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BIOSYNTHESIS OF β-N-OXALYL-L-α,β-DIAMINOPROPIONIC ACID, THE *LATHYRUS SATIVUS* NEUROTOXIN

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Abstract—The biosynthesis of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP) the *Lathyrus sativus* neurotoxin has been found to follow the scheme depicted below:

$$\text{Oxalate} + \text{ATP} + \text{Coenzyme A} \xrightleftharpoons{Mg^{2+}} \text{Oxalyl-CoA} + \text{AMP} + \text{PP}_i$$

$$\text{Oxalyl-CoA} + \text{L-}\alpha,\beta\text{-diaminopropionic acid} \rightarrow \text{ODAP} + \text{CoA}.$$

The first reaction is catalysed by oxalyl-CoA synthetase which has properties similar to that of the enzyme in peas. The second reaction is catalysed by another enzyme which is specific to *L. sativus* and is designated as oxalyl-CoA-α,β-diaminopropionic acid oxalyl transferase. The enzymes have been purified by about 60-fold and their properties studied. A partial resolution of the two enzyme activities has been achieved using CM-sephadex columns.

INTRODUCTION

It was earlier[1] indicated that the biosynthesis of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), HOOC.CO.NH.$CH_2(NH_2)$. COOH, may involve oxalyl activation followed by condensation with L-α,β-diaminopropionic acid. (U-^{14}C) oxalic acid was found to be incorporated as an intact unit into ODAP and an enzyme catalysing the formation of oxalyl-CoA was also detected in the seedlings of *Lathyrus sativus*. Oxalyl-CoA synthetase activity was first detected in *Pisum sativum* and the properties of a 6-fold purified enzyme were described by Giovanelli.[2] ODAP formation could be demonstrated in the crude extracts of *L. sativus* seedlings, but not in *P. sativum*, when L-α,β-diaminopropionic acid was added along with the constituents necessary for oxalyl-CoA formation.

In the present communication the results of the studies on the properties of the enzymes involved in ODAP formation in *L. sativus* are presented. It is concluded that two different enzymes catalyse the following steps leading to ODAP formation.

$$\text{Oxalate} + \text{ATP} + \text{Coenzyme A} \underset{\text{Oxalyl-CoA synthetase}}{\xrightleftharpoons{Mg^{2+}}} \text{Oxalyl-CoA} + \text{AMP} + \text{PP}_i$$

$$\text{Oxalyl} - \text{CoA} + \text{L-}\alpha,\beta\text{-diaminopropionic acid} \xrightarrow[\text{ODAP synthase}]{} \text{ODAP} + \text{Coenzyme A}.$$

RESULTS AND DISCUSSION

Table 1 indicates the steps employed to purify oxalyl-CoA synthetase and ODAP-forming activity from *Lathyrus sativus*. The ratio of the two enzyme activities varies with the

[1] K. MALATHI, G. PADMANABAN, S. L. N. RAO and P. S. SARMA, *Biochim. Biophys. Acta* **141**, 71 (1967).
[2] J. GIOVANELLI, *Biochim. Biophys. Acta* **118**, 124 (1966).

different purification steps. Treatments, such as alcohol and acetone precipitation, completely inhibit ODAP-forming activity without affecting oxalyl-CoA synthetase. The pooled fractions of the Biogel P-200 eluate show an overall 60-fold purification of oxalyl-CoA synthetase with about 4 per cent recovery. The maximal purification obtained in a single fraction was

Table 1. Purification of oxalyl-CoA synthetase and ODAP synthetase from *L. sativus*

Purification step	Protein recovered (mg)	Oxalyl-CoA synthetase: Total activity	Oxalyl-CoA synthetase: Specific* activity A	ODAP synthase: Total activity	ODAP synthase: Specific* activity B	A/B
I Crude	1850·0	555·0	0·3	240·50	0·13	2·3
II 0·4–0·6 $(NH_4)_2SO_4$	335·0	402·0	1·2	83·70	0·25	4·8
III Calcium phosphate gel supernatant	182·6	328·6	1·8	62·08	0·34	5·3
IV Acid precipitation + 0·35–0·55 $(NH_4)_2SO_4$	11·4	99·2	8·7	13·34	1·17	7·4
Alcohol precipitation†	10·5	71·4	6·8	0·21	0·02	340·0
Acetone precipitation†	9·6	51·8	5·4	0·48	0·05	108·0
V Biogel P 200	1·1	21·0	19·1	7·86	7·15	2·7

* Specific activity of oxalyl-CoA synthetase is expressed as μmoles oxalyl monohydroxamate/mg protein. Specific activity of ODAP synthase is expressed as μmoles ODAP/mg protein. The incubation period was 30 min.

† Alcohol and acetone fractionation steps were carried out directly on the gel supernatant.

Table 2. Properties of oxalayl-CoA synthetase

Treatment*	Rate relative to oxalate†
Oxalate (500 μM)	100·0 (1·15 × 10⁵)
No substrate	1·8
Glycollate (500 μM)	3·2
Glyoxylate (500 μM)	1·6
No metal	1·3
Magnesium (5 mM)‡	100·0
Cobalt (5 mM)‡	78·3
GSH (5 mM)	120·0
PHMB (90 μM)	0
PHMB (90 μM) + GSH (5 mM)	117·0
PHMB (90 μM) + cysteine (5 mM)	116·0

* The complete reaction mixture and incubation conditions are described in the Experimental. Each reaction mixture contained 4 μmoles of $^{32}P(PP_i)$ ($2·5 \times 10^6$ counts/min) and the biogel P 200 eluate equivalent to 0·39 mg protein. Tris-HCl buffer (pH 7·5) was used.

† The actual incorporation expressed as counts/min in ATP is given in parentheses.

‡ Added separately to a metal-free reaction mixture.

86-fold. It was not possible to purify the enzyme on CM-cellulose or DEAE-cellulose columns and it was not stable on storage; this explains the low recovery.

The properties of the purified preparation of oxalyl-CoA synthetase are given in Table 2. This enzyme was purified 6-fold from peas by Giovanelli.[2] The *L. sativus* enzyme closely resembles that of *Pisum* in its properties and hence only the salient features are presented.

The enzyme is dependent on ATP, Co-A and Mg^{2+} for activity. It is specific for oxalate, and glyoxylate, which gives 20 per cent activity with the pea enzyme,[2] is not a substrate for the purified *Lathyrus* preparation. The products of the reaction are oxalyl-CoA, AMP and PP_i. The K_m values for oxalate, ATP and CoA are 1·33 mM, 1·20 mM and 100 μM respectively. Giovanelli[2] obtained the corresponding K_m values for the pea enzyme as 2 mM, 4 mM and 70 μM. The *Lathyrus* enzyme also requires sulphydryl groups for activity and Co^{2+} can replace Mg^{2+}, giving 80 per cent of the activity.

The results presented in Table 1 indicate that oxalyl-CoA synthetase activity and ODAP-forming activity are due to different enzymes. While it was not possible to separate them on

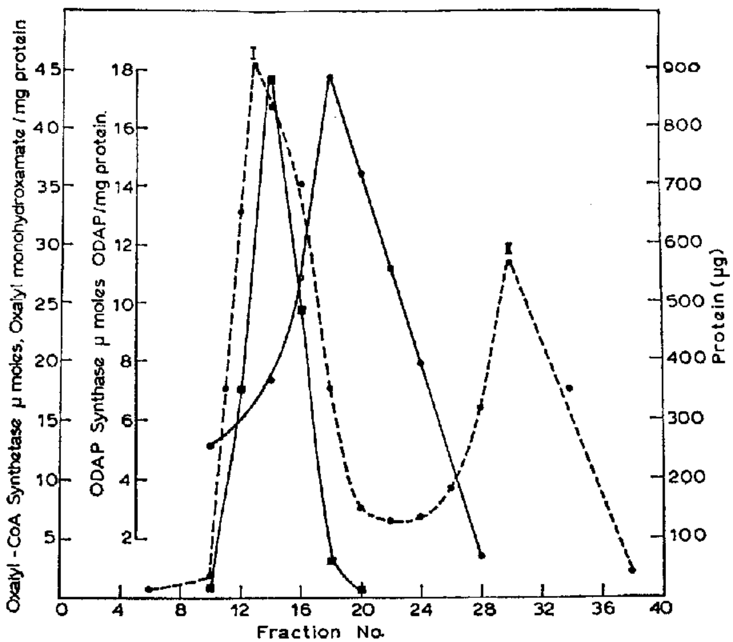

Fig. 1. Fractionation of oxalyl-CoA synthetase and ODAP synthase on CM-sephadex columns.

The experimental details are given in text.

o——o oxalyl CoA synthetase activity

■——■ ODAP synthase activity; ●-----● protein

Biogel P-200, DEAE- or CM-cellulose, they were partly resolved on a CM-Sephadex column (Fig. 1). Polyacrylamide gel electrophoresis of a lyophilized preparation of the pooled fractions under protein peak I showed two bands.

The Biogel P-200 eluate was used to study the properties of the enzyme-catalysing ODAP formation from oxalyl-CoA and L-α,β-diaminopropionic acid. The effect of enzyme concentration on ODAP formation is given in Fig. 2. The reaction shows a linear rate for 30 min and has a broad pH optimum of 7·4–8·0. The K_m values calculated for oxalyl-CoA and DAP from Fig. 3 are 0·45 mM and 0·3 mM, respectively.

The results presented in Table 3 indicate that PHMB does not inhibit ODAP-forming activity. EDTA slightly enhances the activity of the enzyme. The enzyme is not effective when succinyl-CoA or acetyl-CoA is used as the substrate in place of oxalyl-CoA. However, some

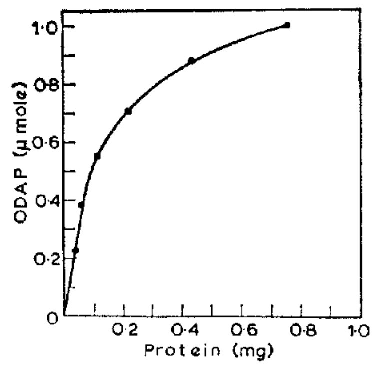

FIG. 2. EFFECT OF PROTEIN CONCENTRATION ON ODAP FORMATION.
The experimental details are given in text.

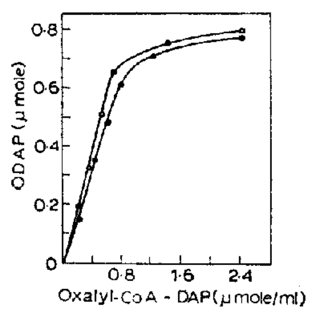

FIG. 3. EFFECT OF SUBSTRATE CONCENTRATION ON ODAP FORMATION.
●——● oxalyl-CoA; ○——○ DAP

TABLE 3. PROPERTIES OF ODAP SYNTHETASE

Treatment*	Product formed (µmoles)
Oxalyl-CoA	0·68
Succinyl-CoA†	0·05
Acetyl-CoA†	0·05
PHMB (90 µM)	0·65
EDTA (500 µM)	0·75

* The complete reaction mixture and incubation conditions are described in the Experimental. Biogel P.200 eluate equivalent to 200 µg protein was added. The incubation period was 30 min.

† A similar yield was obtained when the reaction was carried out in presence of boiled enzyme.

non-enzymic succinylation as well as acetylation of DAP was observed under these conditions. Seneviratne and Fowden[3] have also reported a chemical reaction between acetyl phosphate and DAP.

[3] A. S. SENEVIRATNE and L. FOWDEN, *Phytochem.* 7, 1047 (1968).

The specificity of oxalylation with respect to the amino acid acceptors was studied in detail using oxalyl-CoA as donor. The results presented in Table 4 indicate that the β-amino group of L-α,β-diaminopropionic acid has maximum specificity for the oxalylation reaction.

TABLE 4. FORMATION OF OXALYL AMINO ACIDS FROM OXALYL-CoA*

Amino acid acceptor	Oxalyl amino acid formed† (μmoles)
α,β-Diaminopropionic acid	0·68
α,β-Diaminopropionic acid‡	0·04
α,γ-Diaminobutyric acid	0·13
Homoserine	0·08
Ornithine	T
Lysine	T
Glutathione	0·40
Glutathione‡	0·42

* The experimental conditions are as described in Table 3.
† The ninhydrin colour yields of the oxalyl derivatives of the amino acids indicated are taken to be the same as ODAP.
‡ Heat denatured enzyme was used.

TABLE 5. OXALYLATION OF AMINO ACID ACCEPTORS

Amino acid acceptor	Oxalyl derivatives* (μmoles)	
α,β-Diaminopropionic acid	β-*N*-Oxalyl derivative	3·10
	Dioxalyl derivative	1·90
α,γ-Diaminobutyric acid	γ-*N*-oxalyl derivative	0·60
	Dioxalyl derivative	0·90
Homoserine	*O*-oxalyl derivative	0·30
	N-oxalyl derivative	0·49
	Dioxalyl derivative	0·21
Serine*	*O*-oxalyl derivative	0·02
	N-oxalyl derivative	0·60
	Dioxalyl derivative	0·20
Glycine*	*N*-oxalyl derivative	0·20
Alanine	*N*-oxalyl derivative	0·13
β-Alanine	*N*-oxalyl derivative	0·15
α-Aminobutyric acid	*N*-oxalyl derivative	0·19
γ-Aminobutyric acid	*N*-oxalyl derivative	0·12

* The experimental conditions are given in text. Briefly, (U-^{14}C) oxalic acid incorporation into the oxalyl derivatives was studied using the Biogel P.200 enzyme preparation. The reaction was carried out in 1·5 ml total volume for 2 hr. 200 μg of protein was used. The ninhydrin-positive oxalyl derivatives were analysed after paper electrophoresis and ninhydrin spray. The colour yields of such derivatives are assumed to be the same as that of ODAP. In the case of ninhydrin-negative derivatives, the Dowex 50 column eluate fractions were pooled in areas of radioactive peaks, hydrolysed with 2 N HCl and the liberated parent amino acid was estimated. The amount of the oxalyl derivative was computed from this estimation.
† Experiments were also conducted where (U-^{14}C) serine and (2-^{14}C) glycine were used as the amino acid acceptors in presence of non-radioactive oxalic acid.

The γ-amino and γ-hydroxyl groups of L-α,γ-diaminobutyric acid and L-homoserine respectively are oxalylated to a limited extent. These results agree with our earlier work[4] based on (U-^{14}C) oxalic acid incorporation into oxalyl derivatives of amino acid acceptors obtained with a cruder enzyme preparation. In a similar study, Johnston and Lloyd[5] failed to find quantitative differences in the rates of oxalylation of different amino acids. As earlier indicated,[4] the presence of glutanthione in the reaction mixture leading to oxalyl-CoA formation results in the non-enzymatic reaction between oxalyl-CoA and glutathione giving rise to *S*-oxalylglutathione, especially in the absence of a suitable amino acid acceptor for the oxalyl group. *S*-oxalylglutathione has an electrophoretic mobility similar to those of the other oxalyl amino acids and this in part could have contributed to the results of Johnston and Lloyd.[5] These workers also detected oxalylation of mono amino acids and di-oxalylation of amino acids such as L-α,β-diaminopropionic acid and L-α,γ-diaminobutyric acid. Our present results (Table 4) show that at least in the case of L-α,β-diaminopropionic acid, ODAP is the product obtained in quantitative yield. To investigate the oxalylation of mono amino acids and dioxalylation of diamino acids under conditions of prolonged incubation with excess oxalate in the system, (U-^{14}C) oxalate incorporation was studied using the biogel P 200 eluate in a 2 hr incubation period. In these experiments, mercaptoethanol was used in place of glutathione. The products were analysed using a Dowex 50 H^+ column and electrophoretic methods as indicated in the Experimental. The results presented in Table 5 permit the following conclusions.

(1) A 3-carbon amino acid is an ideal substrate for oxalylation. This is indicated by the fact that amino acids such as glycine, L-α,γ-diaminobutyric acid show lesser affinities for oxalylation. Ornithine and lysine are poor acceptors of the oxalyl group.[1,4]

(2) In a 3-carbon unit, the β-amino group has maximum affinity for the oxalyl group provided the α-carbon also carries an amino group. This is indicated by the fact that whereas the β-amino group of α,β-diaminopropionic acid is the best acceptor of the oxalyl group, β-alanine is a poor acceptor. The α-amino group as such is not a good site for oxalylation, as exemplified by the results with glycine, alanine and α-aminobutyric acid.

(3) In the case of hydroxyamino acids, such as serine and homoserine, the γ-hydroxyl is preferentially oxalylated as compared to the β-hydroxyl group. A typical analysis of the products on Dowex 50 H^+ columns with (U-^{14}C) serine is given in Fig. 4. It can be seen that peak I, which can include both *N*-oxalylserine and dioxalylserine (both ninhydrin negative), is greater than peak II which represents *O*-oxalylserine. Electrophoretic analysis of peak I by the procedure described by Johnston and Lloyd along with an authentic sample of *N*-oxalylserine indicates that this derivative may account for a greater proportion of radioactivity than the dioxalyl derivative. However, the separation of *N*-oxalylserine from the dioxalyl derivative is not sufficient to permit an accurate estimate. Also the possibility of non-enzymatic interconversion between *N*-oxalyl and *O*-oxalyl derivatives by the mechanism proposed by Bell and O'Donovan[6] cannot be ruled out. Thus, the α-amino group can be oxalylated more preferentially than the β- or even the γ-hydroxyl groups; in the absence of hydroxyl groups at the β or γ carbon atoms, the α-amino group is not significantly oxalylated.

[4] K. MALATHI, G. PADMANABAN and P. S. SARMA, *Indian J. Biochem.* **5**, 184 (1968).
[5] G. A. R. JOHNSTON and H. J. LLOYD, *Australian J. Biol. Sci.* **20**, 1241 (1967).
[6] E. A. BELL and J. P. O'DONOVAN, *Phytochem.* **5**, 1211 (1966).

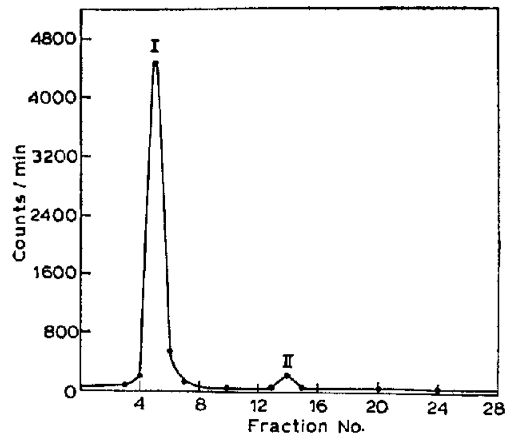

FIG. 4. FRACTIONATION OF THE OXALYL DERIVATIVES OF (U-^{14}C) SERINE ON DOWEX-50(H^+) COLUMN.
The experimental details are given in text. The amount of radioactivity put on the column was $2\cdot5 \times 10^5$ counts/min. Peak I includes dioxalylserine and *N*-oxalylserine. Peak II represents *O*-oxalylserine. With diamino acids as the amino acid acceptor, Peak I would represent the dioxalyl derivative and Peak II the ω-oxalyl derivative. When (U-^{14}C) oxalic acid is used in the assay mixture, Peak I would also include free oxalic acid.

(4) Among diamino acids such as L-α,β-diaminopropionic acid and L-α,γ-diamino butyric acid, the α-amino group has little affinity for the oxalyl group. The mono-oxalyl derivatives in these cases are ninhydrin positive and the products detected are only β-*N*- and γ-*N*-oxalyl derivatives. Electrophoresis in 10 per cent acetic acid showed that the product obtained was the β-, and not the α-isomer.[1] Prolonged incubation gives rise to the dioxalyl derivative as well. The results presented in Table 5 also indicate that ODAP can serve as a substrate for oxalylation giving rise to the di-oxalyl derivative.

To summarize, the affinity of a functional group for enzymatic oxalylation is influenced by other substituents in the molecule and follows the order: β-amino group of α,β-diaminopropionic acid > γ-amino group of α,γ-diaminobutyric acid > α-amino group of homoserine or serine > -γ-hydroxyl group of homoserine > -β-hydroxyl group of serine. Thus, the ODAP-forming enzyme may be designated as oxalyl-CoA: L-α,β-diaminopropionic acid oxalyl transferase and the trival name ODAP synthase given it.

EXPERIMENTAL

Purification of OX-CoA Synthetase and ODAP-Forming Activities

Lathyrus sativus seeds were germinated for 72 hr and then extracted in the cold with 0·05 M potassium phosphate buffer (pH 7·5) containing 1 mM GSH. The extract was centrifuged at 10,000 *g* for 15 min after filtration through muslin (Step I). One hundred millilitres of this crude extract was treated with 2 ml of 1 M $MnSO_4$ (pH adjusted to 7·0) and the extract centrifuged after keeping it stirred for 5 min. The 0·40–0·60 $(NH_4)_2SO_4$ fraction of the supernatant was collected and dialysed (Step II). The dialysed preparation was stirred with calcium phosphate gel. The gel/protein ratio was kept at 0·5. The mixture was centrifuged after 15 min. The Gel supernatant (fraction III) was adjusted to pH 5·0 with 1 M HOAc and centrifuged immediately. The precipitate was triturated with an excess of buffer (pH 7·5) and the insoluble portion removed by centrifugation. The supernatant was again subjected to $(NH_4)_2SO_4$ fractionation. The 0·30–0·50 fraction (Step IV) was loaded on to a Biogel P 200 column (35 cm × 2 cm), equilibrated with potassium phosphate buffer (pH 7·5) and 2-ml fractions were collected. The two enzyme activities, located in fractions 40–60 and the fractions 45–55, were generally pooled.

CM-Sephadex Chromatography

The pooled fractions of the biogel P 200 eluate obtained from two or three batches were subjected to $(NH_4)_2SO_4$ fractionation. The (0–0·6) fraction was collected and loaded on to a CM-Sephadex (15 cm × 1·5 cm) column equilibrated with 0·02 M potassium phosphate buffer (pH 7·5) and 1 ml fractions were collected. The two enzyme activities were assayed in each tube.

Assay of Oxalyl-CoA Synthetase

The hydroxamate assay and $^{32}P(PP_i)$ exchange assay methods were employed.[1,2] In the former procedure, the enzyme was incubated with a mixture containing the following components (in μM) in a final volume of 3 ml: ATP 10; CoA, 0·2; GSH, 10; potassium oxalate 40; $MgCl_2$, 10; hydroxylamine, 800, potassium phosphate buffer, 200. The final pH of the incubation mixture was 7·5. After 30 min incubation at 37° the reaction was stopped by adding 1 ml of $FeCl_3$ reagent and the colour measured using a 54 filter in a Klett colorimeter.

In the $^{32}P(PP_i)$ exchange assay, the enzyme was incubated for 10 min with a mixture of the following components (in μM) in a final volume of 2 ml: ATP, 4; potassium oxalate, 1; $MgCl_2$, 10; $(^{32}P)PP_i$ containing approximately 10^6 counts/min, 4; potassium phosphate or tris, 100. The final pH was 7·5. The reaction was stopped by adding 1 ml of cold 7 per cent (w/w) $HClO_4$. ATP was isolated from the supernatant by charcoal adsorption, elution with 50 per cent ethanol containing 0·3 M NH_4OH followed by circular paper chromatography. The ATP band was eluted and estimated after rechromatography for radioactivity using an end-window GM counter. Its chemical content was calculated based on the absorption in the u.v. The total counts incorporated into ATP were calculated by multiplying the specific activity of the isolated ATP by the total amount of ATP originally present in the reaction mixture.

The identification of the products of the mixture, namely oxalyl monohydroxamate, AMP and PP_i were according to the methods described by Giovanelli.[2]

Formation of Oxalyl Derivatives of Amino Acids

From oxalyl-CoA. The incubation mixture in 1 ml of total volume contained (in μM) oxalyl-CoA, 2; DAP (or other amino acids were indicated) 2; Tris or potassium phosphate buffer (pH 7·5), 10. To permit good separation of the oxalyl derivative formed on the electrophoretic strips, it was found necessary to keep the salt concentration as low as possible. The reaction mixture was incubated for 30 min at 37°. The oxalyl amino acids (ninhydrin positive) were analysed in trichloracetic acid supernatants by paper electrophoresis as described earlier.[1]

Formation from Oxalate

The respective amino acid (7 μM) was included with the components of the oxalyl-CoA synthetase assay mixture. The Biogel P.200 eluates were used as the enzyme sources. After incubation at 37°, the ninhydrin-positive products were analysed in the trichloracetic acid supernatants by electrophoresis.

Analysis for The Oxalyl Derivatives

Column procedure. A Dowex 50 H^+ column (10 cm × 1 cm) was used. In these experiments radioactive oxalyl derivatives were obtained by using (U-^{14}C) oxalic acid and in some cases (U-^{14}C) serine and (2-^{14}C) glycine in the assay mixture. The trichloracetic acid supernatants were extracted with ether, concentrated to dryness *in vacuo* and the residue taken in 0·5 ml water. A small portion was counted for radioactivity and then fed on to the column after adjusting the pH to between 5–6. The column was eluted with water and 2-ml fractions were collected. One millilitre aliquots were plancheted and the radioactivity measured using a Panax Counter. A typical run obtained with (U-^{14}C) serine as the amino acid acceptor is given in Fig. 4. The fractions under the peaks were collected, hydrolysed with 2 N HCl, and the liberated amino acid was estimated after electrophoresis or paper chromatography followed by ninhydrin spray.

Electrophoretic and Chromatographic Procedures

For the analysis of the ninhydrin-positive oxalyl derivatives, paper electrophoresis of the products at pH 3·6, using pyridine:acetic acid:water buffer (1:10:190), was carried out for 2 hr at 800 V. In some cases the procedure employed by Johnston and Lloyd,[5] namely electrophoresis in pH 7·5 buffer at 600 V, was carried out for 1 hr. The amino acids liberated by acid hydrolysis of the ninhydrin-negative oxalyl derivatives were analysed by paper chromatography employing n-BuOH–HOAc–H_2O (4:1:1) or PhOH–H_2O (4:1).

Synthesis of Oxalyl-CoA and Oxalylglutathione

Oxalyl-CoA was synthesized from thiocresyl hydrogen oxalate and Coenzyme A as described by Quayle.[7] It was found that a similar treatment of thiocresyl hydrogen oxalate and glutathione resulted in the formation of *S*-oxalylglutathione which could be isolated by preparative electrophoresis at pH 3·6.

[7] J. R. QUAYLE, *Biochim. Biophys. Acta* 57, 398 (1962).