whereas plasmin splits both the arginine and lysine esters.

The results presented in this paper indicate that the fibrinolytic euglobulin preparations contain plasmin which splits the tosylarginine ester but not the lysine ester; they contain little plasminogen, since there is no increase in the splitting of the arginine esters with the addition of streptokinase; and they contain some proactivator, as is shown by the increased breakdown of lysine esters after addition of streptokinase. The non-fibrinolytic euglobulin contains little plasmin but considerable amounts of proactivator and plasminogen, which can be converted into activator and plasmin respectively, as is shown by the marked increase in esterase activity in both assays on the addition of streptokinase.

When fibrinolytic euglobulins are added to plasma, the lack of increase of esterase activity could be caused by the inhibitors of the plasma which have been shown to inhibit ester-splitting activity of trypsin (Bastian *et al.* 1956). The significant inhibition of the plasma-esterase activity by the non-fibrinolytic euglobulins could be due to the presence in the euglobulins of inhibitors which might have been eliminated from the fibrinolytic euglobulins during their preparation. Such inhibitors have been demonstrated in precipitated euglobulins (Müllertz, 1953). The significant increase of esterase observed when streptokinase is added to the non-fibrinolytic euglobulins in plasma can be explained by the presence of plasminogen and proactivator in the euglobulin preparation.

SUMMARY

1. Little correlation could be found between the fibrinolysis-inducing effect of urokinase and fibrino-

lytic euglobulins as shown by lysis of plasma clots and the plasma-enzyme activity measured by the splitting of synthetic amino acid esters.

2. When urokinase was added to plasma there was no increase in esterase activity, but a marked fibrinolytic activity was observed.

3. Fibrinolytic euglobulins used alone showed an activity towards tosylarginine methyl ester, but not towards lysine ethyl ester. There was no increase in activity when the fibrinolytic euglobulins were added to plasma, but a strong fibrinolysis was obtained.

4. It is concluded that the synthetic-substrate methods are not applicable as tests for fibrinolytic activity in the presence of plasma. Possible reasons for the discrepancies are discussed.

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REFERENCES

- Astrup, T. (1956). *Blood*, **11**, 781.
Astrup, T. & Sterndorff, I. (1952). *Proc. Soc. exp. biol., N.Y.*, **81**, 675.
Bastian, J. W., Hill, R. G. & Ercoli, N. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 800.
Kaulla, K. N. von (1954). *J. Lab. clin. Med.* **44**, 944.
Kaulla, K. N. von (1956). *Acta haemat.* **16**, 315.
Kaulla, K. N. von & Taylor, C. (1957). *Fed. Proc.* **16**, 1466.
Müllertz, S. (1953). *Acta physiol. scand.* **28**, 29.
Müllertz, S. (1955). *Biochem. J.* **61**, 424.
Quick, A. J. (1951). *The Physiology and Pathology of Hemostasis*, p. 82. Philadelphia, Pa.: Lea and Febiger.
Schultz, R. L., Moorman, J. A., Matoush, L. O. & Lincoln, A. F. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 198.
Shulman, N. R. (1955). *J. biol. Chem.* **213**, 655.
Troll, W. & Sherry, S. (1955). *J. biol. Chem.* **213**, 881.
Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.

Studies on the Aspartic Decarboxylase of *Nocardia globerula*

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Specific decarboxylases have been described for several amino acids and have proved useful for the estimation of these amino acids in mixtures (Gale, 1957). The enzyme system from *Clostridium welchii* is much more active on glutamic acid than aspartic acid. The determination of aspartic acid with this preparation depends on the difference between the volume of carbon dioxide produced from glutamic acid plus aspartic acid, and that produced from glutamic acid alone when the aspartic decarboxylase is inhibited (Meister, Sober & Tice, 1951). The

present communication describes a preparation specific for the decarboxylation of L-aspartic acid and completely inactive on all other amino acids tested.

MATERIALS AND METHODS

Organism. The organism was isolated from soil and corresponded closely in properties with *Nocardia globerula* (Gray, 1928). A strain of this organism has been deposited with the National Collection of Industrial Bacteria, Teddington, Middlesex, under the no. 8852. During these studies the organism was maintained on agar slopes

containing 2% (w/v) of Difco peptone and subcultured once a month.

Preparation of the acetone-dried powder. The inoculum for enzyme preparation was taken from a peptone slope culture grown for 24 hr. at 30° and suspended in 2.5 l. of 2% (w/v) peptone in a 5 l. round-bottom flask. Incubation was continued for 60 hr. with occasional shaking. The culture was then harvested by centrifuging, the pellet washed once with and resuspended in 0.85% NaCl, and pipetted rapidly into 20 vol. of acetone at 4°. The coagulum was removed on a Büchner filter, washed once with acetone (4°) and once with ether (4°). The pad was then broken up and allowed to dry in air at room temperature. The yield of acetone-dried powder was about 0.6 g./l. of culture, with a $q\text{CO}_2$ of 50 ($q\text{CO}_2 = \mu\text{l. of CO}_2/\text{mg. of acetone-dried powder/hr.}$) under the conditions given below. For use the powder was suspended in water (10 mg./ml.). The aspartic decarboxylase activity of the dry powder was stable for at least 2 years in a stoppered bottle at room temperature, and that of the suspension for several months at -15°.

Buffers. The buffers used were made up by titrating 100 ml. of the acid (0.3M) with the appropriate alkali to the required pH value and diluting to 150 ml. with water. The molarity of the buffer given refers to the concentration of total acid, dissociated and undissociated.

Manometric methods. Determinations of the rate of the reaction were made in single-side-bulb Warburg manometers at 37° with air as the gas phase. The main compartment contained 0.5 ml. of the suspension of acetone-dried powder, sodium acetate buffer (pH 5.5, 0.1M final concentration) and any other additions required; the side bulb contained 2–10 μmoles of L-aspartic acid in sodium acetate buffer (0.1M, pH 5.5). The total volume of liquid was 3 ml. The substrate was added after equilibration and readings were taken for at least 20 min. to obtain an estimate of the rate of CO_2 production.

Quantitative estimations of aspartic acid were made in a similar manner except that double-side-bulb manometers were used, the second side bulb containing 0.4 ml. of 2N- H_2SO_4 , which was added after completion of the reaction to expel any bound CO_2 . Two controls were set up with the same system but with the aspartic acid-containing solution replaced by water. The first of these contained a paper soaked in KOH soln. in the centre well. The pressure change occurring in this manometer is due mainly to a change in vapour pressure resulting from the addition of the sulphuric acid (Peel, personal communication), and the pressure changes in the other manometers were corrected by subtracting this value. The corrected pressure change in the second control was then converted into a volume and subtracted from the experimental determinations.

RESULTS

Products of the reaction. The gas produced was absorbed by 10% (w/v) KOH and was therefore assumed to be CO_2 . The non-volatile product of the reaction was prepared by the action of the enzyme in ammonium acetate buffer (0.1M, pH 5.5). When CO_2 evolution had ceased acetic acid was added until a flocculent precipitate came down. The precipitate was centrifuged off; the

clear solution was evaporated to dryness at 60° in a stream of air and the residue dissolved in 1 ml. of water and again evaporated to dryness. The resulting substance was identified as α -alanine by its R_f values, in comparison with an authentic sample of α -alanine, on paper chromatograms developed in the ascending direction on Whatman no. 4 paper with the butan-2-ol-formic acid-water and the phenol-aq. NH_3 soln.-water systems of McQuillen & Roberts (1954) and the ethyl methyl ketone-propionic acid-water system of Clayton & Strong (1954). α -Alanine and β -alanine have different R_f values in the last two solvent systems. They also give characteristically different colours when sprayed with a solution of ninhydrin (0.1%, w/v) in acetone containing CoCl_2 (0.3%, w/v) and heated to 100° for a few minutes (modified from Wiggins & Williams, 1952), α -alanine giving a purple colour and β -alanine a reddish-brown colour. The residual aspartic acid in the product was separated on paper chromatograms in the phenol-aq. NH_3 soln.-water system (McQuillen & Roberts, 1954) and estimated by the quantitative ninhydrin reaction (Cocking & Yemm, 1954). Less than 0.1% of the initial aspartic acid remained.

The optical rotation of the product was determined in a solution of 140 mg. in 10 ml. of acetic acid in a 2 dm. tube. The rotation was $[\alpha]_D^{25} + 30^\circ$ ($\pm 2^\circ$) and was similar to that of an authentic sample of L- α -alanine.

Quantitative nature of the reaction. The mean of ten determinations of the CO_2 evolved from 10 μmoles of L-aspartic acid was 9.8 μmoles (S.D. 0.6 μmole). The quantitative ninhydrin reaction (Cocking & Yemm, 1954) of the mixture remained unchanged during the reaction, so that α -alanine was apparently produced quantitatively from aspartic acid. Production of CO_2 from protein hydrolysates was also studied and, as shown by Table 1, the volume of CO_2 produced from hydrolysates of pure edestin was close to that calculated on the basis of its aspartic acid content. The volume of CO_2 produced from DL-aspartic acid was half that produced from an equimolar amount of L-aspartic acid (Fig. 1).

Specificity. The preparation produced CO_2 from L-aspartic acid but not from L-alanine, L-arginine, L-cysteine, L-glutamic acid, glycine, L-histidine, DL-isoleucine, L-leucine, L-lysine, DL-methionine, L-ornithine, DL-phenylalanine, L-proline, L-tyrosine, DL-serine, DL-tryptophan, DL-threonine, L-valine, α -L-glutamyl-L-aspartic acid and γ -L-glutamyl-L-aspartic acid. The addition of each of the above amino acids had no effect on the production of CO_2 from L-aspartic acid. It was concluded that the enzyme was specific for L-aspartic acid. The overall reaction is therefore: L-aspartic acid $\rightarrow$ L- α -alanine + CO_2 .

Table 1. *Estimation of aspartic acid in protein hydrolysates*

Pure edestin [254 mg., aspartic acid content 12% (w/w), Tristram, 1953] was hydrolysed with 6N-HCl in a sealed tube for 18 hr. at 105°. The HCl was removed in a stream of air at 45° and the residue dried *in vacuo* three times. The volume was then made up to 10 ml. with water and samples (0.5 ml.) were taken for aspartic acid determination with the *Nocardia globerulea* aspartic decarboxylase. Each manometer contained 0.5 ml. of the acetone-dried-powder suspension and sodium acetate buffer (pH 5.5, 0.1M final concentration) in the main compartment; one side bulb contained the protein hydrolysate and the other 0.4 ml. of 2N-H₂SO₄. The hydrolysate was added after equilibration and the H₂SO₄ after completion of the reaction. The estimation was carried out at 37° with air as the gas phase. After allowing for ash and moisture content the expected volume of CO₂ from the aspartic acid in the protein was 227 µl. The value given for the observed volume of CO₂ is corrected for the two controls as described in the text. Control 1 (+KOH) = 6 mm.; control 2 = 17 µl.

	(a) Theoretical CO ₂ (µl.)	(b) Observed CO ₂ (µl.)	(b × 100)/a	Mean
1	227	213	94	95
2	227	218	96	
3	227	224	98	
4	227	214	94	
5	227	206	91

Fig. 1. Production of CO₂ from L- and DL-aspartic acid. Each manometer contained 0.5 ml. of acetone-dried-powder suspension in sodium acetate buffer (pH 5.5, 0.1M final concentration) in the main compartment; the side bulb contained 10 µmoles of L-aspartic acid or 10 µmoles of DL-aspartic acid in the same buffer. Total volume of liquid was 3 ml.; gas phase, air; temperature, 37°. The substrate was added at time 0 after equilibration and readings were taken every 5 min. for 60 min. The results have been corrected for the CO₂ produced in the control without substrate.

Fig. 2. Effect of pH value on the rate of CO₂ production. Conditions were as described in Fig. 1 except that sodium acetate buffers of the stated pH values were used (0.1M final concentration) and the side bulb contained 10 µmoles of L-aspartic acid in all cases. The results have been corrected for the control without substrate (qCO₂ = 3.0, pH value 5.5) and the rates were calculated over the first 20 min.

Effect of pH. The optimum pH value for CO₂ production from L-aspartic acid was found to be 5.5 under the conditions given above (Fig. 2).

Effect of inhibitors. The effect of various buffers and other additions on the rate of CO₂ production is shown in Table 2. In general the results are similar to those obtained with the *Cl. welchii* system (Meister *et al.* 1951).

Coenzyme requirements. Many of the amino acid decarboxylases have been shown to require pyridoxal phosphate as coenzyme. The aspartic decarboxylase of *Nocardia globerulea* was stimulated about 40% by the addition of pyridoxal phosphate (3 µg./ml.). However, it was not possible to resolve the preparation into apoenzyme and coenzyme by treatment with ammoniacal ammonium sulphate, in contrast with certain of the other amino acid decarboxylases (Gale, 1946). Ultraviolet irradiation increased the dependence on added pyridoxal phosphate although the enzyme activity was rapidly lost during this treatment. It seems that pyridoxal phosphate may be the coenzyme for this reaction.

Mechanism of the reaction. The effects of semicarbazide and keto acids are consistent with a reaction mechanism involving transamination, possibly:

Table 2. *Effect of buffers and other additions on the rate of production of carbon dioxide from L-aspartic acid*

Each manometer contained 0.5 ml. of acetone-dried-powder suspension in sodium buffer of the acid stated (pH 5.5, 0.1 M final concentration) in the main compartment; the side bulb contained 10 μ moles of L-aspartic acid in the same buffer. Total volume of liquid was 3 ml. Rates were determined at 37° with air as the gas phase. The substrate was added at time 0 after equilibration. Rates are given after subtraction of the control without substrate, relative to acetate buffer = 100. $q\text{CO}_2$ with acetate buffer = 52.

Buffer	Other additions	Relative rate
Phosphate	—	51
Succinate	—	8
Phthalate	—	5
Barbiturate	—	2
Citrate	—	2
Acetate	Citrate (0.01 M)	2
Acetate	Citrate (0.01 M) + CaCl_2 (0.01 M)	2
Acetate	Citrate (0.01 M) + MgCl_2 (0.01 M)	3
Acetate	Ethylenediaminetetra-acetate (0.01 M)	100
Acetate	Semicarbazide (0.006 M)	11
Acetate	Semicarbazide (0.006 M) + pyruvate (0.018 M)	140
Acetate	Semicarbazide (0.006 M) + α -ketoglutarate (0.018 M)	138
Acetate	Cetyltrimethylammonium bromide (0.006 M)	1

Table 3. *Decarboxylation of [^{15}N]aspartic acid*

Six samples of 10 μ moles of [^{15}N]aspartic acid were decarboxylated by the *Nocardia globetula* aspartic decarboxylase in ammonium acetate buffer (pH 5.5, 0.1 M) at 37° in manometers with air as the gas phase. To three of the samples (a) 100 μ moles of L-glutamic acid were added; other samples (b) had no addition. The alanine produced was isolated, the amino nitrogen converted into gaseous nitrogen and the ^{14}N : ^{15}N ratio determined with a mass spectrograph.

	Ratio ^{14}N : ^{15}N	Mean	Atoms % excess
Initial aspartic acid	42.7	—	0.78
Alanine (a) 1	44.0	43.9	0.75
2	46.7		
3	41.1		
Alanine (b) 1	47.1	46.2	0.70
2	43.1		
3	48.4		

If an excess of a suitable keto acid were added to the reaction system it might be expected to give rise to the corresponding amino acid. This was tested by adding an excess of α -ketoglutaric acid and estimating the glutamic acid present by the specific decarboxylase method (Gale, 1946). No increase could be detected during the reaction.

A further study was made with [^{15}N]aspartic acid. If the reaction takes place by a transamination mechanism it might be possible to dilute the ^{15}N appearing in the alanine by carrying out the reaction in the presence of another amino acid. Accordingly, six samples of 10 μ moles of [^{15}N]aspartic acid were decarboxylated by the acetone-dried powder in ammonium acetate buffer (pH 5.5, 0.1 M) at 37° in manometers. To three of the samples 100 μ moles of L-glutamic acid was added. When

CO_2 evolution had ceased each sample was treated as follows. The contents of the manometer cup were washed out, the protein was removed and the reaction product dried as described for the isolation of α -alanine. The alanine was then separated from the reaction mixture on paper chromatograms in the phenol-aq. NH_3 soln.-water system (McQuillen & Roberts, 1954); the alanine spots, located by the position of marker spots, were cut out and eluted with water. The samples were then dried and made alkaline with methanolic sodium borate (Connell, Dixon & Hanes, 1955) and dried again to remove ammonia. The amino nitrogen was converted into ammonia by digestion with H_2SO_4 and a mercury catalyst (Hiller, Plazin & Van Slyke, 1948). The ammonia formed was distilled off in a Rittenberg all-glass still and collected in 0.1 N- H_2SO_4 . After reduction of the volume to 2 ml., ammonium sulphate was added to bring the total ammonium nitrogen to 1 mg./sample. The ammonia was converted into gaseous nitrogen by the method of Sprinson & Rittenberg (1949) with sodium hypobromite. ^{14}N : ^{15}N ratios were determined in the mass spectrometer. The initial [^{15}N]aspartic acid was digested directly in the same way. Table 3 shows that there was little effect of decarboxylation or of the presence of glutamic acid on the ^{14}N : ^{15}N ratio of the reaction product. Had the amino group of the glutamic acid equilibrated completely with that of the alanine formed, the product would have contained about 0.07–0.08 atoms % excess of ^{15}N , as the amount of glutamic acid present was ten times the amount of aspartic acid present, and the ^{14}N : ^{15}N ratio of the product would have been affected proportionately. It therefore remains uncertain whether the reaction takes place by a direct decarboxylation or by a

transamination mechanism. However, if a transamination reaction is taking place it is apparently concerned with some amino acid other than glutamic acid.

SUMMARY

1. Acetone-dried powders of a strain of *Nocardia globnerula* possess aspartic decarboxylase activity but are inactive towards all other amino acids tested.

2. The enzyme can be used for the quantitative estimation of L-aspartic acid in protein hydrolysates and is stable for long periods.

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REFERENCES

- Clayton, R. A. & Strong, F. M. (1954). *Analyt. Chem.* **26**, 1362.
 Cocking, E. F. & Yemm, E. W. (1954). *Biochem. J.* **53**, xii.
 Connell, G. D., Dixon, G. H. & Hanes, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 46.
 Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
 Gale, E. F. (1957). *Methods of Biochemical Analysis* IV, p. 285. New York: Interscience Publishers Inc.
 Gray, P. H. H. (1928). *Proc. Roy. Soc. B.* **102**, 265.
 Hiller, A., Plazin, J. & Van Slyke, D. D. (1948). *J. biol. Chem.* **176**, 1401.
 McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
 Meister, A., Sober, H. A. & Tice, S. V. (1951). *J. biol. Chem.* **189**, 577.
 Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 705.
 Tristram, G. R. (1953). In *The Proteins*, vol. 1, part A, chap. 3. New York: Academic Press Inc.
 Wiggins, L. F. & Williams, J. H. (1952). *Nature, Lond.*, **170**, 279.

Succinate as a Respiratory Inhibitor in *Tenebrio molitor* Pupal-Tissue Preparations

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When changes in the chemical composition of *Tenebrio* tissues during the pupal period were being observed (Patterson, 1956*a*) an effort was made to relate these findings to alterations in enzyme pattern which have been reported by various authors, e.g. Agrell (1953) for holometabolous insects during metamorphosis. Furthermore, as citrate was present in greatest concentration in *Tenebrio* pupal tissues at the stage of development corresponding to the lowest rate of oxygen consumption by the live pupa (Patterson, 1956*b*), any variations in the activities of the enzymes of the tricarboxylic acid cycle during metamorphosis were considered to be of interest.

The oxidation of succinate by *Tenebrio* tissues is the only step in the tricarboxylic acid cycle so far investigated. It has been found, surprisingly, that the addition of succinate to pupal-tissue homogenates does not uniformly stimulate respiration and, indeed, that with tissues obtained late in the pupal period a distinct inhibition is produced by adding succinate.

The investigation reported here shows that this diminished respiration was probably due to the

inhibition by the succinate of enzymes of the phenolase type, which become more active in pupal tissues towards ecdysis (Dennell, 1944; Karlson & Wecker, 1955; Wojtczak, 1956), and may make a quantitatively important contribution to the endogenous respiration by acting as a general end-system for oxidative processes (cf. Wojtczak, 1955). The oxidation of tyrosine by homogenates of *Tenebrio*-pupal tissues has been shown to be inhibited by succinate and, in the absence of phenolase activity (suppressed by the addition of ethylenediaminetetra-acetic acid), it was demonstrated that succinate was oxidized throughout metamorphosis although not at a uniform rate.

The inhibition of tyrosine oxidation by succinate was studied in acetone-dried-powder preparations, in which the endogenous respiration and succinoxidase activity were negligibly small but the tyrosine activity was high.

MATERIALS AND METHODS

Insects. Larval, pupal and adult tissues of *Tenebrio molitor* were used in these experiments. Fresh pupae were collected from a culture of larval insects (mealworms) and the metamorphosis was allowed to proceed in separate glass jars at 24° until the insects were required. The 'period

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