The inhibition of protein-lysine 6-oxidase by various lathyrogens

Evidence for two different mechanisms

Charles I. LEVENE* and Michael J. CARRINGTON
Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PQ, U.K.

Lathyrogens decrease collagen and elastin cross-linking by inhibiting lysine oxidase. The lathyrogens isoniazid and semicarbazide decrease liver pyridoxal phosphate and are teratogenic; all their effects are reversed by pyridoxal. β-Aminopropionitrile, another lathyrogen, does not affect liver pyridoxal phosphate, and its lathyrogenic and teratogenic effects are not reversed by pyridoxal. Time courses of these effects differ greatly, suggesting enzyme inhibition by different mechanisms.

INTRODUCTION

A lathyrogen is a compound which prevents the cross-linking of collagen and elastin in vivo by inhibiting lysine oxidase (protein-lysine 6-oxidase, EC 1.4.3.13). This enzyme acts by oxidatively deaminating the ε-amino groups of certain lysine and hydroxyllysine residues, resulting in the formation of their aldehydic derivatives. These can then cross-link non-enzymically with specific lysine or hydroxyllysine residues in neighbouring collagen chains and molecules, by Schiff-base formation or by more stable aldol condensations, to give collagen its tensile properties (Bailey, 1968; Robins, 1982). Similar reactions occur in the cross-linking of elastin (Partridge et al., 1964). The enzyme has two obligatory cofactors, i.e. copper (Miller et al., 1965; Carnes, 1971; Harris & O'Dell, 1974) and a pyridoxal derivative (Murray & Levene, 1977; Murray et al., 1978; Bird & Levene, 1982, 1983). The changes in a lathyrinic chick embryo include striking fragility of the connective tissues and an increase in the salt-solubility of collagen and elastin; these may result in avulsion of tendon from bones, slipping of epiphyses, subluxation of the lenses of the eyes from their suspensory ligaments, severe skeletal deformity and, often, death from aortic rupture.

During a study of the mode of action of lathyrogens (Levene, 1961a), four separate chemical groups were identified (Table 1). These four groups share the property of being carbonyl-blocking agents. Pyridoxal was able to antagonize the lathyrogenic effect of isoniazid (INAH) in the chick embryo in vivo (Levene, 1961b, 1962) and restore the impaired collagen strength to near normal. The lathyrogenic effect of β-aminopropionitrile (BAPN), however, was not antagonized by pyridoxal; these findings suggested that these two lathyrogens may act differently on the enzyme, both leading, however, to cross-link inhibition and its sequelae. The present study provides new evidence that semicarbazide (SCH), a lathyrogen belonging to the ureide group, acts unlike BAPN, but very like INAH, a member of the hydrazide group, indicating that there are at least two different mechanisms of action of inhibitors on lysine oxidase.

EXPERIMENTAL

Incubation and injection of fertile eggs

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 days before being used. Drugs were dissolved in distilled water, and 0.1 ml of solution was injected through a pin-hole in the shell. Most injections were administered after 14 and 15 days of incubation and the embryos harvested on day 16, except in the time-course experiment, when they were harvested at the stated times. Into each egg 6.0 mg of SCH and 11.0 mg of pyridoxal

<table>
<thead>
<tr>
<th>Table 1. Examples of the four groups of lathyrogens</th>
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<tbody>
<tr>
<td><strong>Lathyrogenic group</strong></td>
</tr>
<tr>
<td>Organic nitriles</td>
</tr>
<tr>
<td>Ureides (C–N bond)</td>
</tr>
<tr>
<td>Hydrazides (C–C bond)</td>
</tr>
<tr>
<td>Hydrazine hydrate and some derivatives</td>
</tr>
</tbody>
</table>

Abbreviations used: PBS, phosphate-buffered saline (0.1 m-sodium phosphate/0.15 m-NaCl, pH 7.6); BAPN, β-aminopropionitrile; INAH, isonicotinic acid hydrazide (isoniazid); SCH, semicarbazide.

*To whom reprint requests should be addressed.
Table 2. Comparison of the effects of INAH and SCH on collagen solubility, lysine oxidase and liver pyridoxal phosphate, and on their reversibility by pyridoxal

Hydroxyproline is expressed as µg extracted into 4 vol. (v/w) of PBS/g wet wt. of bone. Values are means ± half the difference between duplicates. Lysine oxidase was assayed on 0.5 ml samples in a total volume of 1.5 ml. For liquid-scintillation counting 1.0 ml samples were taken, and results were corrected to 1.5 ml. Counting efficiency was approx. 30%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydroxyproline (µg/g wet wt. of bone)</th>
<th>Lysine oxidase (d.p.m./0.5 ml of extract)</th>
<th>Pyridoxal phosphate (nmol/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.80 (100%)</td>
<td>4789 ± 158 (100%)</td>
<td>46.4 ± 1.4 (100%)</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>1.80 (100%)</td>
<td>4589 ± 83 (96%)</td>
<td>60.9 ± 1.1 (131%)</td>
</tr>
<tr>
<td>INAH</td>
<td>3.36 (183%)</td>
<td>1573 ± 83 (33%)</td>
<td>29.7 ± 1.4 (64%)</td>
</tr>
<tr>
<td>INAH + pyridoxal</td>
<td>2.62 (143%)</td>
<td>1971 ± 207 (41%)</td>
<td>43.2 ± 0.7 (93%)</td>
</tr>
<tr>
<td>SCH</td>
<td>4.34 (235%)</td>
<td>1141 ± 72 (24%)</td>
<td>26.0 ± 1.4 (56%)</td>
</tr>
<tr>
<td>SCH + pyridoxal</td>
<td>3.52 (189%)</td>
<td>1692 ± 202 (35%)</td>
<td>38.6 ± 0.7 (83%)</td>
</tr>
</tbody>
</table>

were injected; these are stoichiometric amounts of the two compounds.

Leg bones and cartilage from the embryos were homogenized in PBS. After shaking overnight at 4 °C, the homogenate was centrifuged at 30000 g for 30 min and the supernatant collected.

**Lysine oxidase assay**

PBS extracts of leg bones and their cartilages were assayed as soon as possible after extraction. Lysine oxidase activity was determined by the release of $^3$H$_2$O 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).

**Hydroxyproline assay**

The PBS extracts were hydrolysed at 110 °C overnight in 6 M 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 (1963), and the result was used as a measure of extractable collagen.

**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**

SCH, like INAH and BAPN, increased the PBS extractability of collagen from the leg bones of 16-day embryos which had been treated with 6 mg of SCH/egg at day 14; unlike BAPN, subsequent injection of SCH-treated embryos with pyridoxal at day 15 (11.0 mg/egg) reversed the increase in collagen solubility (Table 2). Lysine oxidase activity (also extracted into PBS) fell greatly after either INAH or SCH treatment and rose after the pyridoxal treatment, although it never recovered to the control value (Table 2). Similarly, liver pyridoxal phosphate content fell after INAH or SCH treatment and rose after pyridoxal treatment (Table 2).

Aortic lysine oxidase is usually extracted into buffers which contain 4–6 M urea; because very little activity can be extracted under non-denaturing conditions and because lysine oxidase is very stable in urea. In contrast, using chick embryo leg bones, we have found that PBS extracts contain 85% of the activity which is extractable by 6 M urea. Furthermore, the proportions of enzyme activity extracted from control and lathyritic leg bones do not vary with the method of extraction (Table 3). Collagenase treatment did not decrease the inhibition of lysine oxidase by the compounds tested. The increased amounts of collagen present in PBS extracts of leg bones...
Lathyrogens act by different mechanisms

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a)

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Groups of removed of BAPN hydroxyproline (a) and dissolved were embryos V C x group cm -C a

extractability of collagen and on in chick embryos (0), 01. (_ -J VX E2 CO Liver ca)

pyridoxal were chick embryos were assayed after injection. All lathyrogens were measured in 0.1 ml of distilled water. Leg bones were removed at intervals, and PBS extracts were assayed for hydroxyproline (a) and lysine oxidase (b).

Fig. 1. Time course of the effect of various lathyrogens on the extractability of collagen and on the activity of lysine oxidase in chick embryos

Groups of 14-day chick embryos were injected with 1.0 mg of BAPN (○), 7.4 mg of INAH (▲) or 6.0 mg of SCH (△). Control embryos (□) were not injected. All lathyrogens were removed at intervals, and PBS extracts were assayed for hydroxyproline (a) and lysine oxidase (b).

Table 4. Liver pyridoxal phosphate contents in normal and lathyrogen-treated chick embryos

Chick embryos were injected at day 14 with SCH (6 mg/egg), INAH (7.4 mg/egg) or BAPN (1 mg/egg). The livers in each group were harvested at the time indicated, and pyridoxal phosphate values were measured as described in the text.

<table>
<thead>
<tr>
<th>Time course (h)</th>
<th>Treatment...</th>
<th>Liver pyridoxal phosphate (nmol/g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCH</td>
</tr>
<tr>
<td>0</td>
<td>51.3</td>
<td>51.3</td>
</tr>
<tr>
<td>6</td>
<td>41.3</td>
<td>17.5</td>
</tr>
<tr>
<td>12</td>
<td>42.5</td>
<td>22.5</td>
</tr>
<tr>
<td>24</td>
<td>50.0</td>
<td>26.3</td>
</tr>
<tr>
<td>36</td>
<td>45.0</td>
<td>23.8</td>
</tr>
<tr>
<td>48</td>
<td>37.5</td>
<td>26.3</td>
</tr>
<tr>
<td>72</td>
<td>41.3</td>
<td>31.3</td>
</tr>
<tr>
<td>Mean value of sum of all time points (±S.D.)</td>
<td>44.1±4.6</td>
<td>24.6±4.2</td>
</tr>
</tbody>
</table>

A 72 h time course of the effect of INAH, SCH and BAPN was performed on groups of embryos to explore any possible variations in behaviour. Extractable hydroxyproline, cartilage and bone lysine oxidase activity and liver pyridoxal phosphate values were estimated at 6, 12, 24, 36, 48 and 72 h after the original 14-day injection. The results (Fig. 1, Table 4) confirm our previous findings (Carrington et al., 1984) that INAH-treated embryos fail to recover anything like as well as BAPN-treated embryos by 24 h, but also show clearly that SCH-treated embryos behave just like INAH-treated embryos in regard to recovery and totally unlike BAPN-treated embryos.

DISCUSSION

As a result of earlier work and these new SCH data, we are now clearer about the mechanisms of action of these three lathyrogens. Our first experiment confirms earlier experiments (Carrington et al., 1984) that INAH both increases collagen solubility in the chick embryo and lowers cartilage and bone lysine oxidase activity and liver pyridoxal phosphate content; treatment with pyridoxal reverse these three effects.

The present study clearly shows that SCH, a hydrazide, behaves just like INAH, a hydrazide; when injected into the chick embryo, pyridoxal phosphate reverses these three effects. BAPN, however, although increasing collagen solubility and lowering cartilage and bone lysine oxidase activity, has no effect on liver pyridoxal phosphate content; moreover, treatment with pyridoxal fails to reverse the increase in collagen solubility and the fall in cartilage lysine oxidase activity.

Certain lathyrogens, when injected into much younger chick embryos (4 days of incubation instead of 14 days) have been shown to produce severe teratogenic changes in the skeleton; these compounds include BAPN, SCH and two members of the hydrazide group (Levene, 1961b). Pyridoxal treatment has been shown to be able to inhibit the deformities produced by the SCH and the hydrazide compounds, but has not effect on BAPN-induced deformities (Levene, 1961a). These results reinforce the view that BAPN acts in a different manner from the other lathyrogens mentioned above, although the
end result on collagen cross-linking is the same for all of
these compounds.

The time-course studies on these various lathyrogens
also indicate a fundamental difference in their behaviour;
our data indicate that the failure of SCH-treated chick
embryos to recover to more than 10% of their original
lysine oxidase activity by 72 h after treatment suggests
that SCH acts in the same way as INAH; the behaviour
of both of these compounds differs markedly from that of
BAPN, where recovery commences within 24 h. One
explanation is that BAPN acts at one site on the enzyme,
whereas SCH and INAH act at a different site. We believe
that the last two compounds act by displacing the
pyridoxal cofactor with which both compounds can form
hydrazones. We have previously shown that loss of an
essential cofactor can lead to the degradation of the
apoenzyme (Murray & Levene, 1977), resulting in a delay
of collagen cross-linking until new enzyme synthesis has
occurred and the essential cofactor has been replaced.
BAPN, on the other hand, appears to block the active
site on the enzyme irreversibly (Pinnell & Martin, 1968;
Tang et al., 1983), and recovery occurs fairly rapidly,
presumably owing to synthesis of the enzyme de novo, the
pyridoxal cofactor still being available; it will be recalled
that BAPN does not decrease liver pyridoxal phosphate.

Tang et al. (1983) have shown that BAPN binds to the
enzyme covalently, inactivation being proportional to the
amount of BAPN bound. They have also shown that
BAPN is not processed to a free aldehyde product on
incubation with the enzyme.

A major difficulty concerning this enzyme has been its
confusion with other amine oxidases; we therefore wish
to make a point about amine oxidases in general. They
occur in several different classes, and one of the major
problems in connection with them is to know their true
substrates; the physiological substrates of lysine oxidase
are collagen and elastin. This is not true of the majority
of monoamine oxidases, which do not effect collagen or
elastin cross-link formation. However, a monoamine
oxidase, previously known as ‘clorgyline-resistant amine
oxidase’ (Lyles et al., 1983), has been isolated from the
brown fat of rats. Unlike other monoamine oxidases, it
is inhibited by SCH (Barrand & Callingham, 1982, 1984),
and has consequently been renamed ‘semicarbazide-
sensitive monoamine oxidase’. Treatment of mice with
SCH has also been shown to lower the amount of
pyridoxal phosphate in the brain (Sakurai et al., 1981).

It would clearly be of interest to see whether an amine
oxidase such as that found in the rat’s brown fat bears
a relationship to lysine oxidase. It is likely that this type
of study would enable one to draw conclusions
concerning mechanism of action.

We gratefully acknowledge the generous support of the
following granting bodies: the Arthritis and Rheumatism
Council (M. J. C.) and the Medical Research Council, of whose
External Staff C. I. L. is a Permanent Member.

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Received 17 July 1985/4 September 1985; accepted 17 September 1985