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Evidence for the Role of Vitamin B-6 as a Cofactor of Lysyl Oxidase

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At 24h after injection of 16-day chick embryos with [G-^{3}H]pyridoxine hydrochloride, some of this label appears in the epiphysial cartilage. Over 35% of this radioactivity appears in the form of [G-^{3}H]pyridoxal and a further 30% as other vitamin B-6 compounds. Partial purification of lysyl oxidase from the labelled epiphysial cartilage reveals a single peak of radioactivity coinciding with a single peak of enzyme activity. On dialysis against phosphate-buffered saline, 75% of this radioactivity is found to be non-diffusible. After incubation with isonicotinic acid hydrazide, a carbonyl reagent that appears to inhibit lysyl oxidase both *in vivo* and *in vitro*, a further 70% of the radioactivity is lost, with a roughly corresponding loss of enzyme activity. It is suggested that a form of vitamin B-6 is required as a cofactor of lysyl oxidase, and that this may have important implications in terms of connective-tissue metabolism.

Lysyl oxidase catalyses the oxidative deamination of the ε-amino group of specific lysine residues in elastin, and of lysine and hydroxylysine residues in collagen (Bailey *et al.*, 1974; Gallop & Paz, 1975; Tanzer, 1976), to form the aldehyde precursors of the cross-links of these structural proteins, and is therefore directly responsible for the ultimate structural integrity of the connective tissues. There is ample evidence to suggest that lysyl oxidase has a specific requirement for Cu^{2+} ions and molecular oxygen *in vitro* (Siegel *et al.*, 1970), and animals raised on copper-deficient diets demonstrate cross-link defects in arterial elastin (O'Dell *et al.*, 1961; Shields *et al.*, 1962). It has also been demonstrated that in copper-deficient pigs, which exhibit these defects, there is a dramatic decrease in plasma amine oxidase activity (Blaschko *et al.*, 1965). On the basis of these findings it was suggested (Page & Benditt, 1967) that lysyl oxidase may belong to that group of amine oxidases, including plasma amine oxidase, which are known to be copper-dependent and probably pyridoxal phosphate-dependent (Yasunobu & Yamada, 1963; Blaschko & Buffoni, 1965). Consistent with this hypothesis was the demonstration (Hill & Kim, 1967; Starcher, 1969) of a derangement of cross-link synthesis in aortic elastin in pyridoxine-deficient chicks. Indirect evidence for the presence of a pyridoxal cofactor has also come from the study of the inhibition characteristics of lysyl oxidase *in vitro* (Harris *et al.*, 1974), which demonstrated the sensitivity of the enzyme to carbonyl reagents. It has also been demonstrated that many of these carbonyl reagents have lathyrogenic activity *in vivo*, resulting in dramatically increased fragility of mesenchymal tissue (Levene, 1961*b*), although the exact mode of action of lathyrogenic compounds is still unclear.

In view of the critical role of cross-linking in connective tissues, it is clearly important to establish the nature of the cofactors of this enzyme. The effects of copper deficiency on the connective tissues are well documented (Carnes, 1971) and, although vitamin B-6 deficiency is rare in animals, there may be situations where suboptimal dietary intakes of this vitamin might have an irreversible effect on the connective tissues (Levene & Murray, 1977), owing to inadequate cross-linking of both collagen and elastin.

We present some new evidence resulting from studies *in vivo* and *in vitro* on lysyl oxidase from embryonic-chick cartilage, suggesting that a form of vitamin B-6 may play a catalytic role in the enzyme.

Experimental

Materials

Fertilized eggs of Light Sussex, Rhode Island Red or cross-bred hens were obtained from the Winter Egg Farm, Thriplow, Cambs., U.K., and were incubated until required at 37°C in a Western incubator.

Pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxal 5′-phosphate, pyridoxamine dihydrochloride, and pyridoxamine 5′-phosphate hydrochloride were obtained from Sigma (London) Chemical Co, Kingston-upon-Thames, Surrey, U.K.

L-[4,5-^{3}H]Lysine hydrochloride (sp. radioactivity 8.7Ci/mmol) and [G-^{3}H]pyridoxine hydrochloride (sp. radioactivity 1.6Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Other reagents were obtained from BDH Chemicals, Poole, Dorset, U.K.

Methods

Partial purification of lysyl oxidase from embryonic-chick cartilage. The method followed was that of Siegel (1974). The epiphysial cartilage from tibiae and femora of 17-day chick embryos was dissected free of surrounding tissue and homogenized in ice-cold phosphate-buffered saline (0.1M-NaH_2PO_4/0.15M-NaCl, pH7.7; 2ml/g of tissue) with a Silverson homogenizer. This suspension was centrifuged (30000$g_{av.}$; 20min; 4°C). The pellet was resuspended in buffer and the process repeated, both supernatants being retained. The pooled supernatants were added directly to a collagen–Sepharose 4B affinity resin (Pharmacia, Uppsala, Sweden) that had been prepared by the addition of neutral salt-soluble rat skin collagen (Bornstein & Piez, 1964) to CNBr-activated Sepharose 4B by using the method of Bauer *et al.* (1971), and equilibrated in 0.05M-Tris/HCl, pH7.5; the mixture was stirred gently for 45 min at 4°C. The resin was eluted sequentially on a Buchner funnel at room temperature (21°C) with 1M-NaCl/0.05M-Tris/HCl, pH7.5, then 0.05M-Tris/HCl, pH7.5, then 6M-urea/0.05M-Tris/HCl, pH7.5, and finally 1M-NaCl/6M-urea/0.05M-Tris/HCl, pH7.5. The 6M-urea/0.05M-Tris/HCl, pH7.5, eluate, containing most of the enzyme activity was then pumped directly in a volume of approximately 50ml on to a column (1.5cm×30cm) of DEAE-cellulose (DE 52; Whatman Biochemicals, W. and R. Balston, Springfield Mill, Maidstone, Kent, U.K.) equilibrated in 6M-urea/0.05M-Tris/HCl, pH7.5, at room temperature. After washing with equilibrating buffer, the column was developed with a linear gradient of 200ml of 0–1M-NaCl in 6M-urea/0.05M-Tris/HCl, pH7.5. The eluate was monitored for A_{280}, and 10ml fractions were collected at a flow rate of 60ml/h. Before assay for lysyl oxidase activity, portions of fractions were dialysed against at least three changes of phosphate-buffered saline at 4°C overnight to remove urea.

Preparation of insoluble substrate for lysyl oxidase. Insoluble elastin substrate was prepared by a modification of the method of Pinnell & Martin (1968). Aortae from 72 17-day chick embryos were dissected and incubated at 37°C for 3h in 10ml of a modified Krebs–Ringer buffer (Manning & Meister, 1966), supplemented with L-[4,5-^{3}H]lysine hydrochloride (25μCi/ml), β-aminopropionitrile fumarate (50μg/ml), ascorbic acid (50μg/ml), benzylpenicillin (Glaxo Laboratories Ltd., Greenford, Middx., U.K.; 60μg/ml) and streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, Middx., U.K.; 150μg/ml). Incubations were carried out in 50ml Erlenmeyer flasks. The stoppered flasks were flushed with O_2/CO_2 (19:1) and incubated in a shaking water bath at 37°C. After incubation, the medium was poured off, the aortae were washed twice with water, freeze-dried and stored at −20°C until required.

To prepare the substrate for assay of lysyl oxidase activity, the required number of aortae was suspended in a suitable volume of phosphate-buffered saline and homogenized by hand with a ground-glass homogenizer. The homogenate was centrifuged (17000$g_{av.}$; 10min; 20°C), the supernatant discarded and the pellet suspended in approx. 0.5ml of phosphate-buffered saline per aorta. This suspension was boiled for 10min to destroy endogenous lysyl oxidase activity, cooled and finally centrifuged (as above). The pellet was suspended once more in phosphate-buffered saline and used as insoluble substrate.

Assay of lysyl oxidase activity. Generally 0.5ml of insoluble substrate suspension containing approx. 400000d.p.m. of ^{3}H was incubated with 1ml of enzyme preparation, previously dialysed against phosphate-buffered saline, in a stoppered test tube. Incubations were carried out at 37°C for 6h in a shaking water bath. The reaction was terminated by the addition of 0.2ml of 50% (w/v) trichloroacetic acid. The ^3H_2O formed by the reaction was vacuum-distilled (Hutton *et al.*, 1966) and 1ml of the distillate was counted for radioactivity in 10ml of Kennedy's (1969) scintillant at an efficiency of approx. 26%. Enzyme controls contained 200μg of β-aminopropionitrile to inhibit lysyl oxidase activity specifically (Pinnell & Martin, 1968). Lysyl oxidase activity is given as the mean of duplicate assays.

Injection of chick embryos with [G-^{3}H]pyridoxine hydrochloride. Ten fertile eggs were injected with an aqueous solution of 100μCi of [G-^{3}H]pyridoxine hydrochloride (13μg) each at day 10 or 16 of incubation, through a small pinhole into the yolk sac (Murray, 1977). The hole was sealed with wax and the eggs were returned to the incubator until 17 days old. At harvest, cartilage was dissected and pooled with cartilage from a further 36 17-day embryos, and enzyme purification carried out as above.

Injection of chick embryos with isonicotinic acid hydrazide. For this 36 fertile eggs were injected after 15 days of incubation with 0.1ml of an aqueous solution containing 5mg of isonicotinic acid hydrazide (isoniazid) through a pinhole via the chorioallantoic membrane (Levene, 1961*a*). The hole was sealed with wax and the eggs were returned to the incubator for a further 48h when they were harvested; at the same time the epiphysial cartilages from 36 chicks of the same age that had not been injected, were used as a control, to compare with the isoniazid-treated cartilage.

Extraction and separation of vitamin B-6 compounds from cartilage. Epiphysial cartilage from 17-day chick embryos that had been injected with [G-^{3}H]-pyridoxine hydrochloride was extracted with 1% metaphosphoric acid (Wada *et al.*, 1957). This method extracted 99% of the radioactivity. From the neutralized supernatant, the methyl-acetal derivatives of the

vitamin B-6 compounds were prepared and chromatographed on thin-layer plates of silica-gel G (Merck, East Molesey, Surrey, U.K.) by the method of Nürnberg (1961), with a two-component solvent system. Plates were first developed in acetone, which was allowed to run half the length of the plate. After drying, plates were developed again in acetone/dioxan/aq. 25% (v/v) NH_3 (9:9:2, by vol.) and allowed to run the full length. Individual compounds were detected with diazotized sulphanilic acid (Maiwald & Maske, 1956). Standard vitamin B-6 compounds were run alongside the extracts. Individual spots were scraped off plates into scintillation vials and counted for radioactivity in the scintillant described above.

Other methods. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of lysyl oxidase preparations was carried out by the method of Weber & Osborn (1969).

Results

Partial purification of lysyl oxidase from embryonic-chick cartilage

Lysyl oxidase activity was eluted from DEAE-cellulose as a single peak. This preparation was approx. 700-fold purified and had a specific radioactivity of 150000 d.p.m. of ^3H/mg of protein (see Table 1). This preparation was not homogeneous on sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis, revealing several protein bands.

Injection of [G-^3H]pyridoxine into fertile eggs

Incorporation of radioactivity into epiphysial cartilage was measured in 17-day embryos injected previously at 10 or 16 days. Incorporation into cartilage of embryos injected at 16 days (1.36 μCi/g of cartilage) was approx. 3 times that into cartilage of embryos injected at 10 days (0.50 μCi/g of cartilage).

T.l.c. of acid extracts of cartilage labelled for 24h indicated that 35% of the injected pyridoxine had been metabolized *in vivo* to pyridoxal, 30% to phosphorylated derivatives and 35% had remained as pyridoxine.

Lysyl oxidase from epiphysial cartilage of ten chicks injected with [G-^3H]pyridoxine hydrochloride at 16 days of incubation, combined with cartilage from 24 unlabelled embryos, was partially purified in a similar manner to the control enzyme previously described. After chromatography on DEAE-cellulose, measurement of both incorporated radioactivity and lysyl oxidase activity indicated a small peak of radioactivity coinciding with a single peak of lysyl oxidase activity. Enzyme activity was eluted at the same position as control enzyme and had a similar specific radioactivity. During assay of lysyl oxidase by the ^3H-release assay, inclusion of β-aminopropionitrile in blanks ensured the specificity of this ^3H release (see Fig. 1).

Effect of isoniazid on lysyl oxidase in vivo and in vitro

Of 30 embryos injected at 15 days of incubation, three died before harvest 48h later. All embryos displayed lathyritic symptoms, such as increased connective-tissue fragility. Phosphate-buffered saline extracts of cartilage from control and isoniazid-treated embryos were partially purified as described under 'Methods'. A small peak of enzyme activity occurred on DEAE-cellulose chromatography of the

Table 1. *Partial purification of lysyl oxidase from embryonic-chick cartilage*

One unit of lysyl oxidase activity catalyses the release of 400 d.p.m. of ^3H in 6h at 37°C, from 400000 d.p.m. of [^3H]lysine-labelled aortic protein substrate.

Purification step	Specific radioactivity (d.p.m./mg of protein)	Purification (-fold)	Yield (units)
Phosphate-buffered saline extract	220	—	1296
Affinity chromatography	46000	209	765
DEAE-cellulose chromatography	150300	683	827

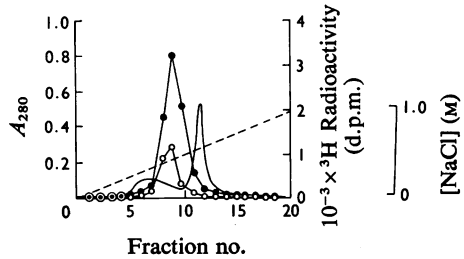

Fig. 1. *Ion-exchange chromatography of lysyl oxidase from 17-day chick embryos injected with [G-^3H]pyridoxine hydrochloride 24h previously*

The 6M-urea eluate from collagen–Sepharose 4B was chromatographed on DEAE-cellulose with a linear gradient of NaCl (----). Elution profiles for lysyl oxidase activity (●), for incorporation of radioactivity (○) and for material absorbing at 280nm (——) are shown. The fraction size was 10ml.

isoniazid-treated chick extract, at a position identical with that of the peak of the control extract. The results of assays on crude and partially purified extracts of epiphysial cartilage showed that crude cartilage extracts from control embryos has an activity of 250 d.p.m. of ^3H/mg of protein, whereas those from isoniazid-treated embryos were devoid of lysyl oxidase activity. After partial purification of these extracts, the specific activity of the isoniazid-treated extracts was 5% of the control value (4400 d.p.m. of ^3H compared with 108000 d.p.m. of ^3H). Attempts to restore activity, by dialysis and by addition of pyridoxal phosphate and metal cations, were unsuccessful.

Assays of partially purified lysyl oxidase from control extracts were carried out in the presence of isoniazid to determine the degree of inhibition of lysyl oxidase by this lathyrogen *in vitro*. The results of these assays are shown in Table 2.

In addition, portions of partially purified lysyl oxidase from cartilage of chick embryos injected with [G-^3H]pyridoxine hydrochloride were dialysed against phosphate-buffered saline and incubated with isoniazid (0.5 mM) before dialysis, and both radioactivity remaining bound and lysyl oxidase activity were assayed. The results of these assays are given in Table 3.

Some 25% of the radioactivity that was eluted at the same position as lysyl oxidase activity from DEAE-cellulose was diffusible after incubation in phosphate-buffered saline at 37°C followed by dialysis. Dialysis of this preparation after incubation with isoniazid resulted in the loss of 70% of the remaining radioactivity, and a corresponding 60% loss of lysyl oxidase activity.

Table 2. *Inhibition of lysyl oxidase in vitro by isoniazid*
Samples of enzyme preparation were preincubated with inhibitor for 30 min at 37°C before addition of substrate.

Inhibitor	Concn. (mM)	Inhibition of lysyl oxidase activity (%)
Isoniazid	1	100
	0.1	15
β-Aminopropionitrile fumarate	0.1	100

Table 3. *Dialysis of radioactively labelled lysyl oxidase*
The treatments involved incubation at 37°C for 30 min in phosphate-buffered saline in the presence or absence of 0.5 mM-isoniazid, followed by dialysis against phosphate-buffered saline overnight at 4°C.

Treatment	Radioactivity remaining (%)	Enzyme activity remaining (%)
None (in 6 M-urea)	100	0
Dialysis against phosphate-buffered saline (to remove urea)	75	100
Incubation with 0.5 mM-isoniazid	12	40

Discussion

Arem & Misiorowski (1976) have shown that lysyl oxidase is inhibited by isoniazid both *in vivo* and *in vitro*. Our observation that isoniazid *in vitro* removes all but 16% of the radioactive pyridoxal and yet leaves an enzyme preparation that is still 40% active may well be due to the concentration of isoniazid used; had a higher concentration been used, it is likely that all radioactivity and enzyme activity would have been lost. Lysyl oxidase is inhibited by many carbonyl reagents (Harris *et al.*, 1974), suggesting a possible pyridoxal cofactor. Isoniazid also possesses lathyrogenic activity (Levene, 1961*a*), producing an increase in the salt-soluble fraction of embryonic-chick collagen. The evidence in the present paper would suggest that, *in vivo*, this effect can be attributed at least in part to inhibition of the cross-linking enzyme. The exact mechanism, however, remains to be elucidated. It is known that isoniazid will form a hydrazone readily with pyridoxal (Braunstein, 1960), and therefore may block the active carbonyl group of a pyridoxal cofactor. However, from our findings it would appear that isoniazid may displace a vitamin B-6 cofactor bound to the enzyme, with a consequent loss of enzyme activity. Levene (1961*a*) showed that the effects of isoniazid injected into chick embryos could be reversed by injection of pyridoxal 24 h later. Although this effect might be explained by complexing of the isoniazid by pyridoxal, it is also possible that reactivation of lysyl oxidase was taking place, thus allowing cross-linking of connective tissues to continue. Alternatively, isoniazid may be inducing vitamin B-6 deficiency through the formation of such hydrazones, resulting in a diminution of lysyl oxidase activity. Indeed, we have found substantial decreases in lysyl oxidase activity in cartilage and aorta of vitamin B-6-deficient chicks. There also appears to be an increase in lysyl oxidase activity in both cartilage and aorta shortly after administration of large doses of vitamin B-6 to vitamin-deficient chicks (J. C. Murray, D. R. Fraser & C. I. Levene, unpublished work).

The available evidence strongly suggests that lysyl oxidase activity is dependent on a form of vitamin B-6, although direct evidence has not been forthcoming. We consider that our findings provide more evidence for the presence of a vitamin B-6 cofactor, and that the use of such a radioactively labelled cofactor may provide a useful tool for further investigations of the catalytic site of lysyl oxidase and the

control mechanisms which may govern its activity *in vivo*.

The presence of a vitamin B-6 cofactor in lysyl oxidase may have profound implications in the metabolism of the connective-tissue proteins, collagen and elastin. In the monkey, 'suboptimal' intakes of dietary vitamin B-6 were shown to be associated with atherosclerotic lesions of the arterial wall (Rinehart & Greenberg, 1956). It is possible that such a situation might arise in the human foetus, where there is a high demand for vitamin B-6 (Contractor & Shane, 1970) and maternal supplies are often suboptimal (Cleary *et al.*, 1975), resulting in focal lesions of the internal elastic lamina in the coronary arteries at sites where atherosclerosis commonly develops during later life (Levene & Murray, 1977).

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