INHIBITION OF D-AMINO ACID OXIDASE BY AROMATIC ACIDS*

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Benzoate competes with D-amino acids for D-amino acid oxidase and thus inhibits the activity of the oxidase (1-3). Monosubstituted benzoates, depending upon the substituent, have higher and lower affinities for the oxidase than has benzoate (2). Kojic acid, a γ-pyron, likewise competitively inhibits the oxidase (4).

The present report describes the inhibition of D-amino acid oxidase by certain acids, particularly heterocyclic acids having aromatic properties; e.g., acid derivatives of furan, pyrrole, and pyrones. Like the benzoic acids (1-3), kojic acid (4), and 4-7-carbon, straight chain, fatty acids (5), the substances compete with D-amino acids for the oxidase and are in contrast to the aromatic nitrogen compounds that compete with the flavin component of the oxidase (3). The findings suggest some analogies between the inhibitors and the amino acids that bear upon the interaction of these substances with the oxidase.

EXPERIMENTAL

The preparations of D-amino acid oxidase were aqueous extracts of acetone-dried pig kidney (6). For each experimental run 1 gm. of the kidney was ground with 10 ml. of water. The mixture was centrifuged and the supernatant fluid collected. The conventional Warburg respirometer was used in assaying the activity of the extract as the oxygen uptake of a mixture containing 0.5 ml. of extract, 1.5 ml. of 0.1 M pyrophosphate buffer, pH 8.3, substrate dissolved in a portion of the pyrophosphate, a solution of potential inhibitor where indicated, and water to give a volume of 2.5 ml. The substrate, Dl-alanine, was added from the side arm of the vessel after temperature equilibrium was reached. Materials tested for inhibi-

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1 The Dow Chemical Company, through Dr. Paul A. Wolf, supplied 2-methyl-3-hydroxy-1,4-pyron, 2-chloromethyl-5-hydroxy-1,4-pyron, 2-methyl-3-hydroxy-1,4-pyron, 3-indolepropionic and -butyric acids. The Eaton Laboratories, Inc., through Dr. R. J. Main and Dr. W. C. Ward, supplied 2-furoic and 5-nitro-2-furoic acids. Merck and Company, Inc., supplied coumalic acid, and, through Dr. Robert L. Peck, quinolinic, pyrazinoic, and 2,5-pyrazinedicarboxylic acids. Dr. Alfred
tion were used as obtained; the solutions of the materials were adjusted to about pH 8. Measurements of uptake were made at 37°C in room air. As usual, L-alanine and other L-amino acids were not oxidized by the extract and did not affect the oxidation of D isomers. The rate of oxidation at a particular concentration of substrate was estimated from the uptake during its period of linearity with respect to time.

Preliminary screening was carried out to distinguish compounds that inhibited the enzyme. In this testing the final concentration of DL-alanine was 0.04 M and the concentration of the compound 0.02 M. Arbitrarily, substances that gave inhibition of less than 0.15 were considered inactive.

For each substance considered active a concentration was determined that gave inhibition of 0.45 to 0.55 in the presence of 0.04 M DL alanine. The actual inhibition found with such a mixture was compared with that found for a second mixture containing substrate and inhibitor, each in one-half the concentration in the first.

The change in degree of inhibition with dilution of inhibitor and substrate is pertinent to consideration of the kind of inhibition. Such consideration is furnished by introducing the results into an equation applicable to competitive inhibition (7),

$$K_I = \frac{K_S (I)}{(K_S + [S]) (V/V_I - 1)}$$

and into an equation for non-competitive inhibition,

$$K_I = \frac{V_I/V (I)}{1 - V_I/V}$$

where $K_S$ is the apparent dissociation (Michaelis) constant for the enzyme-substrate complex, $(I)$ and $([S])$ are the concentrations of inhibitor and substrate, respectively (both assumed equal to the concentration added), $V_I$ and $V$ are, respectively, rates with and without inhibitor present, and $K_I$ is the dissociation constant for the enzyme-inhibitor complex, in the first case for a complex with free enzyme, in the second for a complex with all forms of enzyme. As will be indicated later, the $K_S$ found for alanine was $7.0 \times 10^{-3}$. If the inhibition found for a substance at a particular concentration with 0.04 M alanine was 0.45 to 0.55, i.e. $V_I = 0.55$ to 0.45 $V$, then on the basis of the first equation the inhibition with 0.02 M alanine should be 0.40 to 0.49, whereas the second equation indicates the inhibition should be 0.29 to 0.38, i.e. $V_I = 0.71$ to 0.62 $V$. All data were considered in terms of these equations. For all compounds considered active, the inhibition observed with 0.02 M alanine differed from that predicted on the

Sussman supplied a number of furan derivatives and 2-pyrololecarboxylic acid. The courtesy of the several organizations and individuals is greatly appreciated.
basis of the inhibition with 0.04 M alanine and the first equation by no
more than 10 per cent, but differed from that predicted on the basis of the
second equation by 24 to 52 per cent. Thus, it appears that the compounds
inhibit by competition with the amino acid for the oxidase.

Dissociation constants for the inhibitor-oxidase complexes were calcu-
lated by use of the first equation. The necessary dissociation constant for
the D-alanine-oxidase complex was estimated from a plot of the reciprocal
of the rates of oxygen uptake against the reciprocal of four corresponding
substrate concentrations. A straight line was drawn through the points.
The concentration of substrate determined by the line and corresponding
to twice the intercept with the reciprocal-rate axis was taken as the con-
stant. In eight tests the mean value and its standard deviation were
7.0 ± 0.5 × 10⁻³. The value of the constant is in reasonable agreement
with those, 6.1 and 6.6 × 10⁻³, reported by Hellerman et al. (3). The
inhibitor constants appear in Table I. Values for benzoic and kojic² acids
are given for comparison. The data for the unsubstituted fatty acids
(Acids 19 to 21) lend confirmation to the report that 4-7-carbon, straight
chain, fatty acids compete with D-amino acids for the oxidase, while acids
with fewer or more carbon atoms do not (5).

Compounds tested and considered inactive, i.e., assuming competitive
inhibition, compounds having a dissociation constant greater than 1.7 ×
10⁻³, are barbituric acid, its isoamylethyl, ethyl-1-methylbutyl, and phenyl-
ethyl derivatives; 2-thiobarbituric acid; taurine; adenosine, guanine, xan-
thine, and uric acid; uracil and thymine; 4,5-imidazoledicarboxylic acid;
acetic, propionic, stearic, oleic, linoleic acid, citric, fumaric, succinic, maleic,
and maleic acids; inorganic cyanate and thiocyanate;3 and glutathione.

² It was reported that kojic acid, in addition to its effect on the oxidation of D-
amino acids, also inhibited the oxidation of L-methionine and L-phenylalanine by a
broken cell preparation of rat liver (4). However, as indicated by test with extracts
of acetone-dried pig kidney, it appears that the materials purchased as L-methionine
and L-phenylalanine each contained a relatively large portion of D-amino acids.
With samples of L-methionine and L-phenylalanine from two other sources which
were found not to contain D-acids, no inhibition of the oxidation of these substances
by liver was observed.

³ Pertinent to the result with thiocyanate is the following: It has been reported
that the oxidation of the DL form of a number of amino acids by broken cell prepara-
tions of guinea pig liver is inhibited by thiocyanate (8). When compared, a higher
concentration of thiocyanate was required for a particular degree of inhibition with the
DL mixture than was required to give the same inhibition with the same concen-
tration of the L isomer. This suggests inhibition of D-amino acid oxidase by thio-
cyanate. As indicated above, thiocyanate did not inhibit the oxidase activity of the
pig kidney extract. To test the possibility that guinea pig tissue is unique, the effect
of thiocyanate on the oxidation of D-amino acids by preparations of liver and kidney
was measured. With the broken cell preparation of tissue described (8), except that
In contrast to the inactive heterocyclic compounds, e.g., the barbiturates, those in Table I have aromatic properties. In the case of furoic acid (Acid 5), in which the number of derivatives permits comparison, effects of substitution are similar in some respects to those obtained with benzoic acid. Metasubstitution in the latter with a methyl group, bromine, or chlorine increases affinity for the oxidase (2). A methyl group (Acid 8) or bromine (Acid 11) in the 5 position of furoic acid likewise increases the affinity. In contrast to benzoic in which a nitro group in any position decreases affinity, with furoic a nitro group (Acid 7) gives greater inhibition. Although not strictly comparable with benzoic acid, replacement of the side chain hydroxyl of kojic acid by chloride (Acid 4) increases the affinity markedly. The similarities indicated and variety of structures showing inhibition suggest that all compounds of aromatic properties having a carboxyl or acidic hydroxyl group will inhibit the oxidase in some degree.

All inhibitors of D-amino acid oxidase found so far that compete with D-amino acids are organic acids, although all such acids are not inhibitory. It is noteworthy that addition of an aromatic component to acetic or propionic acid, neither of which does inhibit, gives acids that do (Acids 16, 17, 22, and 23). Also, addition of such a component to a fatty acid giving some inhibition leads to greater inhibition (see Acids 18 and 19). The similar affinities of the unsubstituted and substituted fatty acids for the oxidase suggest that the effect of addition of the aromatic component is comparable to lengthening the aliphatic chain.

With a few exceptions, the affinities of acids with a ring directly attached to the acid group are markedly greater than those of the fatty acids. This indicates factors concerned with inhibition in addition to length of carbon chain. Bartlett (2) suggested that consideration of resonance and induction might provide an explanation for the variation in activity of benzoic acid provided by monosubstitution. Emphasis for a possible role of resonance in the inhibition is provided by the data of Table I for benzoic, phenylacetic, hydrocinnamic, and cinnamic acids. Separation of ring and carboxyl decreases affinity for the oxidase. Introduction of the double bond to give cinnamic acid restores the affinity considerably. The resonance

\[ \text{(I)} \quad \text{(II)} \]

the concentration of kidney was one-half that of liver, and with 0.02 M d-alanine and d-phenylalanine as substrates, it was found that 0.02 M thiocyanate had no effect on the oxidation. Apparently, therefore, the effect of thiocyanate on the oxidation of the D mixtures of amino acids (8) is to be ascribed to an effect other than on the oxidation of the D isomers.
TABLE I

Inhibitory Effect of Aromatic Heterocyclic and Other Acids on D-Amino Acid Oxidase

The results are expressed as the dissociation constants of inhibitor-oxidase complexes. Each constant is the mean of results obtained from at least three comparisons of (a) the inhibition in the presence of 0.04 M Dl-alanine and sufficient concentration of material to give a 45 to 55 per cent decrease in activity with (b) the inhibition obtained in the presence of one-half these concentrations of substrate and inhibitor. The standard deviations,

\[ \left( \frac{\sum (\text{mean} - \text{individual estimate})^2}{\text{No. of estimates} - 1} \right)^{\frac{1}{2}} \]

did not exceed 10 per cent of the mean. Comparison between compounds indicated that the constants as given represent the relative inhibitory capacities of the compounds. The trivial names of some compounds are given in parentheses.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1,2-Pyrone-5-carboxylic acid (coumalic)</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>2. 1,4-Pyrone-2,6-dicarboxylic acid (chelidonic)</td>
<td>$1.5 \times 10^{-2}$</td>
</tr>
<tr>
<td>3. 2-Hydroxymethyl-5-hydroxy-1,4-pyrene (kojic)</td>
<td>$2.1 \times 10^{-6}$</td>
</tr>
<tr>
<td>4. 2-Chloromethyl-5-hydroxy-1,4-pyrene</td>
<td>$4.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>5. 2-Furancarboxylic acid</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>6. 2,5-Diphenyl-3,4-furandicarboxylic acid</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>7. 5-Nitro-2-furancarboxylic acid</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>8. 5-Methyl-2-furancarboxylic acid</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>9. 2-Furanaecrylic acid</td>
<td>$5.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>10. 2-Benzofurancarboxylic acid (coumarilic)</td>
<td>$3.1 \times 10^{-1}$</td>
</tr>
<tr>
<td>11. 5-Bromo-2-furancarboxylic acid</td>
<td>$1.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>12. 2,5-Pyrazinedicarboxylic acid</td>
<td>$1.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>13. Pyrasinecarboxylic acid</td>
<td>$4.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>14. 3-Pyridinecarboxylic acid (nicotinic)</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>15. 2-Pyrrolecarboxylic acid</td>
<td>$2.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>16. 3-Indoleproponic acid</td>
<td>$2.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>17. 3-Indoleacetic acid</td>
<td>$2.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>18. 3-Indolebutyric &quot;</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>19. n-Butyric acid</td>
<td>$1.0 \times 10^{-2}$</td>
</tr>
<tr>
<td>20. n-Caprylic &quot;</td>
<td>$6.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>21. n-Valerie &quot;</td>
<td>$4.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>22. Phenylacetic acid</td>
<td>$6.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>23. Hydrocinnamic acid</td>
<td>$5.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>24. Cinnamic acid</td>
<td>$5.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>25. Benzoic &quot;</td>
<td>$4.8 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

forms for benzoic (I) and cinnamic (II) acids indicate a connection between ring and carboxyl not permitted the substituted or unsubstituted fatty acids.

Although not established, it seems reasonable to suppose that interaction between amino acids and the oxidase involves both amino and carboxyl
groups. Also, simplicity suggests that the acid groups of inhibitors react with the same portion of the oxidase as the carboxyl of amino acids. It might be expected, therefore, that inhibitors with both acid and basic groups would be more effective than those having acid properties alone. As indicated by formula I, benzoic acid, due to resonance, has areas of low electron density that might simulate the amino group. This might explain why benzoic acid is a stronger inhibitor than the fatty acids. A somewhat stronger case can be made for kojic acid, which may be represented by

\[
\text{OH} \\
\text{H} \\
\text{HO-} \\
\text{C} \\
\text{O} \\
\text{H}
\]

This form for kojic acid represents a greater proportion of all possible forms than is the case for the form of benzoic acid given. The more marked basic element in kojic acid may be the reason that it is a stronger inhibitor than benzoic acid.

### SUMMARY

A number of heterocyclic acids having aromatic properties were found to inhibit \(\alpha\)-amino acid oxidase by competing with the substrate. It appears likely that such inhibition will in some degree be exhibited by all such acids. Certain of such inhibitors, namely those with the acid group attached to the ring, in addition to the negative charge attributable to the acid group bear a positive charge arising from resonance. This suggests an analogy to the similar charges of the amino acids and, therefore, a like attachment of amino acids and inhibitors to the oxidase.

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