D-Amino Acids

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The oxidative deamination of α-amino acids has been known since 1909. *Neubauer*[1] and *Knoop*[2] showed that this process consumed oxygen and resulted in the formation of ammonia and α-keto acids. Kidney tissue contains an enzyme, D-amino acid oxidase, which specifically deaminates D-amino acids[3] and requires flavine adenine dinucleotide (FAD)[4] as coenzyme. *Greenstein*[5] used this enzyme to prepare the pure L-isomers from amino acid racemates.

Table 1 shows the activity of a D-amino acid oxidase preparation from sheep kidney with various D-amino acids[6]. We found that an enzyme from pig kidney was also able to deaminate D-aspartic acid and D-glutamic acid, if the enzyme concentration was increased and the reaction time was prolonged.

D-Amino acid oxidase is found in the kidney and liver of all mammals and some other vertebrates, the kidney of sheep and pig being especially rich in the enzyme. The enzyme from the hepatopancreas of *Octopus*[7] is active with D-aspartic acid and D-glutamic acid. D-Amino acid oxidase preparations from *Neurospora crassa*[8], *Aspergillus niger*[9], *Proteus morganii*[10] and other bacteria are significantly less active than those from pig or sheep kidney.

Table 1. The rate of oxidation of D-amino acids by D-amino acid oxidase from sheep kidney.

D-Amino acid	μl O_2 consumed/ hr./mg. dry weight of enzyme preparation	D-Amino acid	μl O_2 consumed/ hr./mg. dry weight of enzyme preparation
Alanine	64	Tyrosine	190
α-Aminobutyric acid	31	Histidine	6.2
α-Aminovaleric acid	20	Tryptophan	37
α-Aminocaproic acid	36	Lysine	0.6
Valine	35	Ornithine	3.1
Leucine	14	Serine	42
Isoleucine	22	Threonine	2.1
Aspartic acid	1.4 *)	Proline	148
Glutamic acid	0 *)	Pipecolinic acid	2.6
α-Amino-adipic acid	0	Methionine	80
Phenylalanine	26	Cystine	1.9

*) see text.

I. Manometric Method

Principle

D-Amino acid oxidase catalyses the reaction:

$$\text{(1)} \qquad \underset{\displaystyle NH_2}{\overset{\displaystyle |}{R-CH-COOH}} + O_2 \longrightarrow \underset{\displaystyle NH}{\overset{\displaystyle \|}{R-C-COOH}} + H_2O_2$$

1) *O. Neubauer*, Dtsch. Arch. klin. Med. *95*, 211 [1909].
2) *F. Knoop*, Hoppe-Seylers Z. physiol. Chem. *67*, 489 [1910].
3) *H. A. Krebs*, Biochem. J. *29*, 1620 [1935].
4) *O. Warburg* and *W. Christian*, Biochem. Z. *296*, 294 [1938]; *298*, 150 [1938].
5) *J. P. Greenstein*, Advances Protein Chem. *9*, 121 [1954]; J. biol. Chemistry *192*, 535 [1951].
6) *H. A. Krebs:* The Enzymes. Academic Press, New York 1951, Vol. II, Part I, p. 508.
7) *H. Blaschko* and *J. Hawkins*, Biochem. J. *52*, 306 [1952].
8) *N. H. Horowitz*, J. biol. Chemistry *154*, 141 [1944].
9) *R. L. Emerson, M. Puziss* and *S. G. Knight*, Arch. Biochem. Biophysics *25*, 299 [1950].
10) *P. K. Stumpf* and *D. E. Green*, Feder. Proc. *5*, 157 [1956].

The imino acid decomposes spontaneously:

$$\text{(2)}\qquad \underset{\underset{\text{NH}}{\|}}{\text{R}-\text{C}-\text{COOH}} + \text{H}_2\text{O} \longrightarrow \underset{\underset{\text{O}}{\|}}{\text{R}-\text{C}-\text{COOH}} + \text{NH}_3$$

If the hydrogen peroxide formed in reaction (1) is destroyed by catalase, the over-all reaction is:

$$\text{(3)}\qquad \underset{\underset{\text{NH}_2}{|}}{\text{R}-\text{CH}-\text{COOH}} + {}^1/_2\,\text{O}_2 \longrightarrow \underset{\underset{\text{O}}{\|}}{\text{R}-\text{C}-\text{COOH}} + \text{NH}_3$$

A mole of D-amino acid yields a mole of α-keto acid and a mole of ammonia, and 0.5 mole of oxygen is consumed. This oxygen consumption is determined and, with the exception D-amino-adipic acid (as well as glutamic acid and lysine, which usually do not react completely), is a measure of the total amount of D-amino acids contained in the sample.

Reagents

1. Sodium pyrophosphate, A. R., $Na_4P_2O_7 \cdot 10H_2O$
2. Potassium hydroxide, A. R., 2 N
3. D-Alanine
   chromatographically pure, free from L-alanine.
4. Catalase
   powder; commercial preparation, see p. 971.
5. D-Amino acid oxidase
   isolation of the enzyme, see p. 371.

Purity of the enzyme preparation

The crude D-amino acid oxidase preparation obtained from sheep or pig kidney (p. 371) satisfies the requirements, as do the commercially available, lyophilized catalase preparations.

Preparation of Solutions

I. Pyrophosphate buffer (0.1 M; pH 8.3):
Dissolve 8.922 g. $Na_4P_2O_7 \cdot 10H_2O$ in 100 ml. doubly distilled water, add 8 ml. 1 N HCl and dilute to 200 ml. with doubly distilled water.

II. D-Alanine (10 μmoles/ml.):
Dissolve 89.1 mg. D-alanine in doubly distilled water and make up to 100 ml.

III. D-Amino acid oxidase:
Use the crude enzyme prepared according to p. 371 directly.

Procedure

Preliminary remarks: The preliminary treatment of the sample depends on its composition. It is useful to confirm the experimental results by determining the amount of ammonia and α-keto acid formed in the reaction mixture.

Preliminary treatment of the sample

A minimum of 10 μmoles D-amino acid, in not more than 2 ml. solution (pH 8.3), are required for the manometric determination. Remove HCl from acid protein hydrolysates by continuous aeration and warming, or better still, by passing the solution through an ion exchange column

and eluting with dilute ammonia solution[11]. Concentrate the eluate at low temperature, preferably lyophilize to avoid racemization of the amino acids. All the ammonia must be removed since it would interfere with the determination of the ammonia liberated on deamination. Solutions containing D-amino acids (*e.g.* biological fluids such as plasma or serum, or enzymatic reaction mixtures) may be used without pretreatment, if they contain sufficient D-amino acids, do not have too high a blank oxygen consumption and do not contain too much ammonia or α-keto acids. Otherwise deproteinize and concentrate the free amino acids by chromatography on an ion exchange resin.

Manometric measurements

The determination is carried out with a Warburg apparatus. Conical flasks with side-arm and centre well; temperature 37°C; gas phase: oxygen; final volume 3.0 ml. 5 Flasks are required for each determination:

Flask 1. Thermobarometer

Flask 2. Enzyme blank for the estimation of O_2 uptake of the crude D-amino acid oxidase solution (omitted if the purified enzyme is used).

Flask 3. Standard to check that the assay is functioning (20 μmoles of D-alanine should be oxidatively deaminated in *ca.* 20 min. with the consumption of 224 μl. O_2).

Flask 4. Sample blank for the estimation of the O_2 uptake of the sample (not always necessary; but the solution serves for the determination of ammonia in the sample before the action of the enzyme).

Flask 5. Sample for determination.

Pipette into the flasks:

	Flask No.				
Main compartment:	1	2	3	4	5
Pyrophosphate buffer (soln. I)	0.75 ml.	0.75 ml.	0.75 ml.	0.75 ml.	0.75 ml.
Catalase	—	0.5 mg.	0.5 mg.	0.5 mg.	0.5 mg.
D-Alanine solution (II)	—	—	2.00 ml.	—	—
Sample	—	—	—	2.00 ml.	2.00 ml.
Water	2.25 ml.	2.00 ml.	—	—	—
Side-arm:					
Water	—	—	—	0.25 ml.	—
D-Amino acid oxidase (soln. III)	—	0.25 ml.	0.25 ml.	—	0.25 ml.
Centre well:					
2 N KOH (on filter paper)	0.20 ml.	0.20 ml.	0.20 ml.	0.20 ml.	0.20 ml.

Gas the flasks for 2 min. with oxygen, equilibrate for 5 to 10 min. and then close the manometer taps. Take an initial reading of the manometers, tip the contents of the side-arms into the main compartment and take readings at 5 min. intervals until the O_2 uptake in all flasks is practically zero. The rate of the reaction varies considerably depending on the nature of

[11] *P. Boulanger, G. Biserte* and *F. Courtot*, Bull. Soc. Chim. biol. France *34*, 366 [1952].

the amino acid and the composition of the sample solution. The reaction time is usually much longer than the 20 min. required for D-alanine.

Use the contents of the flasks for the determination of ammonia and α-keto acids.

Calculations

Correct the manometer readings for changes in the thermobarometer values (flask 1) and multiply by the flask constants (refer to p. 40). This gives the μl. oxygen consumed. Subtract the value obtained for flask 2 (enzyme blank) from the values for flasks 3—5. The O_2 uptake in flask 3 should now be 224 μl. (corresponding to 20 μmoles D-alanine). The value for flask 4 gives the blank oxygen consumption of the sample. Subtraction of this amount from the O_2 uptake for flask 5 gives the amount of oxygen required for the oxidative deamination of the D-amino acids in the sample.

As 1 μmole of D-amino acid corresponds to $^1/_2$ μmole of $O_2 = 11.2$ μl., the D-amino acid content per ml. sample is

$$\mu\text{moles D-amino acid/ml.} = \frac{\mu\text{l. oxygen uptake}}{11.2 \times \text{volume of sample taken for assay}}$$

Determination of the ammonia liberated

Pipette 1.5 ml. of solution from the main compartment of each Warburg vessel with a long, drawn-out pipette into 10 ml. conical centrifuge tubes and mix with 1 ml. 50% (w/v) trichloroacetic acid solution and 2.5 ml. water. Centrifuge at 3000 r.p.m. for 15 min. and use the supernatants for the determination of ammonia by the *Conway* method[12]. The contents of flask 4 serve as a control (NH_3 content before the enzymatic reaction) and the contents of flask 1 as a reagent blank.

Identification of the α-keto acids formed

The α-keto acids formed can be identified by paper chromatography of their 2,4-dinitrophenylhydrazones. The hydrazones can be converted to the original amino acids by hydrogenolysis and these can be identified by paper chromatography. Both methods have the same disadvantage: they are not suitable for the dinitrophenylhydrazones of α-keto acids formed by the deamination of basic D-amino acids (diaminobutyric acid, ornithine, lysine, arginine and histidine), because these hydrazones are not extracted by ether from acid solution.

Carefully remove the KOH-papers from the centre wells of the manometer flasks. Transfer the contents of the flasks to 10 ml. graduated centrifuge tubes, mix with 1 ml. 10% (w/v) sodium tungstate solution and 1 ml. 0.66 N H_2SO_4, dilute to the mark with water and centrifuge. Pour the supernatants into 50 ml. separating funnels and add 2 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. Allow to stand for 1 to 2 hours and then extract the hydrazones several times with peroxide-free ether. Wash the combined ether extracts with a little water and then extract with small amounts of 10% (w/v) sodium carbonate solution until the hydrazones are completely removed.

Carefully acidify the aqueous carbonate solutions with 10% (v/v) H_2SO_4 and re-extract the hydrazones with a little ether. Allow the ether to evaporate in small dishes in the dark. Take up the residues in 0.5 ml. ethanol and use these solutions for the identification of α-keto acids by paper chromatography[13] or reduction.

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[12] *E. J. Conway:* Microdiffusion Analysis and Volumetric Error. Crosby Lockwood and Son Ltd., London 1947.

[13] *D. Cavallini* and *N. Frontali*, Biochim. biophysica Acta *13*, 439 [1954].

II. Micro Methods

If the D-amino acid content of the sample is too low for the manometric method then it can be measured by one of the following enzymatic methods.

1. After paper chromatography

Principle

The amino acids are separated by two dimensional paper chromatography using well-known methods[14] and the chromatogram is sprayed with a solution of purified D-amino acid oxidase and catalase[15]. The keto acids formed are located in UV-light (yellow fluorescence) after spraying with Wieland's reagent[16].

Reagents and Solutions

All solutions required for paper chromatography and

I. D-Amino acid oxidase-catalase:
Dissolve 5 mg. catalase powder in doubly distilled water, add 2 ml. D-amino acid oxidase solution and make up to 100 ml.

II. Wieland's reagent:
Dissolve 100 mg. *o*-phenylenediamine in 100 ml. 5% (w/v) trichloroacetic acid.

Procedure

Spray a two dimensional chromatogram of the sample with D-amino acid oxidase-catalase solution (I), incubate for 1 to 2 hours in a closed container with a high humidity. Spray with Wieland's reagent. Compare the dirty-yellow fluorescence in UV-light or the pink colour obtained on heating with a standard chromatogram.

Sources of Error and Sensitivity

The D-amino acid oxidase solution also has considerable fluorescence, therefore the reaction loses greatly in sensitivity.

2. In combination with ion exchange chromatography[17]

Principle

Half the sample is incubated with highly active D-amino acid oxidase (without addition of catalase) for *ca.* 3 hours at 37.5° C. The difference between the amino acid values (obtained by ion exchange chromatography) before and after incubation corresponds to the D-amino acid content of the sample[18].

Appendix

Isolation of D-amino acid oxidase

Crude extracts from pig kidney have a higher activity with D-aspartic acid and D-glutamic acid, but are less stable than preparations from sheep kidney. They dissociate easily to FAD and inactive protein. To prepare the crude enzyme pig kidney should be used and to obtain a purified preparation sheep kidney should be used.

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14) *E.* and *M. Lederer:* Chromatography. Elsevier, Amsterdam 1953, p. 197.
15) *T. S. G. Jones*, Biochem. J. *42*, LIX, [1949].
16) *T. Wieland*, Angew. Chem. *60*, 171 [1951].
17) *S. Moore, D. H. Spackman* and *W. H. Stein:* Analytic. Chem. *30*, 1185 [1958].
18) *G. Biserte* and *M. Dautrevaux*, Bull. Soc. chim. biol. France *39*, 795 [1957]

Reagents

Ia) Pyrophosphate buffer (0.05 M; pH 8.3):
Dilute solution I (p. 368) in the ratio 1 : 1 with water.

Ib) Pyrophosphate buffer (0.017 M; pH 8.3):
Dilute solution Ia in the ratio 1 : 2 with water.

Ic) Pyrophosphate buffer (0.067 M; pH 8.3):
Dilute solution I (p. 368) in the ratio 2 : 1 with water.

Procedure

a) *Acetone-dried powder*

Remove fat and decapsulate kidneys from freshly slaughtered pigs or sheep (frozen kidney can be used if worked up immediately after thawing), cut into small pieces and homogenize with 3 volumes of acetone at 4° C. Quickly filter the suspension. Suspend the moist residue in the same volume of cold acetone and filter. Wash again with acetone and then three times with the same volume of ether at 4° C. Allow the powder to dry in the air as a thin layer and store, stoppered, at 4° C. The enzyme is stable for several years.

b) *Preparation of the crude enzyme*

Stir 1 g. acetone-dried powder with 4 ml. pyrophosphate buffer (solution Ib) for 20 min. Centrifuge at 3000 r.p.m. for 30 to 40 min., remove the small fatty layer, decant the strongly coloured supernatant and filter if necessary.

c) *Preparation of the purified enzyme*

Proceed according to[19], but use sodium sulphate instead of ammonium sulphate for the fractional precipitation, so that the ammonia liberated under the assay conditions can be determined.

Suspend 10 g. acetone-dried powder from sheep kidney in 250 ml. 0.017 M pyrophosphate buffer (solution Ib), stir for 45 min. at 38° C and then centrifuge at 3000 r.p.m. for 30 min. Decant the supernatant, adjust to pH 5.1, quickly heat to 38° C and then cool to 15° C in ice water. Immediately centrifuge off the precipitate and filter the supernatant. Add 17 g. anhydrous sodium sulphate to every 100 ml. filtrate and stir for 2 hours at room temperature. Centrifuge off the precipitate and dissolve in 10 ml. 0.067 M pyrophosphate buffer (solution Ic). Use this enzyme solution for the determination. The purified enzyme exhibits no deaminating activity with L-amino acids and glycine.

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[19] *E. Negelein* and *H. Brömel*, Biochem. Z. *300*, 225 [1939].