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Inhibition of chick embryo lysyl oxidase by various lathyrogens and the antagonistic effect of pyridoxal

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Summary. Lysyl oxidase, which cross-links collagen and elastin, was obtained from chick embryo bone and cartilage and its substrate, elastin, from aorta. The enzyme was studied using an improved assay which enabled the stability of the substrate to be monitored. The enzyme was fully inhibited *in vivo* by β-aminopropionitrile, semicarbazide, thiosemicarbazide and isoniazid and *in vitro* by β-aminopropionitrile and semicarbazide but only partially by thiosemicarbazide and isoniazid.

Penicillamine, which solubilizes collagen by labilizing Schiff base cross-links *in vivo* and which prevents stable cross-link formation *in vitro* indirectly by binding to aldehyde groups on collagen, was shown to have no direct inhibitory effect on lysyl oxidase *in vivo* or *in vitro*.

Homocysteine, which also solubilizes collagen by a mechanism similar to penicillamine does not inhibit lysyl oxidase either *in vivo* or *in vitro*.

Pyridoxal reversed the inhibition of lysyl oxidase by semicarbazide and isoniazid *in vivo* but was unable to reverse that produced by either β-aminopropionitrile or thiosemicarbazide. These results can be explained by the presence of a sulphydryl group near the active site of lysyl oxidase, which can form a complex with the nitrile group on β-aminopropionitrile or with the thiol group on thiosemicarbazide leading to irreversible inhibition.

Keywords: chick embryo, collagen, cross-links, lathyrogens, lysyl oxidase, pyridoxal

Lysyl oxidase (protein lysine 6-oxidase, E.C 1.4.3.13) is responsible for the formation of important cross-links in collagen and elastin thus ensuring the integrity and tensile strength of the connective tissues (Robins 1982). It catalyses the oxidative deamination of specific lysine and hydroxylysine residues in tropocollagen and tropoelastin to form the corresponding aldehyde derivatives (Pinnell & Martin 1968), which subsequently give rise to a variety of covalent cross-links (Tanzer 1973; Bailey *et al.* 1974; Gallop & Paz 1975; Robins 1981). The presence of the enzyme has been established in chick embryo cartilage (Siegel *et al.* 1970; Stassen 1976), chick aorta (Harris *et al.* 1974), bovine aorta (Kagan *et al.* 1979), turkey aorta (Narayanan *et al.* 1982), bovine ligamentum nuchae (Jordan *et al.* 1977), calf aortic smooth muscle (Ferrera *et al.* 1982), cultured pig aortic endothelium (Levene & Heslop 1977) and human placenta (Kuva-

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niemi *et al.* 1984). The presence of the enzyme is considered to be essential for the normal development of all tissues containing collagen or elastin (see Siegel 1979; Kagan 1986). Lysyl oxidase requires copper as one cofactor (Siegel *et al.* 1970; Kagan *et al.* 1974; Harris *et al.* 1974; Shieh & Yasunobu 1976; Tang *et al.* 1983) as well as a second cofactor whose exact nature is still contested (Levene *et al.* 1988).

In the collagen molecule, M_r 300000, only two lysine residues are oxidized by lysyl oxidase (Siegel 1974). These are situated in the non-helical portions, one in the N-terminal telopeptide and the other in the C-terminal telopeptide. The resultant aldehyde groups on the side-chains then spontaneously condense with other vicinal lysine-derived aldehydes and with ε-amino groups of lysine and hydroxylysine to form the various intra and inter-molecular cross-links required for collagen to fulfil its major stress-bearing function. The enzyme shows a preference for fibrillar collagen as substrate as opposed to collagen chains or peptides (Siegel 1979). In elastin, three or four such lysine-derived aldehydes condense to form the desmosine family of cross-links specific to elastin (Partridge *et al.* 1964, 1966).

The importance of cross-link formation may be seen in the experimental condition oesteolathyrism, in which treatment during early development of the animal with β-aminopropionitrile (BAPN; the 'Lathyrus factor'), results in the irreversible inhibition of lysyl oxidase followed by the failure of cross-link formation. The resultant clinical picture includes severe skeletal deformity, avulsion of tendons, slipping of epiphyses and death following aortic rupture, conditions which resemble Marfan's syndrome of which osteolathyrism is a phenocopy (Geiger *et al.* 1933; Ponseti & Baird 1952; Ponseti & Shepard 1954; Dasler 1954; Bachhuber & Lalich 1955; Churchill *et al.* 1955).

Progress towards a fuller understanding of lysyl oxidase function has been delayed by experimental difficulties encountered with the enzyme and with its assay. The stability of the enzyme is limited. Extraction is difficult because it is almost insoluble in saline buffers although readily soluble in 4–6 M urea (Narayanan *et al.* 1974; Harris *et al.* 1974; Stassen 1976; Shieh & Yasunobu 1976; Siegel & Fu 1976; Jordan *et al.* 1977). The assay of lysyl oxidase, first described by Pinnell and Martin (1968) involves the use of a particulate substrate, chick embryo aortic elastin, which has been labelled with ^{3}H-4,5-lysine. This has a limited life due to spontaneous loss of tritium from the substrate. In an attempt to improve the reliability of this method the present study has incorporated modifications which have permitted a better evaluation of compounds suspected of preventing the action of lysyl oxidase.

This present study is therefore concerned with the effect of lathyrogenic agents on cross-link formation. Normal cross-link synthesis requires lysyl oxidase, its two cofactors (copper and a second cofactor not, as yet, fully identified), as well as the two substrates, collagen and elastin. Absence of any of these components results in a failure of cross-linking which manifests as fragility of the connective tissues and increased collagen solubility. To try to elucidate the modes of action of established lathyrogens we have examined some of these compounds on the developing chick embryo.

Materials and methods

Fertile eggs were purchased from Winter Egg Farm, Thriplow, Cambs, UK. L-4,5-^{3}H-lysine monohydrochloride (specific activity 3.11 TBq/mmol) was purchased from Amersham International, Bucks, UK, methylene aminoacetonitrile (MAAN) and thiosemicarbazide (TSC) from Aldrich Chemical Co., Gillingham, Dorset, UK. All other lathyrogenic compounds, β-aminopropionitrile monofumarate (BAPN), aminoacetonitrile (AAN), semicarbazide HCl (SC), isoniazid (INAH) as well as pyridoxal HCl, penicillamine and homocysteine were standard catalogue items from Sigma Chemical Co. Ltd, Poole,

Dorset, UK and were used without further purification. Emulsifier-Safe liquid scintillation fluid was purchased from Packard Instrument, Reading, Berks, UK. All other reagents were of analytical grade where possible.

Millipore Ultrafree MC 10000 NMWL Filter Units were obtained from Millipore UK, Wembley, Middlesex, UK and Dowex 50W-X8 from BDH Chemicals Ltd, Poole, UK).

Production of lathyrism in the chick embryo

At least 30 fertile chick eggs were injected at 15 days of incubation with 0.054 mmol of each lathyrogenic compound dissolved in 0.1 ml distilled water through a pinhole in the shell of fertile eggs on to the surface of the chorioallantoic membrane. Controls received 0.1 ml water. The hole was sealed with wax and the incubation continued for a further 48 h. At this time the tibiae and femora from live embryos only were dissected on ice and the bones freed of muscle and ligament. The pooled bones and cartilages were frozen in liquid nitrogen, pulverized in a steel mortar, and extracted for 24 h at 4°C with a solution of 6 M urea + 0.05 M $(NH_4)_2 SO_4$ (6 M urea), pH 7.6 (4/1 v/w) with constant shaking. Extracts were separated from the residue by centrifugation at 20000 *g* at 4°C in a Microcentrifuge 154 (Ole Dich, Denmark) and the clear supernatant filtered in the cold through a fine sintered glass funnel.

Modifications to the assay

Tritiated water (^3H_2O) is one of the products of the oxidative deamination of peptidyl-^{3}H-lysine in the aortic substrate. This fact was used by Pinnell and Martin (1968) in the original assay as a measure of the activity of lysyl oxidase. The ^3H_2O formed in the reaction was originally separated from the reaction mixture by distillation. We have replaced this distillation step by centrifugation of the deproteinized supernatant through Millipore Ultrafree MC 10000 NMWL Filter Units. These filters retain material of M_r greater than 10000. The filtrate was then passed through a Dowex 50WX8 cation exchange column (Melet *et al.* 1977) in order to remove ^{3}H-lysine and ^{3}H-lysine peptides present in the substrate as the result of non-specific proteolytic or hydrolytic activity. Melet *et al.* (1977) showed that results obtained by passage of extracts over Dowex columns were almost identical with those obtained by distillation of the extracts. Our control experiments confirmed this observation. ^3H_2O was then eluted from the column with 5% trichloroacetic acid (TCA) and its radioactivity measured (see below).

A second difficulty encountered with this assay was the high background radioactivity, which rose during storage of the substrate before use. This increase in background appeared to be due to tritium exchange and was successfully overcome by washing and resuspending the particulate substrate in fresh phosphate buffered saline (PBS) immediately before use for as many times as was necessary to lower the background to acceptable levels.

Substrate preparation

The radiolabelled 'elastin' substrate was prepared from 17-day-old chick embryo aortae which were incubated for 24 h in Dulbecco's Minimal Eagle's medium together with 0.5 mCi L-4,5-^{3}H-lysine/48 aortae in the presence of penicillin (100 μg/ml), streptomycin (100 μg/ml) and ascorbic acid (150 μg/ml). The substrate, commonly referred to as 'elastin', has been shown to consist of 88% elastin and 12% collagen, based on elastase and collagenase digestion (Siegel 1979). To inhibit endogenous aortic lysyl oxidase, β-aminopropionitrile fumarate (BAPN, 400 μM) was added. At the end of the incubation period, the medium was decanted and the aortae washed twice with 10 ml PBS to remove most of the unreacted BAPN and surplus label. The labelled aortae were then homogenized in 5 ml of PBS in a Polytron homogenizer (Kinematika, Switzerland),

with three bursts of 10 s followed by three bursts of 15 s in an MSE ultrasonicator to give a fine particulate suspension. This suspension was centrifuged for 10 min at 20 000 *g* and 4°C and the resulting pellet resuspended in 5 ml PBS. This procedure was repeated twice to remove any remaining BAPN and free label. The final suspension was adjusted to contain 500 000 d.p.m. in 20–50 μl, divided into five or six portions and stored at −70°C until use. The substrate remained usable for up to 3 weeks or when the background radioactivity in blank assay tubes reached 500 d.p.m. or when additional washing was ineffective in reducing this background.

Lysyl oxidase assay

Prior to assay, the urea extracts of bone and cartilage were dialysed in the cold against three changes of PBS in order to remove all traces of urea which interferes with the assay. Lysyl oxidase was determined by the release of ^3H_2O from the elastin substrate, which had been radioactively labelled with 4,5-^{3}H-lysine as described above. For each assay, 0.3 ml of extract together with a volume of substrate containing approximately 500 000 d.p.m. was made up to 1 ml with PBS. Six estimations together with six blanks were performed for each sample. Blanks contained substrate and PBS but no enzyme. The mixture was incubated for 2 h in a shaking water bath at 45°C (Kagan 1986) and the reaction terminated by the addition of 100 μl 50% TCA.

Precipitated protein was removed by centrifugation at 11 000 *g* for 10 min. The supernatant was then centrifuged through a Millipore Ultrafree filter unit for 15 min at 11 000 *g* (as described above) and 0.3 ml of the filtrate made up to 1 ml with distilled water. 100 μl of this diluted filtrate was counted for radioactivity and the remaining 0.9 ml passed through a 2-cm cation exchange column (Dowex 50W-X8(H), 200–400 mesh) made up in a long Pasteur pipette (i.d. 5mm), plugged loosely with a small glass bead. Before use the column was washed with 1 ml 5% TCA. ^3H_2O was eluted from the column with 1 ml 5% TCA and the radioactivity counted.

Radioactivity in the Millipore filtrate consists of both ^3H_2O and non-water tritium (non-H_2O ^{3}H). The radioactivity in the extract following Dowex treatment is a measure of ^3H_2O only. The difference between these two figures is therefore a measure of what is here designated as non-H_2O ^{3}H and represents ^{3}H-lysine peptides or ^{3}H-lysine originating from the substrate.

Radioactivity in both the 0.9 ml of eluate and the 0.1 ml of filtrate was measured by mixing with 10 ml Emulsifier Safe scintillant in 20 ml scintillation vials followed by counting in a Packard liquid scintillation counter (Tricarb 300) with automatic external standardization.

With the radioactively labelled substrate prepared in the manner described above, even though it was always diluted to contain 500 000 d.p.m. per assay tube, there was a wide variation in the amount of labelled product extracted even in the absence of lathyrogen (control values). This is a commonly seen problem and is, in large part, due to the wide variation in non-substrate radioactivity following from the binding of the label to lysine residues that were not involved in cross-link formation. Nevertheless, it was possible to compare control and treated embryos within each experiment through the use of the same source of substrate. It was therefore important to maintain the uniformity of the enzyme throughout any experiment which would involve comparisons between the effects of several lathyrogens and control. This was achieved by pooling the extracts from all 30 or more embryos used at any one time. As a result, s.e.m. values serve only to estimate the precision of the assay. However, the results of 5 separate experiments are described in Table 1 and are compared by Student's paired *t*-test.

Table 1. Effect of β-aminopropionitrile treatment on the amounts of tritiated water and non-water tritium resulting from the action of chick embryo lysyl oxidase from bone and cartilage on aortic elastin substrate

Expt.		Activity after Millipore filtration (d.p.m./mg protein) Total ^3H	Activity after Dowex treatment (d.p.m./mg protein) ^3H_2O	Non-H_2O ^3H
(1)	Control	6675	1917*	4758
	BAPN-treated	8532	48	8484
(2)	Control	10061	2173*	7881
	BAPN-treated	4227	69	4208
(3)	Control	6514	1149*	5365
	BAPN-treated	4063	87	3976
(4)	Control	6653	1351*	5284
	BAPN-treated	8236	0	8236
(5)	Control	2971	940*	2301
	BAPN-treated	1206	0	1206

The results of five separate experiments (performed in sextuplicate) on normal and chick embryos treated with 0.054 mmol of β-aminopropionitrile (BAPN) showing total radioactivity in protein-free extracts of bone and cartilage after assay for lysyl oxidase. Extracts were filtered through Millipore Ultrafree Units and then passed through Dowex 50X-8 columns to remove non-H_2O ^3H. For details see Methods.

There was no significant difference in non-H_2O ^3H between normal and BAPN-treated embryos.

*Significant difference in each group between normal and BAPN-treated ($P<0.001$, by Student's paired *t*-test).

determined by the method of Lowry *et al.* (1951) and enzyme activity (over 2-hour incubation period) expressed as d.p.m./mg protein.

Results

Initial observations in three preliminary experiments suggested that treatment of chick embryos with BAPN *in vivo* increased by about 30% the amount of non-H_2O ^3H formed during incubation with extracts of bone whilst completely inhibiting lysyl oxidase. A more comprehensive study was then undertaken and the results are shown in Table 1. Bone and cartilage extracts from normal and BAPN-treated chick embryos were assayed for lysyl oxidase activity employing the Millipore filter and Dowex-50 columns to separate non-H_2O ^3H. No significant difference was observed in non-H_2O ^3H between control and BAPN treated extracts. Thus the initial observations could not be confirmed.

Two other organic nitriles, methylene aminoacetonitrile (MAAN) and aminoacetonitrile (AAN), had effects similar to BAPN. Inhibition of lysyl oxidase was complete when 17-day old chick embryos were treated 2 days previously with 0.054 mmol/egg of each compound (Fig. 1).

Semicarbazide (SC), isoniazid (INAH) and thiosemicarbazide (TSC) all inhibited lysyl oxidase completely when 17-day-old chick embryos were treated *in vivo* 2 days previously with 0.054 mmol/egg of these compounds (Fig. 1). When two identical and

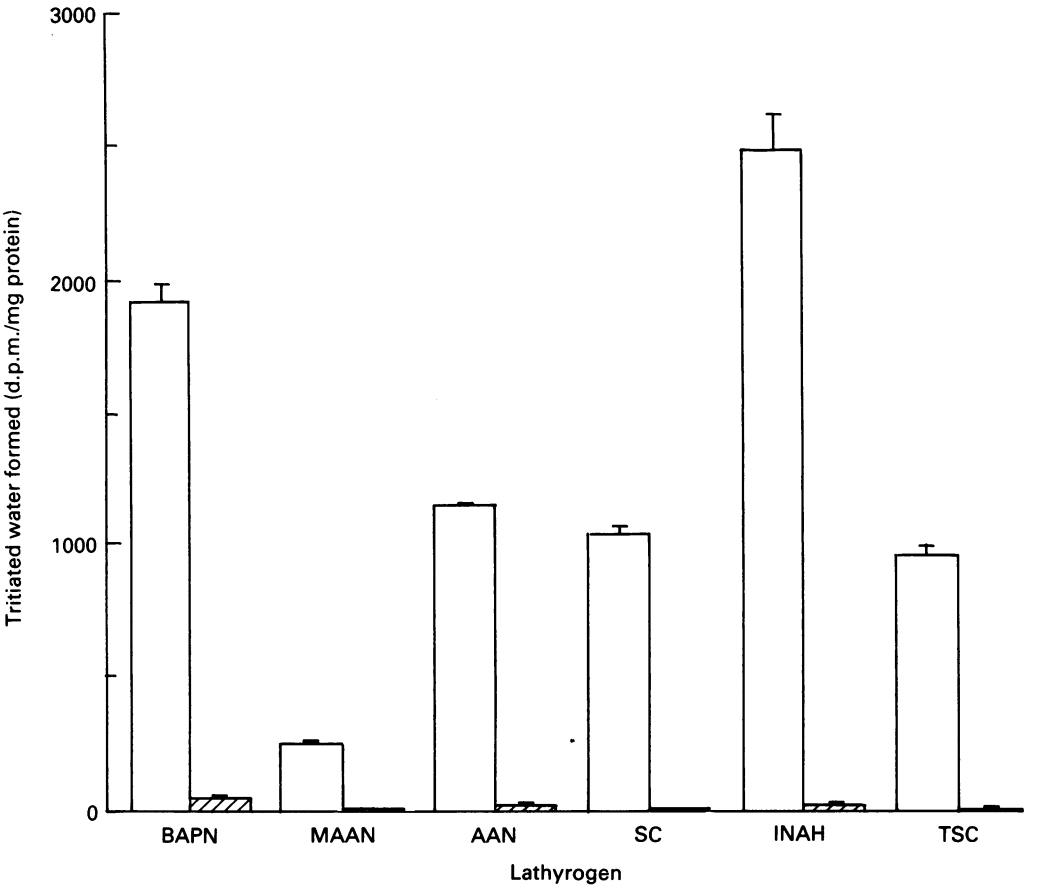

Fig. 1. Effect of lathyrogenic compounds on lysyl oxidase activity from chick embryos treated *in vivo*. Fifteen-day-old chick embryos were treated with 0.054 mmol of each lathyrogenic compound. Extracts from bone and cartilage were assayed for lysyl oxidase activity 48 h later. Following Millipore filtration ^3H_2O was separated from non-H_2O ^3H by passage over a Dowex column. Each value represents the mean ± s.e.m. of six separate estimations of the pooled enzyme source per drug treatment or control, that is, 12 separate homogenates, each from at least 30 normal embryos, were used. □, Normal; ▨, treated.

separate experiments with TSC as the lathyrogen were performed but using the distillation method of Pinnell and Martin (1968) to extract ^3H_2O, the results were (in d.p.m./mg protein) control values: 1920 and 1283, and TSC treated: 220 and 25, respectively.

Preincubation *in vitro* of extracts from normal 17-day-old chick embryo bone and cartilage for 3 h at 37°C with BAPN, SC or TSC at a concentration of 400 μM resulted in complete inhibition of lysyl oxidase. BAPN and SC also fully inhibited the enzyme after preincubation at the lower concentration of 40 M but TSC only partially inhibited the enzyme at this concentration. INAH only partially inhibited at both concentrations (Fig. 2).

TSC fully inhibited lysyl oxidase *in vivo* (Fig. 1) but the other sulphur-containing compounds, homocysteine and D-penicillamine, had no effect *in vivo* (Table 2). *In vitro*, TSC fully inhibited at 400 μM whilst homocysteine and D-penicillamine had no effect at the lower and little if any at the higher concentration (Table 3).

Chick embryos were injected with TSC

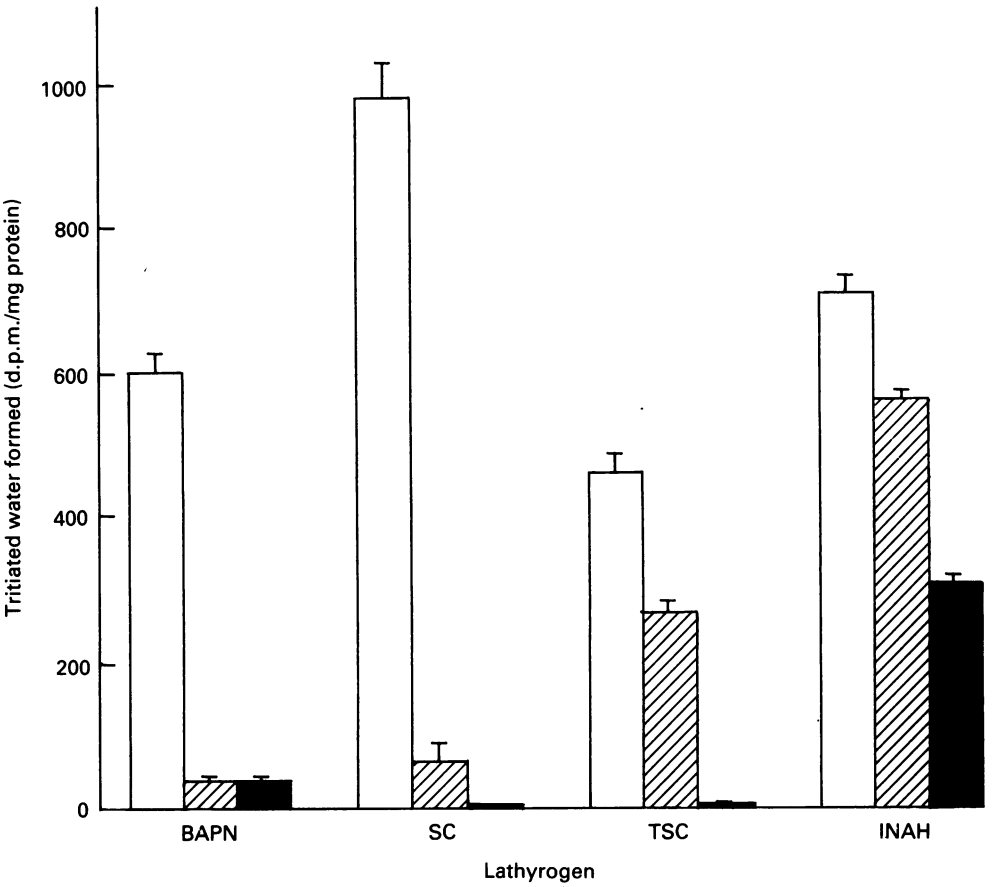

Fig. 2. Effect on lysyl oxidase of preincubation of normal chick embryo bone and cartilage extract with various lathyrogenic compounds *in vitro* at 40 μM and 400 μM final concentration, expressed as d.p.m./mg protein. Bone extracts from 17-day-old normal chick embryos were preincubated for 3 h at 37°C with various lathyrogens at concentrations of 40 and 400 μM and lysyl oxidase activity assayed. Each value represents the mean ± s.e.m. from 6 separate estimations per drug treatment or control from the same sample of enzyme for each comparison, that is four separate homogenates, each from at least 30 normal embryos, were used. □, Normal; ▨, 40; ■, 400 μM.

(0.054 mmol/egg) on day 15, further injected with pyridoxal (0.054 mmol/egg) on day 16, and the bones and cartilages harvested on day 17. In three experiments, pyridoxal was shown to be unable to reverse the total inhibition of lysyl oxidase which TSC had caused.

Discussion

The results described in Table 1 illustrate that, after passage through the ion exchange column to separate ^3H_2O from non-H_2O ^3H, there was virtually no ^3H_2O produced in the bone and cartilage extracts from embryos treated with BAPN, indicating total inhibition of lysyl oxidase. However, no statistical difference could be found between the amounts of non-H_2O ^3H from control and treated embryo preparations. These results therefore failed to support our preliminary results that seemed to suggest that there was an increased amount of non-H_2O ^3H formed

Table 2. Effect of sulphur-containing compounds on lysyl oxidase activity in extracts of bone and cartilage from chick embryos treated *in vivo*

Compound 0.054mM/egg	Lysyl oxidase activity (d.p.m./mg protein)	
	Control	Treated
Thiosemicarbazide	947±45.6	17±5.8
Homocysteine	947±45.6	1423±61
D-Penicillamine	947±45.6	1146±52

17-Day-old chick embryos, injected 48 hours earlier with 0.054 mmol of either compound or distilled water. Assays included Millipore filtration and passage over a Dowex column. Each value represents the mean±s.e.m. of six separate estimations of the pooled enzyme source per drug treatment or control, that is, four separate homogenates, each from at least 30 normal embryos, were used. The whole experiment was performed at the same time with the same control group.

during incubation with extracts of bone although the lysyl oxidase had been completely inhibited by the dose of BAPN that had been chosen. As the sole source of non-H_2O ^3H was the labelled aortic substrate used for the assay of lysyl oxidase, it had seemed possible that, in addition to inhibiting lysyl oxidase, BAPN also caused an increase in the degradation of the substrate leading to an increase in non-H_2O ^3H. However, it does not appear that this hypothesis can be sustained.

The other known lathyrogens tested in this study were the nitrile compounds, MAAN and AAN and the non-nitriles, SC, TSC and INAH. Like BAPN, all these compounds completely inhibited lysyl oxidase *in vivo* at a dose of 0.054 mmol/egg. *In vitro* the non-nitriles exhibited a spectrum of diminishing activity; SC inhibited at both concentrations used, TSC fully inhibited at the higher concentration only, whilst INAH partially inhibited at both concentrations tested. These results obtained here by use of the more stringent assay technique confirmed our earlier observations (Carrington *et al.* 1984; Levene & Carrington 1985).

In this study the effect of penicillamine on lysyl oxidase has been examined *in vivo* as well as *in vitro*, in which latter case it was found to cause negligible inhibition even at 400 μM. Penicillamine *in vitro* has been reported to inhibit lysyl oxidase partially at a concentration of 100 μM and fully at 10 mM (Nimni *et al.* 1972; Misiorowski & Werner

Table 3. Effect of preincubation *in vitro* with sulphur-containing compounds, on lysyl oxidase activity in normal extracts of bone and cartilage from chick embryos

Compound tested	Lysyl oxidase—d.p.m./mg protein		
		Concentration of lathyrogen	
	Control	40 μM	400 μM
Thiosemicarbazide	458±25.9	264±19.6	4±2.6
Homocysteine	490±37.9	460±24.6	300±19.2
D-Penicillamine	455±12.5	460±30	389±20

Extracts from bones of normal 17-day chick embryos pre-incubated with compounds at 40 μM and 400 μM final concentration and assayed for lysyl oxidase. Each value represents the mean±s.e.m. from six separate estimations per drug treatment or control from the same sample of enzyme for each comparison, that is, three separate homogenates, each from at least 30 normal embryos, were used.

1978). This very weak effect *in vitro* was reflected in its lack of inhibition of the enzyme *in vivo*. Penicillamine has been successfully used in the treatment of Wilson's disease, albeit with side-effects, in order to chelate copper which is excessively deposited in the brain (Walshe 1977). This might indicate that this chelation of copper could have led to the inhibition of the copper-dependent lysyl oxidase but this is clearly not the case. The major action of penicillamine is exerted on the collagen itself (see Nimni 1977). It is also used in scleroderma in order to mobilize collagen which has been deposited in various organs and, in particular, in the skin. It has been reported to react with the aldehyde cross-link precursors in collagen (Deshmukh & Nimni 1969) and, through this effect, may wrongly appear to inhibit lysyl oxidase. Nimni's studies (1977) have elucidated the complex effects produced by penicillamine in the rat *in vivo* and *in vitro*. *In vivo*, the solubility of neutral salt-soluble collagen is greatly increased but the aldehyde content of this collagen is not, however, diminished. The increase in collagen solubility is the result of the breaking of pre-existing Schiff base cross-links (Rojkind & Juarez 1966; Deshmukh & Nimni 1969). Although Nimni's studies did not include measurement of lysyl oxidase *in vivo*, it is reasonable to assume from the normal aldehyde content that lysyl oxidase is not inhibited by penicillamine *in vivo*. This is consistent with the present findings on lysyl oxidase.

In addition to breaking down pre-existing cross-links, penicillamine *in vivo* binds to aldehyde groups present on collagen to form a thiazolidine complex between the lysine-derived cross-link precursor, α-aminoadipic-δ-semialdehyde and penicillamine, thus preventing formation of stable cross-links. The resulting complex is in equilibrium with its products and can be completely dissociated by exhaustive dialysis. L-Cysteine, cysteamine and other analogues also bind to free aldehydes on collagen *in vivo* in a similar manner and with similar results (Nimni *et al.* 1969). These authors have further shown that *in vitro*, the formation of stable cross-links in normal collagen is also inhibited by the formation of thiazolidine complexes.

At 40 μM, homocysteine had no inhibitory effect on lysyl oxidase *in vitro* but, at 400 μM, the activity of the enzyme was reduced to about 60% of normal. In an earlier study by Siegel (1975) no support could be found for the possibility that lysyl oxidase might be inhibited by homocysteine *in vivo*. Indeed, he showed an increase in aldehyde-derived cross-links in homocystinuria. In our present study a significant rise (about 50%) was seen in the activity of the enzyme from embryos treated with homocysteine, which would be consistent with an increase in collagen cross-linking. Homocystinuria is an inherited disease in man caused by reduced activity of cystathione synthetase, an enzyme which converts homocysteine and serine to cystathione (McKusick 1972). When the enzyme is deficient, homocysteine, serine and methionine accumulate in the tissues and homocystine appears in the urine. The clinical picture resembles, but is distinct from, Marfan's syndrome and involves the connective tissues. The mechanism underlying the connective tissue disorder is not however clear. Harris and Sjoerdsma (1966) observed that, in homocystinuria, skin collagen was more soluble than normal, suggesting an abnormality in collagen cross-linking. Kang and Trelstad (1973) noted the similarity in the structures of homocysteine and penicillamine and suggested that homocysteine might bind to lysine-derived aldehyde precursors of collagen cross-links and so increase collagen solubility. It will be necessary to examine the effects of homocysteine treatment of chick embryos on the tensile strength of their collagen in order to try to resolve the apparent discrepancies between these latter findings and those showing an increase in aldehyde-derived cross-links and lysyl oxidase activities.

In the present study, good agreement was seen between the distillation method and the more convenient method described here.

Both methods showed that TSC inhibited lysyl oxidase *in vivo*. The modified method was also used to show that TSC inhibited lysyl oxidase *in vitro*. These observations accord with the findings of Tanzer *et al.* (1966) who showed that collagen from chick embryos treated *in vivo* with ^{35}S-TSC contained negligible amounts of aldehyde. Normal calf skin collagen incubated *in vitro* with ^{35}S-TSC was shown to bind two moles of TSC per mole of collagen to form thiosemicarbazones. Rojkind and Gutierrez (1969) have confirmed that TSC treatment *in vivo* results in a collagen which is deficient in aldehydes. Their spectrophotometric evidence supports the view that in-vitro treatment of normal collagen with TSC results in the formation of thiosemicarbazones by reacting with aldehydes to prevent the formation of normal intermolecular cross-links. These studies indicate that a failure to find aldehydes may signify either that they are absent or that their presence is masked by thiosemicarbazone formation.

An attempt was made to reverse the inhibitory actions of TSC with the putative cofactor of the enzyme, pyridoxal, deficiency of which has been shown to lead to the inhibition of lysyl oxidase (Murray & Levene 1977; Murray *et al.* 1978; see Levene 1989). It was found that pyridoxal treatment did not reverse the inhibition of lysyl oxidase produced by TSC *in vivo*. In contrast, previous studies showed that pyridoxal did reverse the inhibition produced by SC (Levene & Carrington 1985), as was that produced by INAH (Carrington *et al.* 1984). In both cases, such treatment also prevented the appearance of osteolathyrism in the chick embryos. However, similar treatment with pyridoxal has been shown to be unable to reverse the inhibition of lysyl oxidase produced by BAPN and also to be unable to prevent the clinical signs of osteolathyrism (Carrington *et al.* 1984). Following the initial observation by Pinnell and Martin (1968) that BAPN inhibits lysyl oxidase irreversibly, Tang *et al.* (1983) showed that BAPN binds to the enzyme but it does not act as a substrate. They have proposed a mechanism of inactivation in which BAPN binds at the active site by Schiff base formation between the β-amino function of BAPN and a putative functional enzyme carbonyl group at the active centre. This is then enzymically processed to a chemically reactive species which covalently binds to the enzyme, typical of enzyme inhibitors categorized as suicide inactivators (Rando 1975). Tang *et al.* (1983) propose that the nitrile group plays an essential electophilic role in the tautomeric changes occurring in the enzyme-bound inhibitor. Consistent with this scheme they have shown that other electrophilic substituents including bromine, chlorine or a nitro moiety were able to substitute for the nitrile group in BAPN to yield new suicide inhibitors of lysyl oxidase (Tang *et al.* 1984).

At present we have no direct evidence in support but consider that TSC may exert its inhibitory action in a similar manner to that of BAPN by attacking a thiol group that may be in the vicinity of the active site. The nitrile group and the thiol group could then react with the sulphydryl group of the TSC causing the inhibitor to be bound tightly to the enzyme and excluding access to pyridoxal. The alternative possibility, discussed earlier in relation to BAPN, that the irreversible action of TSC might be due to a reduction in the effective concentration of the substrate by removal of lysine from the labelled collagen, was examined in this present study and was not found to take place. In addition, it does not seem that the results reported here will have been affected either by the actions of proteolytic enzymes or by any effect of these lathyrogens upon them. Control experiments have shown that BAPN, even at 250 μM, has no effect on the hydrolysis of casein by trypsin, nor has any direct influence on blood clotting by these agents been reported.

The results reported here demonstrate clearly that chick embryo lysyl oxidase is inhibited by the action of the lathyrogens, BAPN, SC, INAH and TSC *in vivo* and *in vitro*. BAPN and TSC were confirmed as irreversible inhibitors while the other agents were

reversible. These results also support the view that penicillamine and homocysteine, which react with collagen, do not inhibit lysyl oxidase but may nevertheless inhibit cross-link formation, most probably by introducing steric hindrance, and so prevent the formation of intra and inter-molecular cross-links in collagen (Nimni 1977).

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