A Study of Amino Acid Oxidase Specificity, Using a New Sensitive Assay*

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A new method for the detection of small amounts of amino acid oxidase activity is described. The method is based on the inactivation of catalase by 3-amino-1,2,4-triazole in the presence of H_2O_2 generated by the amino acid oxidase reaction. The oxidation of 2–5 mμmoles of amino acid is easily detectable. The application of the method to the study of enzyme specificity is discussed. It is shown that D-lysine is a substrate of D-amino acid oxidase and that L-serine and L-threonine are substrates of L-amino acid oxidase.

The classic method for the determination of amino acid oxidase activity is the manometric measurement of oxygen consumption (Krebs, 1935). Although more sensitive methods exist for specific substrates such as phenylalanine (Knox and Pitt, 1957; Wellner and Meister, 1960) and hydroxyproline (Corrigan *et al.*, 1963), these are not applicable to all amino acids. The recent observation of Neims and Hellerman (1962) that glycine is slowly oxidized by D-amino acid oxidase indicated the need for a re-examination of the specificity of amino acid oxidases by the use of an assay procedure capable of detecting very low rates of enzymatic oxidation and applicable to a large number of amino acids. Such a procedure is described. It is shown that some amino acids which were previously thought not to be susceptible to the action of D- and L-amino acid oxidases are oxidized by these enzymes.

The procedure is based on the finding of Margoliash and Novogrodsky (1958) that catalase is irreversibly inhibited by hydrogen peroxide in the presence of 3-amino-1,2,4-triazole. Since hydrogen peroxide is a product of the amino acid oxidase reaction, if the reaction is carried out in the presence of catalase and aminotriazole, the amount of amino acid oxidized may be estimated by measuring the inhibition of catalase. It is possible in this way to measure the oxidation of as little as 2–5 mμmoles of amino acid. The great sensitivity attainable is owing to the catalytic amplification of the effect of the product being measured. Since each molecule of catalase is capable of decomposing several million molecules of hydrogen peroxide, the inactivation of a few catalase molecules produces a greatly amplified and easily measurable effect. The principle of catalytic amplification has been utilized previously by Berger *et al.* (1959) in a sensitive assay for pepsin, using ribonuclease as substrate, in which the inactivation of ribonuclease was used as a measure of pepsin activity.

Experimental Procedure

Materials.—Crystalline beef liver catalase was obtained from the Sigma Chemical Co. D-Amino acid oxidase was prepared from hog kidney by the method of Massey *et al.* (1961), but was not crystallized. Crystalline L-amino acid oxidase was prepared from rattlesnake venom as described previously (Wellner and Meister, 1960). 3-Amino-1,2,4-triazole was obtained from Calbiochem. The amino acids were bought from Mann Research Laboratories, Nutritional Biochemicals Corp., and Schwarz BioResearch. L-3,4-Dehydroproline was synthesized by the method of Robertson and Witkop (1962). DL-α-Methylphenylalanine was obtained from Merck, Sharp and Dohme.

Enzyme Units.—One unit of catalase is defined as the amount of enzyme required to decompose 1 μmole of H_2O_2 per minute at *p*H 7.0 and 25° when the H_2O_2 concentration is 0.01 M. (The catalase activity was determined according to the method given by the Sigma Chemical Co.) One unit of D-amino acid oxidase is defined as the amount of enzyme which catalyzes the uptake of 1 μl of O_2 per minute at *p*H 8.3 and 37° in the presence of 2.25×10^{-2} M D-alanine and catalase (Burton, 1955). One unit of L-amino acid oxidase is defined as the amount of enzyme required to catalyze the uptake of 1μl of O_2 per 30 minutes at *p*H 7.5 and 37° in the presence of 7×10^{-3} M L-leucine (Wellner and Meister, 1960).

Determination of Amino Acid Oxidase Activity.—A reaction mixture containing Tris-HCl buffer (80 μmoles, *p*H 7.3), 3-amino-1,2,4-triazole (10 μmoles), flavin adenine dinucleotide (25 μg), amino acid, amino acid oxidase, and catalase in a total volume of 0.5 ml was incubated with shaking at 37° for 60 minutes. The enzymes were found to be unstable in dilute solution and were therefore diluted immediately before use. At the end of the incubation the reaction mixture was transferred quantitatively to a flask containing 9.5 ml of a solution of $NaBO_3$ (780 μmoles) at *p*H 6.8 and 37° (Feinstein, 1949). After 5 minutes the reaction was stopped by adding 10 ml of 2 N H_2SO_4 and the amount of perborate remaining was titrated with $KMnO_4$. All determinations were carried out in duplicate, and control experiments in which the amino acid and amino acid oxidase were omitted together and separately were also performed. In the experiments reported here, the controls yielded values which were the same within experimental error. The amount of catalase was such that between 80 and 90% of the perborate was destroyed in the uninhibited controls. Determinations of catalase activity were reproducible to ±5% of the uninhibited value. Less than 5% inhibition is reported as zero.

The sensitivity of the assay could be decreased by increasing the catalase concentration, since a given number of inactivated catalase molecules would then represent a smaller fraction of the total amount present. In experiments where more than 170 units of catalase were used, the reaction mixture was suitably diluted with water and an aliquot (0.5 ml) was assayed for catalase activity as described above.

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TABLE I

RELATIVE SUSCEPTIBILITY OF VARIOUS AMINO ACIDS TO OXIDATION BY D-AMINO ACID OXIDASE[a]

Amino Acid	Reported Relative Activity[b]	Catalase Inhibition (%) 0.16 Unit D-OX	0.65 Unit D-OX	6.5 Units D-OX	65 Units D-OX
DL-Proline	(100)	83			
DL-Methionine	77	73			
DL-Isoleucine	73	68			
D-Alanine	52	66	89		
DL-Valine	49	53			
DL-Norvaline	33	63			
D-Allo-4-hydroxyproline	30	41			
DL-Aspartic acid	23	0	0	27	
DL-Homoserine	16	29			
DL-Serine	14	24	75		
DL-Ornithine	8	0	0	78	
DL-Arginine	5	0	27		
DL-Histidine	4	0	0	71	
DL-Threonine	1.5	0	38		
DL-Glutamic acid	1.5	0	0	0	48
Glycine[c]	0.8	0	0	0	87
DL-Lysine	0	0	0	78	

[a] The reaction mixtures contained 8500 units of catalase, D-amino acid oxidase (D-OX) as indicated, and 40 μmoles of amino acid. Other conditions as described in the text. [b] Based on data of Neims and Hellerman (1962) and of Greenstein and Winitz (1961). Proline was assigned a value of 100. [c] 50 μmoles were used.

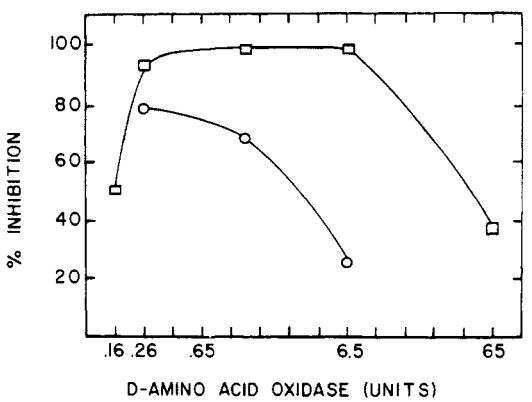

FIG. 1.—Effect of D-amino acid oxidase concentration on catalase inhibition. Upper curve: 850 units of catalase, 0.2 μmole of D-alanine. Lower curve: 170 units of catalase, 0.01 μmole of D-methionine. Other conditions as described in the text.

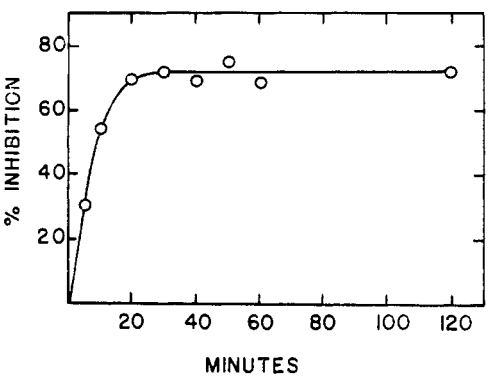

FIG. 2.—Kinetics of catalase inhibition. The reaction mixtures contained 170 units of catalase, 1.3 units of D-amino acid oxidase, and 0.01 μmole of D-methionine. Other conditions as described in the text.

RESULTS

In order to determine the optimal conditions for the method, the effects of D-amino acid oxidase concentration, substrate concentration, and incubation time were investigated. Figure 1 (upper curve) shows that there is an optimal concentration of D-amino acid oxidase above which the inhibition of catalase decreases. This is because at high concentrations of D-amino acid oxidase the hydrogen peroxide is produced in a burst, and much of it is decomposed by catalase before inhibition can occur. The irreversible inhibition of catalase by hydrogen peroxide and aminotriazole has been shown to be a relatively slow reaction and it is therefore favored by continuous generation of small amounts of hydrogen peroxide. A single addition of a large amount of hydrogen peroxide produces little inhibition (Margoliash and Novogrodsky, 1958; Margoliash *et al.*, 1960). In an experiment in which the substrate concentration was smaller (Fig. 1, lower curve), less amino acid oxidase was required for maximal inhibition.

Figure 2 shows the time course of catalase inhibition in the presence of 0.01 μmole of D-methionine and 1.3 units of D-amino acid oxidase. It may be seen that after 30 minutes, when all the substrate is presumably oxidized, no further inhibition takes place.

The sensitivity of the method is indicated in Figure 3. Under the conditions used, 50% inhibition of catalase was obtained with about 3.5 mμmoles of either D-proline or D-alanine. The oxidation of 1 mμmole of amino acid produced a detectable amount of inhibition.

When a number of amino acids were tested as substrates for D-amino acid oxidase under assay conditions of low sensitivity (high catalase concentration), the results shown in Table I were obtained. For most of the amino acids, a good correlation was found between the amount of catalase inhibited and their reported susceptibility to attack by D-amino acid oxidase as determined by other methods. Very small amounts of D-amino acid oxidase were required to effect observable oxidation of good substrates such as D-alanine, D-proline, and D-methionine, but much more oxidase was required to detect the oxidation of D-aspartic acid, D-ornithine, D-histidine, D-glutamic acid, glycine, and D-lysine. D-Lysine, which has been reported not to be attacked, is shown here to be a substrate of D-amino acid oxidase. Under similar conditions, using 65 units of D-amino acid oxidase, no inhibition of catalase was found with L-alanine, L-phenylalanine, L-proline, L-4-hydroxyproline, or L-serine (50 μmoles of each), but 92% inhibition was observed with L-3,4-dehydroproline (10 μmoles) (see Discussion).

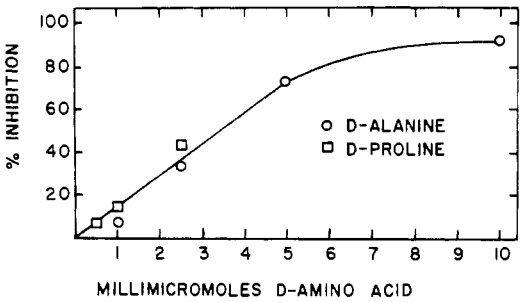

FIG. 3.—Sensitivity of the catalase-inhibition method for the detection of D-amino acids. The reaction mixtures contained 170 units of catalase and 1.3 units of D-amino acid oxidase. Other conditions as described in the text.

TABLE II

INHIBITION OF CATALASE WITH SLOWLY OXIDIZABLE SUBSTRATES OF D-AMINO ACID OXIDASE

Amino Acid[a]	Catalase Inhibition (%)
D-Alanine (0.2 μmole)	95
DL-Lysine (2 μmoles)	92
DL-Histidine (2 μmoles)	91
DL-Threonine (2 μmoles)	91
D-Aspartic acid (1 μmole)	90
DL-Ornithine (2 μmoles)	89
D-Glutamic acid (1 μmole)	0
D-Glutamic acid (10 μmoles)	70
Glycine (1 μmole)	0
Glycine (10 μmoles)	13

[a] The reaction mixtures contained 170 units of catalase and 1.3 units of D-amino acid oxidase. Other conditions as described in the text.

TABLE III

INHIBITION OF CATALASE WITH SLOWLY OXIDIZABLE SUBSTRATES OF L-AMINO ACID OXIDASE

Amino Acid[a]	Reported Relative Activity[b]	Catalase Inhibition (%)
DL-Lysine (100 μmoles)	0.2	95
L-Glutamic acid (100 μmoles)	0.08	83
L-Serine (10 μmoles)	0	94
L-Threonine (50 μmoles)	0	94
L-Proline (50 μmoles)	0	0
L-4-Hydroxyproline (50 μmoles)	0	0
Glycine (50 μmoles)		0

[a] The reaction mixtures contained 8500 units of catalase and 1000 units of L-amino acid oxidase. Other conditions as described in the text. [b] Based on data of Greenstein and Winitz (1961). Methionine was assigned a value of 100.

When the sensitivity of the assay was increased by decreasing the catalase concentration (Table II), substantial inhibition of catalase was observed with lower concentrations of substrates and D-amino acid oxidase than in the experiments described in Table I. D-Glutamic acid and glycine, unlike D-lysine and other slowly oxidized substrates of D-amino acid oxidase, produced no measurable effect in this sytem at a concentration of 2×10^{-3} M. However, at ten times this concentration, activity was observed.

Table III shows the results of experiments carried out with L-amino acid oxidase. It may be seen that, in confirmation of previous data, L-proline, L-4-hydroxyproline, and glycine are not substrates of the enzyme. However, L-serine and L-threonine, which were thought not to be oxidized by L-amino acid oxidase, are shown here to be substrates.

TABLE IV

DETECTION OF SMALL AMOUNTS OF D-ISOMER IN L-AMINO ACID PREPARATIONS

Substrates[a]	Catalase Inhibition (%)
D-Methionine (5 mμmoles)	49
L-Methionine (10 μmoles)	0
D-Methionine (5 mμmoles) + L-Methionine (10 μmoles)	36
D-Alanine (5 mμmoles)	82
L-Alanine[b] (75 μmoles)	19
D-Alanine (5 mμmoles) + L-Alanine[b] (75 μmoles)	94
D-Proline (5 mμmoles)	94
L-Proline[b] (75 μmoles)	7
D-Proline (5 mμmoles) + L-Proline[b] (75 μmoles)	94

[a] The reaction mixtures contained 170 units of catalase, and 0.26 unit of D-amino acid oxidase (in the experiments with methionine) or 1.3 units of D-amino acid oxidase (in the experiments with alanine and proline). Other conditions as described in the text. [b] Purified by treatment with D-amino acid oxidase (see text).

TABLE V

TEST OF VARIOUS L-AMINO ACID AND DL-α-METHYLAMINO ACID PREPARATIONS FOR SUSCEPTIBILITY TO D-AMINO ACID OXIDASE

Amino Acid[a]	Catalase Inhibition (%)
D-Alanine (0.2 μmole)	92
DL-α-Methylphenylalanine (10 μmoles)	0
DL-Isovaline (10 μmoles)	0
L-Aspartic acid (50 μmoles)	79
L-Arginine (50 μmoles)	65
L-Histidine (50 μmoles)	0
L-Valine (50 μmoles)	0
L-Leucine (25 μmoles)	0
L-Tryptophan (10 μmoles)	0
L-Tyrosine (1 μmole)	0

[a] The reaction mixtures contained 170 units of catalase and 6.5 units of D-amino acid oxidase. Other conditions as described in the text.

The high sensitivity of the method suggested that it could be used for detecting contamination of L-amino acid preparations by small amounts of the D isomer. Table IV shows that 0.05% of D-methionine can easily be detected in a preparation of L-methionine. It may be seen, furthermore, that the L-methionine preparation used contained much less than 0.05% of the D isomer.

The available commercial preparations of L-alanine and L-proline, however, contained appreciable quantities of the corresponding D isomers. These amino acids were therefore purified as follows: L-Proline or L-alanine (2 g) was dissolved in 15 ml of water and the *p*H was adjusted to 8.0 with a few drops of 2 N NH_4OH. To this was added 50 μg of flavin adenine dinucleotide, 1 ml of a D-amino acid oxidase solution (650 units), and a few drops of chloroform. After a 6-hour incubation at 37° a further addition of 1 ml of D-amino acid oxidase (650 units) was made and the mixture was incubated at 37° for an additional 12 hours. Protein and nucleotide were removed by the addition of activated charcoal, followed by centrifugation. The amino acid was recrystallized twice (alanine from ethanol-water and proline from ethanol), and the crystals were thoroughly dried in a vacuum desiccator over H_2SO_4.

Even after the above treatment, both L-alanine and L-proline produced some inhibition of catalase when incubated in high concentrations with D-amino acid oxidase (Table IV). However, the data indicate that there was less than 1 part in 15,000 of the D isomer in each of these amino acid preparations.

A number of amino acids which were not thought to be substrates for D-amino acid oxidase were also tested by the above procedure (see Table V). Of these, only L-aspartic acid and L-arginine gave positive results. However, since these amino acids had not been pretreated with D-amino acid oxidase or recrystallized, it is possible that they may have been contaminated with small amounts of the corresponding D isomers. The presence of 0.02% of the D isomers would explain these results.

Discussion

The method described for the detection of amino acid oxidase activity is applicable in principle to any reaction in which hydrogen peroxide is formed. It may therefore prove to be useful in the study of a number of flavoprotein-catalyzed reactions. It should be noted, however, that two types of compounds which occur in biological preparations may interfere with the procedure. These are (a) autoxidizable compounds (e.g., ascorbic acid), which generate hydrogen peroxide nonenzymatically, and (b) compounds which act as electron donors for catalase-H_2O_2 complex I (e.g., ethanol), which protect catalase from inactivation (Margoliash *et al.*, 1960). It is therefore important to carry out suitable control experiments, especially when working with unfractionated biological materials.

Although D-lysine, D-glutamic acid, and glycine are oxidized extremely slowly by D-amino acid oxidase, their oxidation can be detected readily by the present procedure. Glycine has been reported by Neims and Hellerman (1962) to have a K_m of about 6×10^{-2} M. The finding that, under the conditions of Table II, glycine and D-glutamic acid caused inhibition of catalase when present at a concentration of 2×10^{-2} M, but not at 2×10^{-3} M, is consistent with high K_m values for these amino acids. Under similar conditions, however, D-lysine caused 92% inhibition of catalase at a concentration of 2×10^{-3} M. This is consistent with the finding of Murachi and Tashiro (1958) that D-amino acid oxidase has approximately the same affinity for D-lysine as for D-alanine. (Although D-lysine was reported not to be a substrate, its affinity for the enzyme was measured by its ability to inhibit the oxidation of D-alanine.) It may be concluded, therefore, that whereas glycine and D-glutamic acid are oxidized slowly by D-amino acid oxidase because they have a low affinity for the enzyme, D-lysine is attacked slowly because it binds to the enzyme in a manner which is unfavorable for further reaction (presumably because of the positively charged ϵ-amino group).

The fact that, under assay conditions of low sensitivity, L-3,4-dehydroproline produced almost complete inhibition of catalase in the presence of D-amino acid oxidase suggested that this amino acid might be a substrate of the oxidase. The accompanying paper (Wellner and Scannone, 1964) presents evidence that both L-3,4-dehydroproline and L-proline are substrates of D-amino acid oxidase. It is shown in Table IV that both L-alanine and L-proline preparations reacted in the assay even after extensive efforts to remove D-amino acids. Although it is difficult to exclude the possibility that a trace of D-alanine remained in the L-alanine preparation (less than 1 part in 15,000), the data suggest the possibility that L-alanine may be slowly attacked by D-amino acid oxidase.

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References

Berger, A., Neumann, H., and Sela, M. (1959), *Biochim. Biophys. Acta 33*, 249.

Burton, K. (1955), *Methods Enzymol. 2*, 199.

Corrigan, J. J., Wellner, D., and Meister, A. (1963), *Biochim. Biophys. Acta 73*, 50.

Feinstein, R. N. (1949), *J. Biol. Chem. 180*, 1197.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, Wiley, p. 1785.

Knox, W. E., and Pitt, B. M. (1957), *J. Biol. Chem. 225*, 675.

Krebs, H. A. (1935), *Biochem. J. 29*, 1620.

Margoliash, E., and Novogrodsky, A. (1958), *Biochem. J. 68*, 468.

Margoliash, E., Novogrodsky, A., and Schejter, A. (1960), *Biochem. J. 74*, 339.

Massey, V., Palmer, G., and Bennett, R. (1961), *Biochim. Biophys. Acta 48*, 1.

Murachi, T., and Tashiro, M. (1958), *Biochim. Biophys. Acta 29*, 645.

Neims, A. H., and Hellerman, L. (1962), *J. Biol. Chem. 237*, PC976.

Robertson, A. V., and Witkop, B. (1962), *J. Am. Chem. Soc. 84*, 1697.

Scannone, H., Wellner, D., Novogrodsky, A., and Meister, A. (1963), Abstracts of Papers, 145th Meeting, Am. Chem. Soc., New York, September, 1963, Abstract No. 176, p. 82C.

Wellner, D., and Meister, A. (1960), *J. Biol. Chem. 235*, 2013.

Wellner, D., and Scannone, H. (1964), *Biochemistry 3*, 1746. (this issue; following paper).