The effect of a vitamin B-6 antagonist, 4-deoxypyridoxine, on the cross-linking of collagen in the developing chick embryo

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The vitamin B-6 antimetabolite 4-deoxypyridoxine, when injected into 13-day chick embryos, has the effect of increasing the amount of collagen solubilized from the leg bones by buffered saline solutions, 24 h after the injection. This effect is similar to, but less marked than, that produced by the administration of the lathyrogen β -aminopropionitrile. Since that fraction of the total collagen which is solubilized by saline represents the least-cross-linked pool, it is concluded that 4-deoxypyridoxine is a lathyrogen, decreasing the cross-linking in the developing embryo, and confirming the importance of vitamin B-6 in that process. Lysyl oxidase, the cross-linking enzyme, was measured in extracts made from the epiphyseal cartilages of embryos 24 h after the injection of either 4-deoxypyridoxine or β -aminopropionitrile. The injection of 5 mg of β -aminopropionitrile causes the lysyl oxidase activity to fall to 61% of that of saline-injected controls; after treatment with 4-deoxypyridoxine, the activity is 74% of the control value. In the latter case, full activity is not restored to the extracts by preincubation with pyridoxal phosphate. The results are discussed in relation to the early development of the connective tissues.

Lysyl oxidase (protein lysine 6-oxidase, EC 1.4.3.13) catalyses the oxidative deamination of specific lysine and hydroxylysine residues in both tropoelastin and tropocollagen to form the corresponding aldehyde derivatives (Pinnell & Martin, 1968). These aldehydes, through a series of non-enzymic condensations, subsequently give rise to various covalent cross-links (for reviews see Bailey *et al.*, 1974; Gallop & Paz, 1975; Tanzer, 1976), which decrease the solubility of these proteins and thus ensure the integrity of the connective tissues. Lysyl oxidase has a requirement for bivalent copper and molecular oxygen (for review see Siegel, 1979), and therefore cross-link defects are seen in copper-deficient animals (O'Dell & Buffoni, 1961; Shields *et al.*, 1962), along with a decrease in plasma amine oxidase activity (Blaschko *et al.*, 1965). These findings prompted the hypothesis (Page & Benditt, 1967) that the cross-linking enzyme belonged to the class of amine oxidases that are dependent on copper (for review see Yasunobu *et al.*, 1976).

Before the discovery of lysyl oxidase, free carbonyl groups were shown to be involved in the cross-linking process (Levene, 1962). In the experimental disease osteolathyrism certain compounds dramatically increase the fragility of the connective

tissues by preventing the cross-linking of collagen and elastin (Levene, 1973). It was subsequently proposed that in lathyrism the carbonyl groups were either blocked or absent (Levene, 1962). Later work showed that lysyl oxidase was inhibited *in vitro* by the carbonyl reagents phenylhydrazine (Harris *et al.*, 1974; Kagan *et al.*, 1974), hydroxylamine (Harris *et al.*, 1974) and Isoniazid (Kagan *et al.*, 1974; Arem & Misirowski, 1976). Since these compounds are all lathyrogenic, the essential carbonyl function affected in lathyrism therefore appeared to be a cofactor of lysyl oxidase.

Both Hill & Kim (1967) and Starcher (1969) have shown that, in the pyridoxine-deficient chick, there is a decrease in the conversion of lysine into the elastin cross-links desmosine and isodesmosine. Moreover, the lathyrogenic effect of Isoniazid in the chick embryo had been shown to be reversible *in vivo* by pyridoxal treatment (Levene, 1961). Direct measurements of the effect of vitamin B-6 deficiency on the lysyl oxidase activity of chick cartilage and aorta were made by Murray *et al.* (1978), by using pair-fed controls; pyridoxine-deficient chicks had significantly lowered enzyme activities and showed subtle histological changes in a range of connective tissues. A 150 μ g dose of pyridoxine, given orally 14 h before each chick was killed, restored the enzyme activity. Murray & Levene (1977) have

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reported biochemical evidence that pyridoxal phosphate is present in lysyl oxidase.

In the present study, the antivitamin activity of 4-deoxypyridoxine (Ott, 1946) was used as a model of functional vitamin B-6 deficiency in the developing chick embryo, during which the cross-linking process was examined.

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 at 37°C in a Westernette incubator.

4-Deoxypyridoxine, pyridoxal 5'-phosphate, L-proline and L-4-hydroxyproline were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

L-[4,5-³H]Lysine hydrochloride (sp. radioactivity 100 mCi/mmol) and L-[5-³H]proline (sp. radioactivity 24 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Injection of eggs

On the 13th day of incubation, injection of β -aminopropionitrile or 4-deoxypyridoxine in 0.2 ml of saline (0.9% NaCl), or 0.2 ml of saline alone, was made on to the chorioallantoic membrane via a pinhole drilled through the shell. After the injection sites had been sealed with molten paraffin wax, the eggs were returned to the incubator for a further 24 h. In some cases, [³H]proline was injected into three eggs from each group 6 h before the injection of the test compound: 250 μ Ci of L-[5-³H]proline was injected on to the chorioallantois in a volume of 0.1 ml.

Extraction of collagen

The tibia and femora of surviving embryos were dissected 24 h after the injection of the test compound or sterile saline. The bones were pooled and minced on ice with fine scissors. Collagen was extracted by an adaptation of the method of Gross *et al.* (1960) by shaking the minced bone for 24 h at 4°C in exactly 2 vol. (v/w) of ice-cold 0.5 M-NaCl buffered to pH 7.6 with 0.2 M-sodium phosphate. The pelleted residues obtained after centrifugation at 50 000 g for 1 h were resuspended in further 2 vol. lots of 0.5 M-acetic acid, then extracted and centrifuged as before. Saline and acetic acid extracts, together with the residues remaining after extraction, were dialysed against large volumes (2000-fold) and frequent changes of the appropriate extractant. At this stage, viscometry was performed with a 0.5 ml-capacity Ostwald viscometer maintained at 5°C (Levene & Gross, 1959). Measured samples of the diffusates were hydrolysed with 2 vol. of 6 M-HCl at 110°C for 24 h. The hydrolysates were assayed

for hydroxyproline (Bergman & Loxley, 1963), and the values obtained were multiplied by the factor 7.46 to obtain the collagen content. In the experiment in which the collagen was labelled *in vivo* with [³H]proline, the radioactive proline was separated from its metabolite hydroxy[³H]proline by passage of measured samples of the hydrolysates through a column (1.2 cm $\times$ 12 cm) of Dowex 50 (X8) resin maintained at 37.5°C and eluted with 0.2 M-sodium citrate buffer, pH 3.41 (Moore & Stein, 1951). The radioactivities of successive 1 ml fractions of the effluent were counted in a Beckman LS-2 liquid-scintillation counter, after being mixed with 10 ml of PCS scintillant (Amersham-Searle) diluted with xylene in accordance with the manufacturers' recommendations. The column was calibrated by the passage of a solution of L-proline and L-4-hydroxyproline containing 1 mg of each amino acid. The elution of proline was monitored by a colorimetric method (Troll & Lindsley, 1955), and hydroxyproline was detected by the method described by Bergman & Loxley (1963).

Preparation of insoluble substrate for lysyl oxidase

Insoluble elastin substrate was prepared by an adaptation of the method of Pinnell & Martin (1968). Freshly dissected aortae from 17-day chick embryos were incubated at 37°C for 24 h in the Dulbecco & Vogt (1954) modification of Eagle's Minimal Essential Medium lacking lysine, supplemented with L-[4,5-³H]lysine hydrochloride (25 μ Ci/ml), β -aminopropionitrile fumarate (50 μ g/ml), ascorbic acid (50 μ g/ml), and benzylpenicillin and streptomycin sulphate (Glaxo Laboratories, Greenford, Middx., U.K.) each at 150 μ g/ml. The incubations were performed in stoppered Erlenmeyer flasks at a ratio of 60 aortae per 25 ml of medium. After incubation the used medium was poured off, and the aortae were washed twice with water, freeze-dried and stored at -20°C until required. Before use, the required number of aortae (1 $\frac{1}{2}$, or about 100 000 c.p.m. of ³H per assay) were homogenized, with a hand-held Teflon/glass homogenizer, in two changes of 0.5 M-NaCl/0.12 M-potassium phosphate buffer, pH 7.6, with centrifugation at 17 000 g for 20 min after each extraction. The pellet was retained and homogenized once in 1 M-HCl (to inactivate endogenous lysyl oxidase activity), then twice in the buffered saline. Finally, the pellet was taken up in a suitable volume of 0.15 M-NaCl/0.12 M-potassium phosphate buffer, pH 7.6 ('assay buffer'), and designated 'insoluble substrate'.

Assay of enzyme activities

Lysyl oxidase activity was assayed in the epiphyseal cartilage of surviving chick embryos 24 h after injection of the test compound. The cartilages,

freed of surrounding tissues, were pooled and homogenized in 2 vol. (v/w) of ice-cold assay buffer in a VirTis 45 homogenizer run at full speed for a total of 90 s. The homogenates were centrifuged at 17 000 *g* for 20 min, the supernatants were retained, and the pellets were re-homogenized in 2 vol. of 4 M-urea/50 mM-Tris/HCl buffer, pH 7.6. After this homogenate had been stirred for 2 h the residues were pelleted by centrifugation, at 17 000 *g* for 20 min, the supernatants being retained and exhaustively dialysed against assay buffer overnight to remove urea. Lysyl oxidase activity was measured in both the assay buffer extracts and the dialysed urea extracts. A 0.5 ml portion of insoluble substrate was mixed with 1 ml of cartilage extract in a stoppered test tube. The mixture was incubated for 4 h at 37°C in a shaking bath. Each assay was done in triplicate. The reaction was terminated by rapidly freezing the contents of the tubes. After the addition of 1 drop of octan-1-ol (to prevent excessive foaming), the ³H₂O formed during the reaction was collected by vacuum-distillation (Hutton *et al.*, 1966) and the radioactivity in 1 ml of the distillate was counted in 10 ml of 66% (v/v) PCS scintillant in xylene, by using a Beckman LS-2 counter. Counting efficiency was typically about 34%, and enzyme activities were corrected for ³H release occurring in appropriate enzyme-free blanks.

Lactate dehydrogenase activity was assayed in pooled sera by the method of Wroblewski & LaDue (1955). Serum aspartate aminotransferase (glutamate-oxaloacetate transaminase) activity was determined by the method of Karmen (1955). Serine dehydratase activity was measured (Freedland & Avery, 1964) in the 20 000 *g* supernatants of livers homogenized in 2 vol. (v/w) of 0.14 M-KCl, pH 8.5. The livers from four to six embryos were pooled in each group.

Results

Effect of 4-deoxypyridoxine on the growth of chick embryos

The effect on 17-day embryos of the injection of various doses of 4-deoxypyridoxine at day 6 is shown in Table 1. Surviving embryos appeared normal; no evidence of histological change was found in the bones or aortae. However, in one embryo that had received 2.5 mg of 4-deoxypyridoxine, the end of a femur was seen to be protruding through the skin of the leg; this lesion is frequently seen after administration of lathyrogens to chick embryos in the early part of their development (Levene, 1973). Examination of such embryos at a later stage reveals no increase in the collagen solubility, and so these early defects probably represent the acute phase of a self-healing cross-link failure.

Table 1. *Effect of injection of 4-deoxypyridoxine in 6-day chick embryos on survival, body weight and bone collagen solubility at day 17*

For experimental details see the text. $\eta_{rel.}$ values are given as means $\pm$ s.d. for three determinations on extracts pooled from the surviving embryos in each group.

Dose of 4-deoxypyridoxine injected (mg)	Mean body wt. (g)	Survival	$\eta_{rel.}$ of neutral salt extracts
None (saline)	17.3 $\pm$ 0.51	11/11	1.43 $\pm$ 0.02
0.2	17.7 $\pm$ 0.53	8/10	1.49 $\pm$ 0.003
0.5	15.1 $\pm$ 0.90	5/8	1.48 $\pm$ 0.03
1.0	17.7 $\pm$ 0.60	7/12	1.49 $\pm$ 0.001
2.5	11.9 $\pm$ 0.20	2/11	1.57 $\pm$ 0.02
3.0	All died	—	—

Table 2. *Effect of injection of 4-deoxypyridoxine in 13-day chick embryos on survival and body weight at day 15*

For experimental details see the text.

Dose of 4-deoxypyridoxine injected (mg)	Mean body wt. (g)	Survival
None (saline)	12.66 $\pm$ 1.25	12/12
2	12.89 $\pm$ 1.47	10/10
5	11.47 $\pm$ 1.76	10/10
10	11.93 $\pm$ 1.27	9/12
14	9.82 $\pm$ 1.22	12/17
28	All died	0/18

4-Deoxypyridoxine, injected in fairly large doses at day 13 of incubation, prevented the gain in body weight seen in normal embryos (Table 2). This inhibition of growth may be regarded as a non-specific indication of vitamin B-6 deficiency.

Effect of 4-deoxypyridoxine on the extractability of collagen

The dose of 4-deoxypyridoxine approximating to the LD₅₀ when injected at day 13 was 17 mg; this amount was used in the subsequent experiments. The influence of this treatment on the extractability of hydroxy[³H]proline-labelled collagen 24 h after injection was compared with that of the known lathyrogen β -aminopropionitrile at a dose of 5 mg per egg (the LD₅₀ of β -aminopropionitrile for 13-day embryos).

The amount of salt-extractable collagen (which represents the least cross-linked pool) was increased by β -aminopropionitrile, and to a smaller extent by 4-deoxypyridoxine (Table 3). There was a decrease in the total incorporation of radioactivity found as hydroxy[³H]proline in the 4-deoxypyridoxine-treated groups. It is not clear whether this reflects decreased synthesis or increased degradation.

Table 3. *Extraction of radioactively labelled collagen from 14-day embryos injected at day 13 with 250 μ Ci of L-[5- 3 H]proline*

For experimental details see the text. Values are obtained by pooling the radiolabelled bones from three embryos in each group.

Extract	Incorporation (c.p.m. of hydroxy[3 H]proline/g dry wt. of bone)		
	Control	β -Aminopropionitrile-injected	4-Deoxypyridoxine-injected
Neutral saline	1000 (0.7%)	30 000 (21%)	5300 (4.2%)
Acetic acid	3400 (2.3%)	3000 (2.1%)	1800 (1.4%)
Residue	145 000 (97.3%)	108 000 (77%)	119 800 (94.3%)
Total	149 000	141 000	127 000

Hydroxyproline was determined in unlabelled bone extracts; these values were used to estimate the total collagen extractable into each solvent after injection of 4-deoxypyridoxine or β -aminopropionitrile (Table 4).

4-Deoxypyridoxine again increased the amount of extractable collagen, whereas overall collagen content was decreased. The viscosities of bone extracts made from embryos treated with a range of compounds suspected of having lathyrogenic activity have been compared (Levene, 1962). In that study, extracts from control chicks never had relative viscosities in excess of 3.0; compounds that produced a higher value were considered to be lathyrogens. The saline extracts from the leg bones of each group were therefore analysed by viscometry (Table 5). By this criterion, 4-deoxypyridoxine qualifies as a lathyrogen, though its potency is less than that reported for the least active of the classically defined lathyrogens, dimethylhydrazine ($\eta_{rel.} = 5.0$).

Effect of 4-deoxypyridoxine on the activity of lysyl oxidase and other enzymes in vivo

As well as lysyl oxidase, three other enzymes were studied; serum aspartate aminotransferase, serum lactate dehydrogenase and liver serine dehydratase; the last-mentioned enzyme was included because of its sensitivity to vitamin B-6 deficiency (Hunter & Harper, 1976); aspartate aminotransferase activity has been used to assess vitamin B-6 status in the growing chick (Murray *et al.*, 1978), and lactate dehydrogenase activity was assayed as a convenient non-pyridoxal-dependent enzyme. The effect of 4-deoxypyridoxine injected at day 13 on the enzyme activities is shown in Table 6.

Aspartate aminotransferase activity was not significantly affected after the administration of β -aminopropionitrile, but fell to 57% of the control value 24 h after injection of 4-deoxypyridoxine. Serine dehydratase activity was so low in both the control and 4-deoxypyridoxine-treated groups that the observed 34% loss of activity in the latter may have little significance. Lactate dehydrogenase ac-

Table 4. *Extraction of collagen from the bones of chick embryos 24 h after the injection of 4-deoxypyridoxine or β -aminopropionitrile*

Experimental details are given in the text. Values are expressed as means $\pm$ s.d. for triplicate assays performed on samples of the hydrolysates of bones pooled from eight embryos from each group.

	Collagen (mg/g dry wt. of bone)		
	Control	β -Amino- propionitrile- injected	4-Deoxy- pyridoxine- injected
Neutral-saline extract	6.12 $\pm$ 0.27 (6.1%)	31.63 $\pm$ 0.54 (31%)	8.7 $\pm$ 0.15 (9.7%)
Residue	93.7 $\pm$ 5.3	70.2 $\pm$ 2.2	80.5 $\pm$ 6.3
Total	99.8	101.8	89.2

Table 5. *Viscometry of bone extracts*

For experimental details see the text. Values are expressed as means $\pm$ s.d. for three determinations made with the pooled extracts from eight embryos per group.

Injection	$\eta_{rel.}$ of neutral saline extracts
Saline	2.13 $\pm$ 0.016
β -Aminopropionitrile	26.63 $\pm$ 1.20
4-Deoxypyridoxine	3.55 $\pm$ 0.025

tivity, as expected, remained unchanged after injection of either compound.

Lysyl oxidase activities were decreased to 61% of control values 24 h after the administration of β -aminopropionitrile. In the cartilage of chicks given 4-deoxypyridoxine the enzyme activity had fallen to 74% of control values; here, the lost activity was not restored by incubation of the cartilage extracts with 10 μ M-pyridoxal 5'-phosphate at 37°C for 1 h before the assay.

Discussion

The data presented here provide additional evidence that pyridoxal phosphate, as a cofactor of lysyl oxidase, is required for the cross-linking of collagen and elastin.

Table 6. *Effect on enzymes of the 14-day chick embryo of the injection, 24 h earlier, of β -aminopropionitrile or 4-deoxypyridoxine*

For experimental details see the text. Values are expressed as means $\pm$ S.D. for three (lysyl oxidase) or two (other enzymes) determinations.

Injection	Aspartate aminotransferase activity [Karmen (1955) units/ml]	Serine dehydratase activity (μ mol/h per g dry wt.)	Lactate dehydrogenase activity (μ mol/min per ml)	Lysyl oxidase activity (c.p.m./g dry wt.)		
				Salt- extractable	4-M-Urea- extractable	Total
Saline	28.05 $\pm$ 0	8.4 $\pm$ 3.6	674 $\pm$ 0	168 $\pm$ 4	876 $\pm$ 17	1044
β -Aminopropionitrile	30.6 $\pm$ 2.6	Not determined	701 $\pm$ 9.5	36 $\pm$ 3	624 $\pm$ 25	636
4-Deoxypyridoxine	15.9 $\pm$ 0.6	5.6 $\pm$ 0.25	690 $\pm$ 19	16 $\pm$ 3	732 $\pm$ 25	768

That 4-deoxypyridoxine behaves as a lathyrogen *in vivo* is supported by our findings of increased solubility of collagen, reflected both in viscometric analysis and in the recovery of elevated amounts of hydroxy[3 H]proline from saline extracts of 4-deoxypyridoxine-treated embryos.

Despite the inhibition of growth induced by injections of β -aminopropionitrile and 4-deoxypyridoxine and the fact that one might reasonably query the significance of the enzymic data (Table 6), examination of the relatively large fall in lysyl oxidase activities, as opposed, for example, to the virtual constancy of that of lactate dehydrogenase, favours a specific cross-link defect rather than a non-specific weight-loss effect.

The lathyrogenic properties of 4-deoxypyridoxine presumably have their basis in the antivitamin activity; the compound shows a high affinity for pyridoxal kinase (Rabinowitz & Snell, 1952), and may thereby as readily become phosphorylated as is pyridoxine. The main pathway to pyridoxal phosphate is via pyridoxine phosphate (Wada *et al.*, 1959; Wada & Snell, 1961), and it is probable that 4-deoxypyridoxine competes at this stage. 4-Deoxypyridoxine also inhibits the reconstitution with pyridoxal phosphate of various apoenzymes, including those of aspartate aminotransferase (Snell & Ayling, 1968), tyrosine decarboxylase (Hurwitz, 1952) and other pyridoxal-dependent enzymes (Dietrich & Borries, 1956). When assessing the effect of vitamin B-6 deficiency on pyridoxal-dependent enzymes it should be borne in mind that the apoenzyme may itself be affected during the deficiency, so that, for example, a relationship may develop whereby the availability of the necessary form of the vitamin dictates the rate of apoenzyme synthesis or breakdown (Hope, 1955; Brin *et al.*, 1960; Kominami & Katunuma, 1976). There is evidence to suggest that the stability of some enzymes *in vivo* may be determined, in part, by the holoenzyme/apoenzyme ratio (Hunter & Harper, 1976).

Because of the slow kinetics of the reaction between lysyl oxidase and its precipitated substrate *in vivo*, and also because of the rapid rate of collagen synthesis occurring in certain tissues of the 13–17-day embryo, lysyl oxidase activity may well be at a premium during this stage of development. In this laboratory it has been shown (Murray & Levene, 1977) that considerable amounts of a radioactive metabolite of injected [3 H]pyridoxine become incorporated into embryonic cartilage lysyl oxidase over a 24 h period.

The half-life of lysyl oxidase has been estimated, on the basis of the inhibition *in vivo* by β -aminopropionitrile, as 16–18 h (Harris *et al.*, 1977). Although lysyl oxidase activity may be completely inhibited by administration of this lathyrogen, the inhibition is transient: recovery takes place rapidly until normal activity values are attained. In the present study the dramatically increased collagen solubility seen after the injection of β -aminopropionitrile indicates an initial cessation of lysyl oxidase activity; the activity 24 h after the injection was, however, only 39% below the control value, and hence considerable recovery had taken place. In contrast, 4-deoxypyridoxine diminished the lysyl oxidase activity by 26% 24 h after the injection but only slightly increased the collagen solubility.

These observations can be reconciled if one assumes that the ways in which the two compounds inhibit lysyl oxidase *in vivo* are different. On the one hand, β -aminopropionitrile binds irreversibly to lysyl oxidase, causing widespread disruption of cross-linking for as long as the lathyrogen remains in the tissues. 4-Deoxypyridoxine, on the other hand, lacking a functional group that could displace (or complex with) bound cofactor, instead induces a gradual decline in activity by either limiting the amount of cofactor available to newly synthesized lysyl oxidase apoenzyme, or by competing with the cofactor for binding sites. In the absence of pyridoxal phosphate the lysyl oxidase apoenzyme might be expected to become the subject of

proteolysis or conformational instability, or both, and to be unable to be re-activated *in vitro* by reconstitution with pyridoxal phosphate.

Relatively massive doses of 4-deoxypyridoxine are required to produce a detectable increase in the solubility of bone collagen after 24 h; the associated inhibition of growth signifies that the embryos are near death. It may reasonably be concluded that more severe manifestations of cross-link failure would not be seen after the injection of 4-deoxypyridoxine simply because the embryos would die of a severe deficiency before lysyl oxidase activity was sufficiently depressed. In the case of less severe but prolonged functional deficiencies, such as that seen during human pregnancy (Cleary *et al.*, 1975), when foetal demand for the vitamin is high (Contractor & Shane, 1970), it is possible that certain lesions may develop. As a result of their work, Levene & Murray (1977) have suggested that the aetiology of early focal lesions in the intima of the coronary artery, characterized by fraying and splitting of the internal elastic lamina (Levene, 1956), may have such an origin. The potentially serious implications of cross-linking defects *in utero* warrant further investigation.

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