L-Amino Acids

L-Lysine, L-Arginine, L-Ornithine, L-Tyrosine, L-Histidine, L-Glutamic Acid, L-Aspartic Acid

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Certain bacteria, grown under suitable conditions, produce specific L-amino acid decarboxylases[1-3]. In most cases, the pH optima of these carboxylases are in the acid range, so that the CO_2 produced can be measured manometrically.

Principle

Amino acid decarboxylases catalyse reactions of the type:

(1) $$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H \longrightarrow R-CH_2-NH_2 + CO_2$$

The CO_2 produced is determined in a Warburg manometer and is a measure of the amino acid content of the sample. Specific decarboxylases are available for the following amino acids:

a)	L-Lysine	cadaverine	(pH optimum: 6.0)
b)	L-Arginine	agmatine	(pH optimum: 5.2)
c)	L-Ornithine	putrescine	(pH optimum: 5.5)
d)	L-Tyrosine	tyramine	(pH optimum: 5.5)
e)	L-Histidine	histamine	(pH optimum: 4.5)
f)	L-Glutamic acid	γ-aminobutyric acid	(pH optimum: 4.5)
g)	L-Aspartic acid	α-alanine	(pH optimum: 5.5)

Reagents

The letters a) to g) correspond to the seven amino acids listed above and indicate the reagents which are required for the determination of the respective amino acids.

1. Reagents for buffers
   - a) Potassium dihydrogen phosphate, KH_2PO_4
   - a–d) Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$
   - b–d) Citric acid
   - e–g) Sodium acetate, anhydrous
   - e–g) Acetic acid
2. a, c, g) Sulphuric acid, *ca.* 2 N
3. Amino acid decarboxylases
   - a) L-Lysine decarboxylase
     acetone-dried powder of *Bacterium cadaveris* (NCIB *) No. 6578). For conditions of growth and preparation of the acetone powder[2], see the Appendix, p. 377.
   - b) L-Arginine decarboxylase
     acetone-dried powder from *Escherichia coli* (NCIB No. 7020). For conditions of growth and preparation of the acetone powder[2], see the Appendix, p. 377.

*) National Collection of Industrial Bacteria, Address: Torry Research Station, Aberdeen, Scotland.

1) *E. F. Gale*, Advances in Enzymology 6, 1 [1946].
2) *E. F. Gale* in *D. Glick:* Methods of Biochemical Analysis. Interscience, New York 1957, Vol. IV, p. 285.

c) L-Ornithine decarboxylase
washed cells of *Clostridium septicum* Pasteur (NCIB No. 547). For conditions of growth[2], see the Appendix, p. 377.

d) L-Tyrosine decarboxylase
acetone-dried powder of *Streptococcus faecalis* (NCIB No. 6782). For conditions of growth and preparation of the acetone-dried powder, see the Appendix, p. 377.

e) L-Histidine decarboxylase
acetone-dried powder from *Clostridium welchii* BW 21 (NCIB No. 6785). For conditions of growth and preparation of the acetone-dried powder, see the Appendix, p. 377.

f) L-Glutamic acid decarboxylase
washed cells of *Clostridium welchii* SR 12*) (NCIB No. 6784). For conditions of growth, see the Appendix, p. 377.

g) L-Aspartic acid decarboxylase
acetone-dried powder from *Nocardia globerula* (NCIB No. 8852). For conditions of growth and preparation of the acetone-dried powder[3], see the Appendix, p. 377.

Stability of the solutions

To obtain preparations of the required specificity, the correct strain of organism must be used, and the conditions of growth and method of preparation of the acetone-dried powders must be strictly adhered to. The lysine decarboxylase preparation may contain traces of arginine decarboxylase; however, the activity of the latter disappears if the acetone-dried preparation is kept for 2–3 days at 0 to 4° C. Likewise, the histidine decarboxylase preparations from *Cl. welchii* occasionally have weak glutamic acid decarboxylase activity. In this case, suspend the acetone-dried powder in 0.05 M borate buffer (pH 8.5) (40 mg./ml.) and incubate overnight at 37° C. Centrifuge for 30 min. at 4000 g and use the clear, yellow supernatant as the histidine decarboxylase preparation[4].

Preparation of Solutions

The letters a) to g) correspond to the seven amino acids listed in the order given on p. 373 and indicate the solutions required for the determination of the respective amino acids. Prepare all solutions with freshly distilled water.

I. Buffer solutions

a) Phosphate buffer (0.2 M; pH 6.0):
Mix 13.0 ml. 0.2 M Na_2HPO_4 solution (35.6 g. $Na_2HPO_4 \cdot 2H_2O$/1000 ml.) with 87.0 ml. 0.2 M KH_2PO_4 solution (27.2 g. KH_2PO_4/1000 ml.).

b) Phosphate-citrate buffer (pH 5.2):
Mix 46.4 ml. 0.1 M citric acid (19.2 g./1000 ml.) with 53.6 ml. 0.2 M Na_2HPO_4 solution (35.6 g. Na_2HPO_4/1000 ml.).

c,d) Phosphate-citrate buffer (pH 5.5):
Mix 43.1 ml. 0.1 M citric acid (19.2 g./1000 ml.) with 65.9 ml. 0.2 M Na_2HPO_4 solution (35.6 g. $Na_2HPO_4 \cdot 2H_2O$/1000 ml.).

e,f) Acetate buffer (0.2 M; pH 4.5):
Mix 42.5 ml. 0.2 M Na acetate solution (16.4 g./1000 ml.) with 57.5 ml. 0.2 N acetic acid (12.0 g. acetic acid/1000 ml.).

*) It is very important that this particular strain is used.

3) *L. V. Crawford*, Biochem. J. *68*, 221 [1958].

4) *H. M. R. Epps*, Biochem. J. *39*, 42 [1945].

g) Acetate buffer (0.1 M; pH 5.5):
Mix 88.0 ml. 0.1 M Na acetate solution (8.2 g./1000 ml.) with 12.0 ml. 0.1 N acetic acid (6.0 g. acetic acid/1000 ml.).

II. Enzyme suspensions

a) L-Lysine decarboxylase
Suspend 100 mg. acetone-dried powder in 5 ml. buffer (solution Ia).

b) L-Arginine decarboxylase
Suspend 100 mg. acetone-dried powder in 5 ml. buffer (solution Ib).

c) L-Ornithine decarboxylase
Suspend 250 mg. washed cells (dry weight) in 5 ml. buffer (solution Ic,d).

d) L-Tyrosine decarboxylase
Suspend 100 mg. acetone-dried powder in 5 ml. buffer (solution Ic,d).

e) L-Histidine decarboxylase
Suspend 300 mg. acetone-dried powder in 5 ml. buffer (solution Ie,f).

f) L-Glutamic acid decarboxylase
Suspend 200 mg. washed cells (dry weight) in 5 ml. buffer (solution Ie,f).

g) L-Aspartic acid decarboxylase
Suspend 50 mg. acetone-dried powder in 5 ml. buffer (solution Ig).

Stability of the solutions

The buffer solutions keep indefinitely in stoppered bottles at 0 to 4° C. The stability of the acetone-dried powders varies from preparation to preparation. Normally, they retain their activity for 2—3 months (sometimes years) when stored in a desiccator. Occasionally the preparations lose their activity within a few days. Suspensions of *Cl. welchii* SR 12 keep for several weeks at 4° C. In contrast, the ornithine decarboxylase activity of suspensions of *Cl. septicum* is much less stable and may be lost within 2—3 days at 4° C. It is best to use a freshly prepared suspension for each estimation.

Procedure

Experimental material

The amino acid solution to be analysed must not contain any inhibitors of the respective amino acid decarboxylase preparations. The pH of the sample must be sufficiently near to the pH optimum of the enzyme, so that when buffer is added the optimum pH is attained. Decarboxylase preparations do not usually attack carbohydrates. However, if the sample contains fermentable sugars, for example, glucose, it is advisable to include a control containing the same concentration of sugar (this is especially important when washed cells are used).

Manometric measurements

Warburg manometers; vessels with side-arms; temperature: 37° C; gas phase: air.
For each estimation 3—4 vessels are necessary: 1—2 experimental vessels, 1 control vessel (without substrate) and 1 thermobarometer. Prepare the vessels as follows:

		Experimental vessel	Control vessel	Thermo-barometer
Main compartment	sample	0.5—1.0 ml.	—	—
	distilled water	—	0.5—1.0 ml.	2.5 ml.
	buffer	1.5—1.0 ml.	1.5—1.0 ml.	—
Side-arm	enzyme preparation	0.5 ml.	0.5 ml.	—

Equilibrate the vessels for 5 – 10 min. Close the taps and read the manometers. Tip the enzyme preparation into the main compartment and record the increase in pressure until the reaction ceases (10 – 30 min.).

In the determination of lysine, ornithine and aspartic acid the pH at the end of the reaction is 5.8 and therefore some CO_2 is retained. To determine this retention use manometer vessels with double side-arms. Prepare the second side-arm with

0.4 ml. 2 N H_2SO_4.

At the end of the enzymatic reaction tip the acid into the main compartment and read the increase in pressure.

Calculations *)

The volume of CO_2 produced is calculated from the manometer readings (mm. manometer fluid) (after correction for the thermobarometer changes) by multiplication by the manometer constant k[5]:

$$k = \frac{V_g \frac{273}{T} + V_f \times \alpha}{P_0}$$

where

V_g = volume of the gas phase in the manometer [ml.]
V_f = volume of fluid in the manometer [ml.]
α = solubility [ml./ml.] of CO_2 in water at 760 mm. and temperature T
T = absolute temperature of the reaction [°K]
P_0 = 760 mm. Hg pressure expressed in terms of manometric fluid (usually P_0 = 10 m.).

The CO_2 production is usually somewhat less than 100% theory. Assays on standard solutions gave the following values (last column: factor with which volume of CO_2 produced in the experimental vessel must be multiplied to obtain the mg. amino acid in the reaction mixture):

	100 µl. CO_2 are produced by	Yield %	Factor
a)	0.652 mg. lysine	98	$\frac{0.652}{98}$
b)	0.775 mg. arginine	95	$\frac{0.775}{95}$
c)	0.590 mg. ornithine	98	$\frac{0.590}{98}$
d)	0.810 mg. tyrosine	96	$\frac{0.810}{96}$
e)	0.692 mg. histidine	96	$\frac{0.692}{96}$
f)	0.656 mg. glutamic acid	98	$\frac{0.656}{98}$
g)	0.596 mg. aspartic acid	97	$\frac{0.596}{97}$

*) Refer to p. 40.

5) *W. W. Umbreit, R. H. Burris* and *J. F. Stauffer:* Manometric Techniques. Burgess Publ. Co., Minneapolis, Minn., 1949.

Example

Determination of L-lysine. Experimental protocol (manometer readings corrected for the thermobarometer changes):

	Control vessel	Experimental vessel
Increase in pressure during the enzymatic reaction	2 mm.	154 mm.
Increase in pressure after tipping acid	13 mm.	25 mm.
Total increase in pressure	15 mm.	179 mm.
Manometer constant	1.73	1.89
Volume of CO_2 evolved	$15 \times 1.73 = 26$ µl.	$179 \times 1.89 = 338$ µl.
Volume of CO_2 liberated from lysine		$338 - 26 = 312$ µl.

$$312 \times \frac{0.652}{98} = 2.08 \text{ mg. lysine/reaction mixture.}$$

Specificity

Each enzyme preparation is specific for its respective L-amino acid substrate. The carboxyl and α-amino group of the amino acid must not be substituted[1]. Occasionally an amino acid derivative with an OH group in the rest of the molecule is attacked: lysine decarboxylase reacts slowly with hydroxylysine; tyrosine decarboxylase attacks phenylalanine[6] at 5—10% of the rate at which tyrosine is decarboxylated and it also reacts with L-3,4-dihydroxyphenylalanine[7]. Glutamic acid preparations may liberate CO_2 from certain isomers of β-hydroxyglutamic acid[8], and also from L-aspartic acid if traces of pyruvate, α-oxoglutarate or other keto acids are present in the sample[9]. The reaction with L-aspartic acid is prevented by the addition of cetyltrimethylammonium bromide (0.25% w/v).

Appendix

Enzyme Preparations

It is essential for the success of the determination that the conditions of growth described in the original publications[2–4] are strictly adhered to. The following information only serves to give an outline of the methods. The letters a) to g) correspond to those on p. 373.

Conditions of growth

a, b) 30 hours at 25°C in a medium containing 3% casein hydrolysate and 2% glucose.

c, e, f) 16 hours at 37°C in a medium containing 3% casein hydrolysate, 2% glucose, 0.1% yeast extract and heart muscle particles. Anaerobic conditions.

d) 16 hours at 37°C in a medium containing 3% casein hydrolysate, 2% glucose and 0.1% yeast extract.

g) 60 hours at 30°C in a medium containing 2% peptone. Aerobic conditions.

With a), b) and d) the bacteria are grown in a flask filled to the neck, but not stoppered. Under these conditions the culture is partially anaerobic.

Preparation of the acetone-dried powders

Suspend the cells in distilled water so that they form a thick suspension or a cream. With stirring, quickly pour into this suspension five volumes of acetone at 15°C. Continue stirring until the cells coagulate. Collect the precipitate (Buchner funnel) and wash once with acetone and ether, and dry in the air.

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6) *R. W. McGilvery* and *P. P. Cohen*, J. biol. Chemistry *174*, 813 [1948].
7) *H. M. R. Epps*, Biochem. J. *47*, 605 [1944].
8) *W. W. Umbreit* and *P. Heneage*, J. biol. Chemistry *201*, 15 [1953].
9) *A. Meister*, *H. A. Sober* and *S. V. Tice*, J. biol. Chemistry *189*, 577, 591 [1951].