volume of 1.5 ml, for 4 h at 37°C. The enzyme is stable for at least 4 h under these conditions. For liquid-scintillation counting 1.0 ml samples were taken, and results were corrected to 1.5 ml. Efficiency of radioactivity counting was 20–30%.

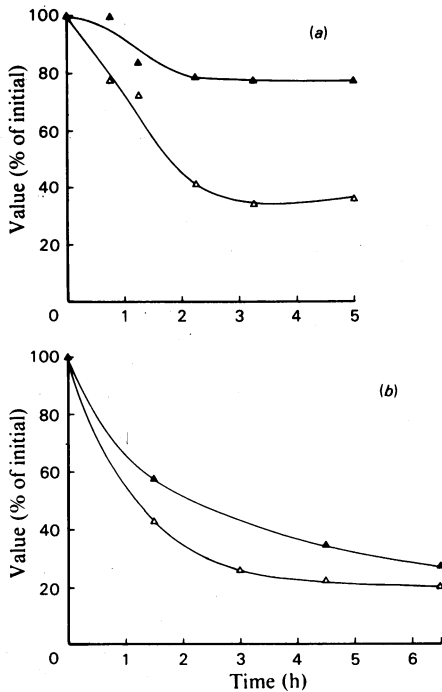| Group	Method of extraction	Lysyl oxidase activity (d.p.m.)	(% of control)	Liver pyridoxal phosphate (%)
Control	PBS	3930 ± 220	100	100
Control	4 M-Urea	5690 ± 320	100	–
Isoniazid	PBS	2670 ± 310	68	35
Isoniazid	4 M-Urea	4760 ± 150	84	–

Fig. 1. *Time course of the depletion of liver pyridoxal phosphate and the decrease in activity of lysyl oxidase after injection of isoniazid*

▲, Lysyl oxidase activity; △, pyridoxal phosphate content of the liver. In (*a*), 10 mg of isoniazid was injected into 16-day embryos, and the activity of lysyl oxidase in urea extracts from chick leg bones and the concentration of pyridoxal phosphate in livers were measured at timed intervals. In (*b*), experimental procedure was as in (*a*), except that 15 mg of isoniazid was injected. Results are given as percentages of the zero-time control values. Preliminary experiments had shown no variation in lysyl oxidase activity over this period in untreated chick embryo bones. The changes caused by isoniazid were not reversed by the injection of pyridoxal (*a*; at 15 mg/egg) or pyridoxine (*b*; at 20 mg/egg) within 4.5 h of the injection of the B-6 vitamins.

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performed (Fig. 2). The decrease in pyridoxal phosphate occurred more rapidly than the inhibition of lysyl oxidase or the increase in collagen solubility. The recoveries of both lysyl oxidase activity and liver pyridoxal phosphate contents were slower than their decrease, as expected. Surprisingly, however, the recovery of collagen cross-linking, as estimated by extractable hydroxyproline, was quite rapid.

Effect of cycloheximide on the changes induced in collagen cross-linking by isoniazid and pyridoxal

The results presented above do not give any information about the reversibility of lysyl oxidase
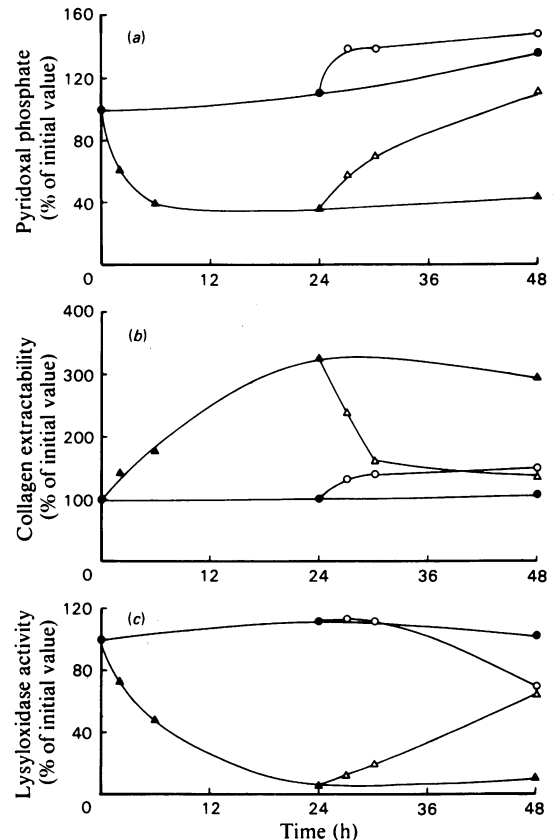

Fig. 2. *Recovery of lysyl oxidase activity in 16-day chick-embryo leg bones after injection of isoniazid and subsequent treatment with pyridoxal*

(*a*) Effect of isoniazid (7.4 mg injected after 14 days incubation) and pyridoxal (11.0 mg injected at 15 days) on the liver content of pyridoxal phosphate; ●, control, untreated embryos; ▲, isoniazid-treated embryos; △, isoniazid-plus pyridoxal-treated embryos; ○, pyridoxal-treated embryos. Results are given as percentages of control values. (*b*) Effect of isoniazid and pyridoxal treatment on PBS-extractable collagen (as extractable hydroxyproline); details as in (*a*). (*c*) Effect of isoniazid and pyridoxal treatment on PBS-extractable lysyl oxidase activity; details as in (*a*).

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inhibition by isoniazid, since recovery may require enzyme synthesis *de novo* or simply the replacement of a defective cofactor with an active one. In an attempt to determine the method of inhibition by isoniazid, the effect of cycloheximide on the recovery of lysyl oxidase activity was studied. As high a dose of cycloheximide as possible was used in these experiments. We found that, after 15 days incubation, 5 μg of cycloheximide injected into eggs which had been treated with isoniazid 24 h

Table 4. *Effect of cycloheximide on the recovery of lysyl oxidase activity after the treatment of chick embryos with isoniazid and pyridoxal*

Isoniazid was injected after 14 days incubation (7.4 mg/egg) BAPN (1 mg/egg) was also injected at this time. After 24 h eggs were treated with cycloheximide at 5 μg/egg, and 2 h later pyridoxal (11.0 mg/egg) were injected. Embryos were harvested at 16 days of incubation. Livers were removed for determination of pyridoxal phosphate, and leg bones were taken for measurement of extractable collagen and lysyl oxidase activity. All results are given as percentages of control values: ND, not determined. I, II and III represent the results from three separate experiments.

Group	Lysyl oxidase activity			Pyridoxal phosphate in liver			Extractable collagen	
Control	100	100	100	100	100	100	100	100
Isoniazid	9	11	9	51	31	37	234	280
Isoniazid + cycloheximide	9	13	6	45	32	35	254	264
Pyridoxal	123	138	ND	130	130	ND	85	ND
Pyridoxal + cycloheximide	92	102	ND	122	132	ND	73	ND
Isoniazid + pyridoxal	29	41	23	84	65	64	105	176
Isoniazid + pyridoxal + cycloheximide	31	27	15	88	75	76	180	173
Cycloheximide	97	96	ND	95	89	ND	87	ND
BAPN	ND	51	78	ND	87	95	151	143
BAPN + cycloheximide	ND	48	77	ND	84	96	106	98

previously, or into eggs subsequently treated with pyridoxal, decreased embryo survival to approx. 60%. Mortality was unaffected by the administration of cycloheximide alone. As the incorporation of radiolabelled amino acids into protein in 15-day embryos was extremely low and the background was very high, the evidence that protein synthesis had been inhibited was inconclusive. Although 20 μg of cycloheximide caused a decrease in [^{3}H]-lysine incorporation from 516 to 163 d.p.m. over the background, the actual radioactivity measured was too low to give an accurate estimate of protein synthesis. However, the much higher incorporation of radiolabelled lysine into untreated 7-day embryos enabled us to show that in the chick embryo protein synthesis can be inhibited by cycloheximide; in the 7-day embryo controls, trichloroacetic acid precipitates contained 116300 d.p.m., whereas the injection of 20 μg of cycloheximide decreased this to only 22300 d.p.m. (less than 20% of the control). BAPN was also injected in these experiments (at 1 mg/egg), since it has been reported to inhibit lysyl oxidase irreversibly (Pinnell & Martin, 1968; Tang *et al.*, 1983) *in vitro*, and recovery of activity should therefore depend on enzyme synthesis *de novo*. Preliminary experiments (Fig. 3) showed that the inhibition of lysyl oxidase by BAPN was rapid (approx. 10% activity remaining 10 h after treatment with BAPN). In the absence of cycloheximide, activity had recovered to 50% of the control value in 48 h. By contrast, inhibition by isoniazid appeared to occur slightly more slowly, and no recovery was evident 48 h after the injection of isoniazid. Our results (Table 4) show that cycloheximide (at 5 μg/egg) had very little effect on the
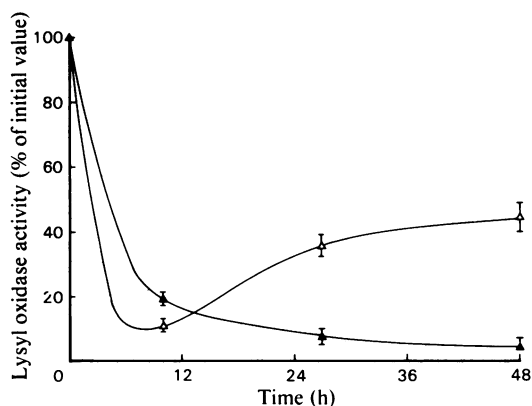

Fig. 3. *Inhibition of lysyl oxidase by isoniazid and BAPN in 14-day chick embryos*

Isoniazid (7.4 mg/egg) and BAPN (1 mg/egg) were injected into eggs which had been incubated for 14 days. The leg bones were removed at intervals, and PBS extracts were assayed for lysyl oxidase activity. Results are means $\pm$ half the difference of two experiments. Curves represent changes in lysyl oxidase activity after treatment with BAPN ($\triangle$) or isoniazid ($\blacktriangle$).

recovery of lysyl oxidase activity after either treatment with isoniazid or BAPN, although it seemed to block the increase in enzyme activity that had been found to occur after treatment with pyridoxal alone.

Discussion

We have confirmed that the injection of isoniazid into chick embryos causes a large increase in

extractable hydroxyproline. Since the measurement of hydroxyproline is used to estimate collagen, it appears that this drug is making the collagen in chick leg bones more soluble. We have now shown that the probable explanation for this increase in collagen solubility when embryos are treated with isoniazid is the inhibition of lysyl oxidase, the enzyme responsible for the initiation of the synthesis of the intra- and inter-molecular cross-links that stabilize collagen fibrils.

Our data also show a highly significant decrease in liver pyridoxal phosphate after treatment of chick embryos with isoniazid. This change appears to precede the increase in collagen solubility and the fall in lysyl oxidase activity, which change synchronously. It seems probable that the changes detailed above are related: that the fall in pyridoxal phosphate concentration causes the inhibition of lysyl oxidase, and that this in turn decreases the extent to which the recently synthesized collagen is cross-linked, resulting in a more soluble product.

The injection of pyridoxal causes the reversal of all the changes elicited by isoniazid. We found that an approximately equimolar amount of pyridoxal was required to restore almost completely the vitamin B-6 content in the liver as well as the bone lysyl oxidase activity. The recovery of pyridoxal phosphate was considerably slower than its depletion; this could be due to the more rapid action of isoniazid or to the slow conversion of pyridoxal into pyridoxal phosphate. As would be expected if the inhibition of lysyl oxidase was due to the depletion of pyridoxal phosphate, the recovery of enzyme activity occurred after the increase in pyridoxal phosphate concentration.

The incubation *in vitro* of isoniazid and pyridoxal under reasonably physiological conditions (i.e. pH 7.5, 37°C in PBS) caused the immediate precipitation of an orange product, which is the hydrazone produced by the reaction of the hydrazide group in isoniazid with the carbonyl group of pyridoxal (Levene, 1961*b*). This reaction also occurs with pyridoxal phosphate (C. I. Levene, unpublished work), but not with pyridoxine or pyridoxamine, both of which lack the carbonyl group. It seems quite probable that this hydrazone formation occurs *in vivo* (Levene, 1962), and that the inhibition of lysyl oxidase, increase in collagen solubility and depletion of pyridoxal phosphate are the results of a complex formed between isoniazid and the pyridoxal cofactor of lysyl oxidase. Our attempts to isolate the hydrazone have so far been unsuccessful.

The experiments in which cycloheximide was employed to inhibit protein synthesis are difficult to interpret, as there was no difference in the lysyl oxidase activity of embryos treated with BAPN

plus cycloheximide and those treated with BAPN alone. Since the injection of cycloheximide, in conjunction with isoniazid or pyridoxal, caused an increase in chick-embryo mortality, it seems likely that it was having some effect on protein synthesis. In addition, we had shown that protein synthesis was inhibited by cycloheximide in 7-day chick embryos. If protein synthesis was significantly inhibited in these experiments, the inhibition by both isoniazid and BAPN must be reversible. From these results it is impossible to determine whether protein synthesis is required for the recovery of lysyl oxidase activity after it has been inhibited with isoniazid. It is probable, however, that the modes of action of isoniazid and BAPN differ *in vivo*, since, as described in the Results section, the enzyme recovered rapidly after inhibition by BAPN, whereas, with isoniazid, enzyme activity did not even begin to recover within 48 h of injection of this compound. The kinetics of inhibition by isoniazid in this closed system are consistent with the depletion of a vitamin cofactor which the chick embryo is unable to replace. In addition, BAPN has no effect on liver pyridoxal phosphate at a dose which severely inhibits lysyl oxidase, whereas isoniazid causes severe depletion, to approx. 40% of control. The differences in the effects of isoniazid and BAPN strongly indicate that they act on lysyl oxidase in very different ways. The identification of inhibitors acting at different sites of the enzyme will be of use in elucidating its reaction mechanism.

We have described the inhibition of lysyl oxidase by isoniazid and the reversal of this effect by pyridoxal. In addition, we have shown that pyridoxal alone causes some enhancement of the basal lysyl oxidase activity. It is possible that these two compounds could be used either to enhance or to delay the rate of collagen deposition in a variety of clinical situations. For example, the 'healing' stage of adult pulmonary tuberculosis is characterized by a massive fibrotic reaction. It is curious that isoniazid should possess both anti-tubercular activity and the capacity to inhibit cross-linking of collagen, the major component of fibrotic tissue. Is it possible that the therapeutic effect of isoniazid involves an effect on the host's mesenchyme as well as on the metabolism of the invading organism? Papers on the therapeutic possibilities for the regulation of fibrosis include those by Levene (1978), Fuller (1981), Moorhead (1981) and Uitto *et al.* (1982).

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