[CONTRIBUTION FROM THE SCRIPPS METABOLIC CLINIC]

The Inhibition of D-Amino Acid Oxidase by Benzoic Acid and Various Monosubstituted Benzoic Acid Derivatives

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Benzoic acid has been reported to give a marked and selective inhibition of the enzyme D-amino acid oxidase.[1] This inhibition is apparently due to a reaction with the activating protein rather than with the flavine-nucleotide prosthetic group, and involves a competitive reversibility with substrate amino acid.[1,2]

In this paper enzyme inhibition is used as a tool to study the influence of ring substitution on the reaction between benzoate and amino acid oxidase protein. There is also presented a more rigorous proof of the substrate competitive nature of the benzoate inhibition and more information on the specificity of the reaction.

Experimental

The D-amino acid oxidase preparation was essentially that described by Krebs.[3]

Pig kidney acetone powder was extracted with five volumes of water in a Waring Blendor, centrifuged and the supernatant lyophilized. The dry powder retains its activity for at least six months at room temperature. Enzyme activity was assayed by oxygen consumption with DL-alanine as substrate in standard Warburg manometric equipment. Unless otherwise specified, conditions were as follows: 1.0 ml. containing 10 mg. of lyophilized pig kidney extract, 0.5 ml. 0.2 *M* pH 7.6 pyrophosphate buffer, 0.3 ml. of 0.3 *M* DL-alanine added from side arm at −3 min., 0.3 ml. of inhibitor solution, water to 3.0 ml. total volume in main part of vessel, 0.2 ml. of 20% potassium hydroxide in center cup, air as gas phase, oxygen consumption measured from 0 to thirty minutes, temperature 38°. Substrate, inhibitor and other solutions were neutralized before adding to vessels.

To check the degree of inhibition each benzoate was tested at 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} molar concentrations. Inhibitory values between 10 and 90%, when plotted against the log of the inhibitor concentration fell on a straight line. There was some deviation at each end of the curve. The slopes for all benzoates tested were identical; 50% inhibition values were calculated from the log benzoate–per cent. inhibition curves. A typical analysis is given in Table I, demonstrating the relationship of inhibitory capacity to concentration of benzoate and showing the reproducibility of data obtainable with two different enzyme preparations.

TABLE I

SAMPLE ANALYSIS. INHIBITION OF D-AMINO ACID OXIDASE BY *p*-TOLUIC ACID[a]

Exp.	Concn. of *p*-toluic	μl. O_2 consumed per 30 min.	% Inhib.
1	0	170	...
	10^{-5} *M*	169	0
	10^{-4} *M*	122	28
	10^{-3} *M*	32	81
	10^{-2} *M*	0	100
2	0	178	...
	10^{-5} *M*	178	0
	10^{-4} *M*	126	29
	10^{-3} *M*	38	79
	10^{-2} *M*	0	100

Calcd. 50% inhibition: 2.5×10^{-4} *M*

[a] Warburg manometric analyses, see text for details.

All benzoates used were commercial products, which when necessary were recrystallized until melting points and titration equivalents checked with accepted values. *o*-Fluorobenzoic acid was kindly supplied by Dr. M. Kilpatrick. The author is indebted to Mr. and Mrs. Francis E. Fowler, Jr., and members of their family, whose generosity has made these studies possible. The helpful encouragement and advice of Dr. E. M. MacKay is gratefully acknowledged.

Results and Discussion

Benzoic acid inhibited D-amino acid oxidase approximately 50% at 10^{-4} *M*, less than 10% at 10^{-5} *M*, and more than 90% at 10^{-3} *M*; see Table II for 50% inhibition values for benzoic acid and a number of ring monosubstituted derivatives. A wide variety of other enzyme systems were inhibited less than 10% by the relatively high benzoate concentration of 10^{-2} *M*; oxidation of acetate by baker's yeast, hydrolysis of edestin by pepsin, decarboxylation of pyruvate by dried brewer's yeast, hydrolysis of acetylcholine by serum, hydrolysis of urea by urease, glycolysis of hexosediphosphate by muscle acetone powder, hydrolysis of monobutyrin by liver esterase, decomposition of hydrogen peroxide by catalase, and oxidation of succinate, choline, uricate and tyramine by a minced, washed rat liver preparation. These results provide a more extensive verification of Klein and Kamin's[1] observations on the highly selective action of benzoate toward D-amino acid oxidase.

From the viewpoint of structural similarity one would not expect benzoate to compete reversibly with the substrate amino acid. However, such a relationship does occur. Concentrations of D-alanine from 0.0032 to 0.316 *M* were tested with 10^{-3} and 10^{-4} *M* benzoate. In Fig. 1, the per cent. inhibitions are plotted against the log concentration of D-alanine. At all ratios of alanine to benzoate there was strict reversible competition. The inhibition is independent of the absolute amount of benzoate and a function of the ratio of alanine to benzoate. Fifty per cent. inhibition results at a ratio of D-alanine to benzoate of 100 to 1. High concentrations (0.1 *M*) of potassium chloride, glycine or of L-alanine had no reversing effect on the inhibition of the oxidation of D-alanine by benzoate. Glycine and L-amino acids are not acted upon by the enzyme nor do they affect the oxidation of D-amino acids. The competition

(1) Klein and Kamin, *J. Biol. Chem.*, **138**, 507 (1941).
(2) Hellerman, Lindsay and Bovarnick, *ibid.*, **163**, 553 (1946).
(3) Krebs, *Biochem. J.*, **29**, 1620 (1935).

experiments prove that substrate displacement adequately accounts for the inhibition: probably both substrate and inhibitor having a high affinity for the same position on the protein.

Various other compounds were tested in order to give some definition of the relationship of structure to enzyme inhibition. Phenyl carboxylate ion proved to be the essential structural unit. At the *p*H of the test system more than 99% of any of the benzoates used would exist as carboxylate ion. Substitutions on carboxyl, replacement of carboxyl by another acidic radical, replacement with alkyl carboxylates, all resulted in negative activity. For example, the following compounds gave less than 10% inhibition at 10^{-2} *M* concentrations: benzamide, ethyl benzoate, phenyl acetate, phenyl propionate, mandelate, sulfanilate, phenyl phosphate, benzenesulfonate.

Although alteration of carboxyl completely destroys the toxicity, ring substitution results in a wide variation in the inhibiting capacity, probably by altering the electron configuration at carboxyl carbon or in the case of *ortho* substituents, giving rise to steric hindrance. The inhibiting power of a series of monosubstituted benzoates is given in Table II. The following points can be emphasized. Except for hydroxy and amino, *meta* gives the greatest inhibition, *para* is intermediate and *ortho* is very low. With hydroxy and amino there is not a marked *ortho* effect, the *para* position giving the lowest inhibitions. In general, halogen, methyl and nitro are stronger inhibitors than hydroxy, amino, methyl and a second carboxyl. The dicarboxylic acids give practically no inhibition. The halogens show a striking series of increasing inhibition in order of increasing electronegativities. *Ortho* substitution apparently blocks access of phenyl carboxylate to the reacting position on protein. It is felt that this can best be looked upon as a geometrical steric hindrance following the notions originally postulated by Victor Meyer for the *ortho* blocking of esterification of benzoates. No analysis will be given here of the relationship of structure to the benzoate protein reaction in terms of current notions on the influence of inductive and resonance effects. However, it is suggested that the quantitative data on the monosubstituted benzoates should provide an interesting series for checking such theories.

TABLE II

THE REACTIVITY OF MONOSUBSTITUTED BENZOIC ACIDS TOWARD D-AMINO ACID OXIDASE

Benzoic acid 1.2×10^{-4} [a]

Substituent group	Ring position *Para*	*Meta*	*Ortho*
I	1.8×10^{-3}	1.6×10^{-4}	1.0×10^{-2}
Br	2.8×10^{-4}	3.1×10^{-5}	3.3×10^{-2}
CL	1.6×10^{-4}	1.6×10^{-5}	5.0×10^{-3}
F	4.0×10^{-5}	.....	1.0×10^{-3}
CH_3	2.5×10^{-4}	4.0×10^{-5}	3.3×10^{-2}
NO_2	3.6×10^{-4}	2.5×10^{-4}	1.7×10^{-2}
OH	5.0×10^{-3}	2.2×10^{-4}	6.3×10^{-4}
NH_2	1.0×10^{-2}	1.0×10^{-3}	1.0×10^{-3}
OCH_3	2.2×10^{-3}	1.8×10^{-3}	3.3×10^{-2}
COOH	1.0×10^{-2}	3.3×10^{-2}	5.0×10^{-2}

[a] Figures represent the molar concentration giving 50% inhibition.

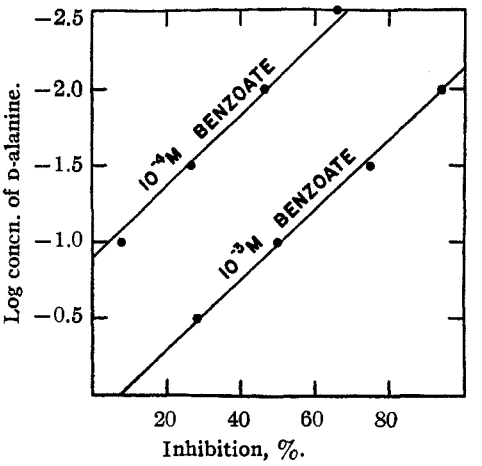

Fig. 1.—Substrate competitive nature of the inhibition of D-amino acid oxidase by benzoate.

Summary

Benzoate was incubated with a variety of enzyme systems and found to be highly specific for inhibiting D-amino acid oxidase.

Benzoate and D-alanine showed a reversible competition for protein over a wide range of concentrations, 50% inhibition of enzyme resulting at a ratio of alanine to benzoate of 100 to 1.

Phenyl carboxylate proved to be the essential structural unit necessary for the D-amino acid oxidase inhibition. Quantitative data is given on the affinities for D-amino acid oxidase of a number of ring monosubstituted derivatives of benzoic acid.

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