The Biochemical Pathway for the Breakdown of *N*⁴-Ethyl-L-asparagine in the Bacterium *Pseudomonas stutzeri*

By ROBERT W. DINEEN* and DAVID O. GRAY

Department of Botany and Biochemistry, Westfield College, Kidderpore Avenue, Hampstead, London NW3 7ST, U.K.

(Received 11 July 1977)

*N*⁴-Ethyl-L-[U-¹⁴C]asparagine and L-[U-¹⁴C]aspartate give identical metabolites, mainly intermediates of the tricarboxylic acid cycle and related amino acids, in whole cells of *Pseudomonas stutzeri*. The labelled asparagine derivative is converted into [¹⁴C]-aspartate by cell-free extracts, and this reaction, which has an optimum pH of 8.8±0.2, is neither inhibited by unlabelled asparagine nor enhanced by unlabelled 2-oxoglutarate. No labelled keto acid corresponding to *N*⁴-ethylasparagine was detected in either whole cells or cell-free extracts. Thus *N*⁴-ethyl-L-asparagine, like asparagine, must be broken down by hydrolysis, at least in this bacterium.

The biochemistry of the protein amino acids is well known, but that of their naturally occurring homologues and analogues, though of considerable comparative interest, has received less attention.

This paper is concerned with the metabolism of the *N*¹⁴-substituted asparagines, a group of asparagine analogues characteristic of the family Cucurbitaceae. The methyl, ethyl and hydroxyethyl derivatives are natural products, first isolated by Dunnill & Fowden (1965), Gray & Fowden (1961) and Fowden (1961) respectively. These *N*⁴-alkyl-L-asparagines are pro-

* Present address: Brewing and Fermenting Department, Allied Breweries (U.K.) Ltd., Burton-on-Trent DE13 0JF, Staffordshire, U.K.

bably synthesized from asparagine by a transferase reaction involving the corresponding amine (Frisch *et al.*, 1967), but nothing has been published about their breakdown. Here we describe the catabolism of the representative compound *N*⁴-ethyl-L-asparagine (*N*-ethyl-4-amido-L-aspartate, β-aspartoethylamide) in the bacterium *Pseudomonas stutzeri*, which can use it as sole carbon and nitrogen source.

Three pathways are possible (Scheme 1). The first route is analogous to the transamination of asparagine and the second to its hydrolysis by asparaginase (Lea & Fowden, 1975). Reaction 3 is the reverse of one of those catalysed by the asparagine *N*⁴-alkylase (asparagine-alkylamine β-transferase) of higher

Scheme 1. Possible pathways of *N*⁴-ethylasparagine breakdown

plants (Frisch *et al.*, 1967). Thus in the present work we have used ^{14}C -labelled substrates to decide whether the first metabolite of N^4 -ethylasparagine is N -ethyl- α -oxosuccinamate, aspartate or asparagine.

Experimental

Chemicals

N^4 -Ethyl-L-asparagine of 99% purity was synthesized by the method of Dineen & Gray (1977). Other chemicals were of commercial reagent grade unless otherwise specified.

Paper chromatography

Descending chromatography on Whatman 3 MM paper was used, the temperature range being $25 \pm 5^\circ\text{C}$. The solvents were: A, phenol/water (3:1, w/v); B, phenol/water (100:39, w/v); C, phenol/50% (v/v) formic acid/water (100:10:29, w/v/v); D, butan-1-ol/acetic acid/water (90:10:29, by vol.); E, butan-1-ol/propionic acid/water (92:47:61, by vol.); F, organic phase of 2-methylbutan-2-ol/90% (v/v) formic acid/water (8:1:4, by vol.); G, 2-methylpropan-2-ol/methanol/water (4:5:1, by vol.).

Chromatograms were dried at 30°C for 2–5 h except those run in phenol-containing solvents, which were dried for 30 h. Amino acids were located by dipping the papers through 0.2% (w/v) ninhydrin (indanetrione hydrate) in ethanol, and heating at 60 – 100°C for 15 min. Organic phosphates and acids were detected with 0.004 M-FeCl_3 in acetone followed by $0.33\text{ M-ammonium thiocyanate}$ in acetone (Firmin & Gray, 1974). Sugars were revealed with an alkaline silver reagent (Trevelyan *et al.*, 1950).

Radioactive substrates

L-[U- ^{14}C]Aspartic acid (229 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Part of this material was used for the synthesis of N^4 -ethyl-L-[U- ^{14}C]asparagine as described by Dineen & Gray (1975). Both ^{14}C -labelled substrates were stored at -20°C either as solids (long-term storage) or in aqueous solution (for less than 7 days). Radiochemical purities, determined immediately before use by paper chromatography in solvents D and G, were: [^{14}C]aspartic acid, 98%; N^4 -ethyl[^{14}C]asparagine, 99%. Initially the ethyl-[^{14}C]asparagine was contaminated with 0.4% of N^1 -ethyl[^{14}C]isoasparagine (N -ethyl-L-amidoaspartate), 0.06% of [^{14}C]aspartate and less than 0.005% of [^{14}C]asparagine. However, when the preparation was mixed with a typical extract of *Ps. stutzeri* and fractionated by ion-exchange chromatography as described under 'Identification of labelled metabolites in bacterial extracts' six new organic cations

accounting for approx. 2.3% of the label appeared, and the proportion of [^{14}C]asparagine increased to 0.2%. These labelled artifacts were negligible in the experiments corresponding to Fig. 2, since ethylasparagine did not accumulate within intact cells.

Isolation and culture of *Ps. stutzeri*

Unless otherwise specified, the medium used throughout was a modified minimal one (Cruickshank *et al.*, 1965) containing 0.1% (w/v) N^4 -ethyl-L-asparagine as sole carbon and nitrogen source, together with K_2HPO_4 (7 g/l), KH_2PO_4 (3 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (100 mg/l), CaCl_2 (10 mg/l), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.5 mg/l), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2.5 mg/l), $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$ (2.5 mg/l) and H_2SO_4 (0.25 mg/l). The inorganic components were sterilized by autoclaving at 103 kPa (15 lb/in 2) for 15 min. However, N^4 -ethyl-L-asparagine partially decomposes under these conditions and was sterilized separately by passing it, in aqueous 1% (w/v) solution, through a membrane filter with $0.45\text{ }\mu\text{m}$ pores.

The bacterium obtained by exposing this medium to laboratory air for 5 min was purified and shown to be an axenic strain of *Ps. stutzeri*. It was maintained at 4°C , in the liquid medium specified, and was subcultured every 7 weeks. Cultures used for metabolic experiments were kept well aerated at 20°C , when the mean generation time was 3.3 h, and were harvested when their turbidities were 35–50% of the stationary-state value.

Preparation of cell-free extracts of *Ps. stutzeri*

Cultures (800 ml) were harvested by centrifugation ($25000g$, 5 min, 5°C). The cells were washed by taking them up in 100 ml of minimal medium containing no N^4 -ethylasparagine and re-centrifuging as above. Next they were dispersed in 20 ml of the appropriate buffer and disrupted by sonication (2 min at 5.5 A) with a Soniprobe (Dawe Instruments, London W.3, U.K.). Cell debris and unbroken cells were removed by centrifugation ($35000g$, 5 min, 5°C), leaving a clear supernatant. Control samples were autoclaved for 15 min at 103 kPa (15 lb/in 2).

Whole cells: incubation with labelled substrates

Typically the cells from 150 ml of medium were recovered by centrifugation ($25000g$, 5 min, 10°C) and resuspended in 45 ml of minimal medium containing no N^4 -ethylasparagine. After exposing them to the relevant ^{14}C -labelled substrate (5–12 μCi in 0.25–0.5 ml of water) for 1 min at 20°C , they were centrifuged down as above and fixed with 50 ml of 70% (v/v) ethanol. Further details are given in the Table legends.

Experiments were replicated whether these involved whole cells or cell-free extracts.

Identification of labelled metabolites in bacterial extracts

The ethanolic cell extracts were initially fractionated by the scheme detailed by Firmin & Gray (1976). In principle, the solutions were passed through two columns (10cm long×1cm diameter) in series. The first, containing Zeo-Karb (Zerolit) 225 (H⁺ form), bound the weakly basic organic cations, which were eluted with aq. NH₃, whereas the second, containing Dowex 1 (X8; acetate form) (BDH Chemicals, Enfield, Middx., U.K.) captured the anions, which were later displaced with formic acid. Uncharged molecules passed through both columns.

Unless otherwise stated, uncharged compounds (sugars) were separated by two-dimensional paper chromatography in solvents B and E, the anions (organic acids) in solvents C and F and the cations (amino acids) in A and D. Radioactive compounds on chromatograms were located by radioautography by using X-ray film (Kodirex; Kodak, Hemel Hempstead, Herts., U.K.) exposed for 2–3 months. They were identified by comparison with internal standards detected as described under 'Paper chromatography'. Though 5-oxoproline (5-pyrroline-2-carboxylic acid) is a cation, it was revealed by the FeCl₃/ammonium thiocyanate reagent.

Measurement of ¹⁴C

¹⁴C was determined by liquid-scintillation spectrometry. Spots from paper chromatograms were normally cut out and immersed in 20ml of scintillation-grade toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)-benzene (Rogers *et al.*, 1966) to give an overall counting efficiency of 43%. This fell to 40 and 18.5% for papers treated with ninhydrin and FeCl₃/ammonium thiocyanate respectively.

Aqueous solutions were mixed with KL 372 scintillation fluid (Koch-Light, Colnbrook, Bucks, U.K.) giving a net efficiency of 86%. All counts have been corrected for quenching, counter efficiency and background, so they are given in absolute terms. Approximate standard errors are: 0.01 nCi, 10%; 0.1 nCi, 2%; 1 nCi, 0.5%.

Results

Metabolites of N⁴-ethyl-L-asparagine in intact cells

When N⁴-ethyl-L-[U-¹⁴C]asparagine was incubated with *Ps. stutzeri* for 1 min, the major metabolites positively identified, apart from CO₂, were aspartate, glutamate, glutamine, 5-oxoproline, fumarate, succi-

nate, 2-oxoglutarate, malate and citrate. There were also radioactive spots in chromatographic positions corresponding to *cis*-aconitate, lactate and oxaloacetate; Fig. 2 (see below) illustrates the relative ratios of these products. Less than 0.2% of the label supplied entered the sugar fraction.

5-Oxo[¹⁴C]proline behaved abnormally in these experiments, because it partially or completely decomposed to glutamic acid when chromatograms, run in the first dimension in solvent A, were dried and then re-run in solvent D. Its identity was confirmed as follows: a sample of the labelled metabolite, purified by preparative paper chromatography, not only co-chromatographed with authentic 5-oxoproline in solvents A and D, but when hydrolysed with acid under mild conditions (1.0M-HCl, 10 min, 100°C) gave [¹⁴C]glutamate.

Thus the nature of the labelled metabolites, demonstrated in at least 12 separate experiments, indicated that ¹⁴C from N¹⁴-ethyl[¹⁴C]asparagine entered the tricarboxylic acid cycle. The kinetics of labelling (Fig. 1a), which showed that aspartate was one of the earliest metabolites of N-ethyl-asparagine, suggested that ¹⁴C was entering the cycle via aspartate and oxaloacetate and leaving it through glutamine and 5-oxoproline. A longer-term kinetic experiment (Fig. 1b) showed that [¹⁴C]asparagine, unlike [¹⁴C]aspartic acid, did not respond to the concentration of ethyl[¹⁴C]asparagine within the cells. This is strong evidence that asparagine is not directly involved in the breakdown of the substituted amide.

Attempts to define the pathway more precisely by treating cultures with ethyl[¹⁴C]asparagine (0.004 μM) in the presence of an unlabelled competitor compound (0.43 μM) were unsuccessful. Unlabelled aspartate, glutamate and malate increased the ¹⁴C present in all the intermediates assayed by an average factor of 2.0.

However, radioautograms showed that L-[U-¹⁴C]-aspartate and N⁴-ethyl-L-[U-¹⁴C]asparagine were metabolized to exactly the same 33 compounds, whose relative labelling was similar in both cases (Fig. 2), strongly supporting the concept that aspartate was an early product of ethylasparagine breakdown. Aspartate was apparently metabolized five times as rapidly as N⁴-ethylasparagine, and this explained why so little labelled aspartate accumulated during the time-course experiments (Fig. 1a). Asparagine was a very minor metabolite, accounting, as expected, for the same proportion (0.07%) of the total ¹⁴C recorded in both experiments.

Metabolites of N⁴-ethyl-L-asparagine in cell-free extracts of Ps. stutzeri

Aspartate was the only major metabolite of N⁴-ethyl[U-¹⁴C]asparagine detected in this simpler

Fig. 1. Time course of ^{14}C accumulation by metabolites of N^4 -ethyl-L-[U- ^{14}C]asparagine in whole cells of *Ps. stutzeri* (a) Short-term experiments: (i) results for amino acids; (ii) results for intermediates of the tricarboxylic acid cycle. Each result represents the metabolite extracted from 10 ml of an unconcentrated culture growing in the medium specified and incubated with $0.86\ \mu\text{Ci}$ of N^4 -ethyl-L-[U- ^{14}C]asparagine ($229\ \text{mCi/mmol}$) at 22°C . Although ensuring steady-state conditions throughout the experiment, unlabelled N^4 -ethyl-L-asparagine from the growth medium obviously lowered the specific radioactivity of the ^{14}C substrate supplied. The cells were fixed by mixing the cell suspension directly with 25 ml of ethanol. Thus the extracts contained residual labelled substrate, which contaminated most chromatographic spots with traces of ^{14}C and prevented many of these graphs from passing through the origin at zero time. The organic cations were separated in solvents D and G and the anions in solvents B and E. Here solvent B was used mixed with 1% (v/v) 18 M-ammonia. Key: (i) ●, aspartate; △, glutamate; ▲, 5-oxoproline; (ii) △, citrate and isocitrate; □, malate; ○, 2-oxoglutarate; ●, succinate. (b) Longer-term experiments. The bacteria from 200 ml of an unconcentrated culture were resuspended in 150 ml of minimal medium containing no ethylasparagine. Samples (10 ml) of this suspension were incubated with $0.1\ \mu\text{Ci}$ of N^4 -ethyl-L-[U- ^{14}C]asparagine ($299\ \text{mCi/mmol}$) at 20°C , and passed through a membrane filter with $0.45\ \mu\text{m}$ pores. The cells, recovered thus within 1.5 min, were extracted with 70% ethanol (10 ml). These extracts, unlike those corresponding to (a), contained very little ethyl[^{14}C]asparagine, so negligible quantities of [^{14}C]asparagine were generated as an artifact during their fractionation by ion exchange. Here, the amino acid fraction was separated by double development in solvent D. Key: ○, asparagine; ●, aspartate; △, ethylasparagine.

biochemical system (Table 1). Unlabelled asparagine neither trapped ^{14}C itself nor inhibited the transfer of ^{14}C to aspartate (Table 1). This competitor experiment confirmed that asparagine was not an intermediate in the breakdown of N^4 -ethylasparagine to aspartate.

As expected, unlabelled 2-oxoglutarate increased the ^{14}C found in intermediates of the tricarboxylic acid cycle and decreased that in glutamate. It also decreased the accumulation of [^{14}C]aspartate, suggesting that aminotransferase reactions involving 2-oxoglutarate were responsible for the breakdown and not the production of this amino acid.

Fig. 3 shows the relationship between pH and the rate of production of [^{14}C]aspartate in cell-free

extracts. This is the simple relationship that would be expected if the ethylasparagine/aspartate reaction were catalysed by a single enzyme.

Discussion

These observations indicate that aspartate is an early metabolite of ethylasparagine and that asparagine is not. The only remaining doubt is whether aspartate is produced directly by the hydrolysis of the substituted amide or indirectly by a route involving two transaminations.

Transamination of N^4 -ethyl-L-[U- ^{14}C]asparagine would yield N -ethyl- α -oxo[U- ^{14}C]succinamate. Even if this keto acid existed in two forms, like α -oxo-

Fig. 2. Comparison of labelling of metabolites of N⁴-ethyl-L-[U-¹⁴C]asparagine (a) and [U-¹⁴C]aspartate (b) in whole cells of *Pseudomonas stutzeri*

Each result represents the metabolite extracted from 9 ml of a concentrated bacterial suspension after this had been incubated with 2 μ Ci of either N⁴-ethyl-L-[U-¹⁴C]asparagine (229 mCi/mmol) or L-[U-¹⁴C]aspartate for 1 min at 20°C. One sample of each cationic fraction was separated in solvents A and D and a duplicate in solvents G and D. The height of the line representing each compound is proportional to the logarithm of its ¹⁴C content, to allow extreme results to be shown on the same diagram. The specific radioactivity of aspartate and that of the aspartic residue of ethylasparagine were initially identical, but these substrates may have been differentially diluted by unlabelled material associated with the cells. Key: ¹⁴Glu, 5-oxoproline; cAn, *cis*-aconitate; Cit, citrate; iCit, isocitrate; Fum, fumarate; Lac, lactate; Mal, malate; OxAc, oxaloacetate; OxGlu, 2-oxoglutarate; Suc, succinate.

succinamate (Meister, 1953), at least one unidentified highly labelled intermediate would have been present in the organic acid fraction. No such compound was detected by radioautography in any of the cell-free extracts. Moreover, even in intact cells, ethyl[¹⁴C]asparagine gave no additional organic acids that were not also metabolites of [¹⁴C]aspartate. This strong though negative evidence that little if any N⁴-ethylasparagine is transaminated in *Ps. stutzeri* supports the results obtained when cell-free extracts were supplemented with unlabelled α -oxoglutarate. Thus all or most of the substituted amide must be broken down by hydrolysis.

The enzyme involved, which has a pH optimum of approx. 9.0 (Fig. 3), is soluble: only 11 $\pm$ 12% of the total activity, as measured by [¹⁴C]aspartate production at pH 7.0, was present in the cell debris precipitated by centrifuging the crude homogenate at 35000g (5°C, 5 min). Cell-free extracts, even at pH 9.0, only metabolized ethylasparagine at 2% of the rate of equivalent whole-cell cultures. This discrepancy may be due to the presence of an inhibitor or the absence of an activator from the cell-free extract. Alternatively, the intact cells may concentrate the substrate, say 100-fold, at the enzymically active sites.

Table 1. Effect of unlabelled asparagine and 2-oxoglutarate on the labelling of the metabolites of *N*⁴-ethyl-L-[U-¹⁴C]-asparagine in cell free extracts of *Ps. stutzeri*

Samples (3.0 ml) of an extract obtained by disrupting cells in minimal bacterial growth medium containing no *N*⁴-ethylasparagine (pH 7.2) were incubated with 0.64 μ Ci of *N*⁴-ethyl-L-[U-¹⁴C]asparagine (0.0027 μ mol) in 0.3 ml of water and, where appropriate, with 0.27 μ mol of one of the unlabelled substrates, for 20 min at 25°C. The reaction mixtures (3.45 ml) were then mixed with ethanol (8 ml) and analysed as specified. All values have been corrected by subtracting those given by appropriate autoclaved controls, and uncertainty was estimated from the experimental variation actually observed.

(a) Effect on the amino acids.

Metabolite	¹⁴ C present in each metabolite (nCi)			Uncertainty (nCi)
	Untreated extract	Extract+ asparagine	Extract+2-oxoglutarate	
Aspartate	8.0	11.2	5.4	±0.9
Asparagine	-2.1	-3.5	0.2	±0.6
Glutamate	0.1	0.16	-0.07	±0.07

(b) Effect on intermediates of the tricarboxylic acid cycle

These contained so little ¹⁴C that diffuse zones of labelled artifacts, partially overlapping the positions of the organic acids on the chromatograms, seriously distorted results obtained by the direct counting method. Thus the relative labelling of the central 3 mm² of each spot was determined by measuring the *A*₅₂₀ of the relevant part of the radioautogram. A spot containing 1.0 nCi gave an approximate reading of 0.47 *A*₅₂₀ unit and the uncertainty was ±0.02 *A*₅₂₀ unit.

Metabolite	Relative amount of label (<i>A</i> values)		
	Untreated extract	Extract+ asparagine	Extract+2-oxoglutarate
Citrate/isocitrate	0.05	0.08	0.11
2-Oxo-glutarate	-0.02	0.01	0.34
Succinate	0.00	0.04	0.45

The enzyme may be specific for *N*⁴-ethylasparagine, but is probably an asparaginase. Certainly, the asparaginase of *Erwinia cartovora* will hydrolyse *N*⁴-ethylasparagine at 0.4% of the rate at which it attacks its normal substrate (Howard & Carpenter, 1972). Moreover, asparaginase may be present in *Ps. stutzeri*, since cell-free extracts destroyed [¹⁴C]-asparagine, present as an impurity in the ethyl-[¹⁴C]asparagine preparations, as shown in Table 1(a); compared with autoclaved controls, labelled asparagine decreased by 29% when incubated with extracts for 20 min at 25°C (average of three experiments).

Fig. 3. Effect of pH on the production of [¹⁴C]aspartate from ethyl[¹⁴C]asparagine in cell-free extracts of *Pseudomonas stutzeri*

The pH was stabilized with a mixture containing citric acid (6.008 g), KH₂PO₄ (3.893 g), H₃BO₄ (1.796 g) and water (1 litre). Samples (1.3 ml) of an extract obtained by disrupting cells in this mixture at pH 7.0 were adjusted to the required pH with 2M-HCl or -NaOH and incubated with 0.31 μ Ci of *N*⁴-ethyl-L-[U-¹⁴C]asparagine (229 mCi/mmol) for 20 min at 25°C. After adding ethanol (5 ml) the cation fraction was isolated and chromatographed as usual. The aspartate region was excised from each chromatogram and its ¹⁴C content determined. The values recorded have been corrected by subtracting those given by equivalent autoclaved controls, and the uncertainty of each result, estimated from the observed experimental variation, is ±0.4 nCi (2 × S.D.).

Whether or not asparagine and *N*⁴-ethylasparagine are broken down by the same enzyme, both compounds are evidently metabolized in the same way by bacteria using them as sole carbon sources. Enzymes hydrolysing L-asparagine have been frequently reported from bacteria (Ramadan *et al.*, 1964; Wade *et al.*, 1968; Wriston, 1971; Howard & Carpenter, 1972), whereas those transaminating the amide occur mainly in higher plants and animals (Meister *et al.*, 1952; Meister & Fraser, 1954; Guitton, 1963; Činčerová, 1969; Lea & Fowden, 1975). It remains to be seen if the same is true for its *N*-ethyl analogue.

Comparisons can also be made between the metabolism of *N*⁴-ethylasparagine and that of *N*⁵-ethylglutamine. The glutamine analogue, too, is hydro-

lysed by bacteria and the enzyme responsible has been purified from *Pseudomonas aeruginosa* (Soda *et al.*, 1965, 1966). However, its immediate fate in higher plants is uncertain, though Kito *et al.* (1966, 1968) have studied the breakdown of N⁵-[Et-¹⁴C]ethyl-L-glutamine in *Thea sinensis*.

We have made no serious attempt to investigate the metabolism of ethylamine, the second product of ethylasparagine hydrolysis. However, *Ps. stutzeri* cells do not detectably incorporate ¹⁴C from externally supplied ethylamine into any of the cell fractions investigated. The free amine is presumably unable to pass through the cell membrane.

Thanks are due to Mrs. J. E. P. Young of the National Collection of Industrial Bacteria, Torry, Scotland, who confirmed the identity and purity of the *Ps. stutzeri* culture used in this work. We are also grateful to the Science Research Council, who supported R. W. D. during these studies.

References

- Činčerová, A. (1969) *Biol. Plant* **11**, 139–148
- Cruickshank, R., Duguid, J. P. & Swain, R. H. A. (eds.) (1965) *Medical Microbiology*, 11th edn, p. 739, E. and S. Livingstone, Edinburgh
- Dineen, R. W. & Gray, D. O. (1975) *Radiochem. Radioanal. Lett.*, **22**, 205–207
- Dineen, R. W. & Gray, D. O. (1977) *Org. Prep. Proced. Int.* **9**, 39–41
- Dunnill, P. M. & Fowden, L. (1965) *Phytochemistry* **4**, 933–944
- Firmin, J. L. & Gray, D. O. (1974) *J. Chromatogr.* **94**, 294–297
- Firmin, J. L. & Gray, D. O. (1976) *Biochem. J.* **158**, 223–229
- Fowden, L. (1961) *Biochem. J.* **81**, 154–156
- Frisch, D. M., Dunnill, P. M., Smith, A. & Fowden, L. (1967) *Phytochemistry* **6**, 921–931
- Gray, D. O. & Fowden, L. (1961) *Nature (London)* **189**, 401–402
- Guitton, Y. (1963) *C. R. Hebd. Séances Acad. Sci.* **257**, 506–507
- Howard, J. B. & Carpenter, F. H. (1972) *J. Biol. Chem.* **247**, 1020–1030
- Kito, M., Kokura, H., Izaki, J. & Sasaoka, K. (1966) *Agric. Biol. Chem.* **30**, 623–624
- Kito, M., Kokura, H., Izaki, J. & Sasaoka, K. (1968) *Phytochemistry* **7**, 599–603
- Lea, P. J. & Fowden, L. (1975) *Biochem. Physiol. Pflanz.* **168**, 3–14
- Meister, A. (1953) *J. Biol. Chem.* **200**, 571–589
- Meister, A. & Fraser, P. E. (1954) *J. Biol. Chem.* **210**, 37–43
- Meister, A., Sober, H. A., Tice, S. V. & Fraser, P. E. (1952) *J. Biol. Chem.* **197**, 319–330
- Ramadan, M. E. A., El Asmar, F. & Greenberg, D. M. (1964) *Arch. Biochem. Biophys.* **108**, 143–149
- Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) *Biochem. J.* **99**, 381–388
- Soda, K., Uchiyama, K. & Ogata, K. (1965) *Bull. Inst. Chem. Res. Kyoto Univ.* **43**, 480–498
- Soda, K., Uchiyama, K. & Ogata, K. (1966) *Agric. Biol. Chem.* **30**, 541–557
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444
- Wade, H. E., Elsworth, R., Herbert, D., Keppie, J. & Sargeant, K. (1968) *Lancet* **ii**, 776–777
- Wriston, J. C., Jr. (1971) *Enzymes 3rd Ed.* **4**, 101–121