SUMMARY

1. A purification of glyoxylic dehydrogenase, obtained from oxalate-grown *Pseudomonas oxalaticus*, is described. The purification involves ammonium sulphate precipitation, gel absorption and ion-exchange chromatography.

2. The pH optimum for the coenzyme A- and triphosphopyridine nucleotide-linked oxidation of glyoxylate to oxalate is 8·6.

3. The glyoxylic dehydrogenase is free of the following enzymic activities: reduced pyridine nucleotide oxidase, pyridine nucleotide-linked glyoxylic acid reductase or glycollic acid dehydrogenase, and glyoxylic acid carboligase.

4. The enzyme is specific for both triphosphopyridine nucleotide and coenzyme A. Diphosphopyridine nucleotide will not replace the former nor will cysteine, glutathione, thioethanolamine or pantetheine replace the latter. In the presence of triphosphopyridine nucleotide and coenzyme A the enzyme will not oxidize formaldehyde, acetaldehyde, glycolaldehyde, pyruvate, oxaloacetate, α-oxoglutarate or hydroxypyruvate. The presence of any of these compounds at 1 mM concentration does not inhibit the oxidation of glyoxylic acid catalysed by the enzyme.

5. No metal-ion requirement for the action of glyoxylic dehydrogenase has been found.

6. K_m values of $5{\cdot}7 \times 10^{-4}$ M and $3{\cdot}4 \times 10^{-5}$ M, at pH 8·6 and 25°, have been found for glyoxylate and triphosphopyridine nucleotide respectively. Coenzyme A is required for the oxidation in catalytic quantities.

7. The free-energy changes involved in the oxidation of glyoxylate are discussed.

8. Glyoxylic dehydrogenase has also been found in extracts of oxalate-grown *Pseudomonas* OD1. This activity is decreased to one-sixth when the same organism is grown on malate.

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Glutamic–Oxaloacetic Transaminase of Cauliflower

1. PURIFICATION AND SPECIFICITY

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Although the scope of the transamination reaction in extracts of higher plants has been amply demonstrated (Wilson, King & Burris, 1954), little information is available on the enzymology of plant transaminases. Cruickshank & Isherwood (1958) reported the partial purification of glutamic–oxaloacetic and glutamic–pyruvic transaminases from wheat-germ without giving details of the purification obtained; although the two activities were not separated, evidence was obtained which indicated that different enzymes were responsible for the two activities, as is the case in animal

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tissues. Patwardhan (1960) has purified glutamic–oxaloacetic transaminase 23-fold from green beans, and demonstrated an activating effect by ferrous ions; this preparation also contained glutamic–pyruvic-transaminase activity. In neither of these two papers was any study reported of the specificity of the transaminases. In view of the suggested importance of transamination in amino acid metabolism in plants (cf. Eberts, Burris & Riker, 1954), the present study was initiated to determine the substrate specificity, cofactor requirements and kinetic properties of glutamic–oxaloacetic transaminase from a higher plant.

MATERIALS AND METHODS

Substrates. L-γ-Hydroxyglutamic acid, β-aminoglutaric acid and DL-γ-methyleneglutamic acid were gifts from Dr L. Fowden. Dihydroxyfumaric acid and hydroxyaspartic acid were gifts from Dr H. J. Sallach. All other substrates were obtained commercially. With the exception of cysteinesulphinic acid, which contained a minor impurity running just behind the main spot in propan-1-ol–formic acid–water (10:1:4, by vol.), all the amino acids were chromatographically pure. Oxaloacetic acid, which gave a molar extinction coefficient of 518 at 280 mμ when dissolved in phosphate buffer (0·2M, pH 7·0), and α-oxoglutaric acid were free from other keto acids when analysed as their 2:4-dinitrophenylhydrazones. Uniformly labelled L-[^{14}C]glutamic acid was obtained from The Radiochemical Centre, Amersham, Bucks.

Gel. Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938), with the modification that the gel was washed with glass-distilled water instead of with tap water.

Diethylaminoethylcellulose. Diethylaminoethylcellulose (DEAE-cellulose) powder with a large pore size (capacity 1·0 m-equiv./g.) was obtained from Brown and Co., Berlin, New Hampshire, U.S.A. This material was poured as a slurry into a glass column (1·7 cm. × 29 cm.) and washed with water; just before use, the column was equilibrated with dipotassium hydrogen phosphate (2 mM).

Protein. Protein was determined by the method of Warburg & Christian (1942). On a few occasions, when turbid solutions had to be assayed, the biuret method of Gornall, Bardawill & David (1949) was employed.

Chromatography. Amino acids were spotted on Whatman no. 1 paper, and run in the descending direction with water-saturated phenol as the usual solvent. The amino acids were detected by the ninhydrin method, and the colour was stabilized by spraying with nickel sulphate (Khabas & El'kin, 1956). Keto acids were chromatographed as the 2:4-dinitrophenylhydrazones by the method of Wager, described by Isherwood & Cruickshank (1954).

Determination of enzymic activity. The initial rate of transamination was measured by the spectrophotometric assay of oxaloacetate at 280 mμ (Green, Leloir & Nocito, 1945; Nisonoff & Barnes, 1952). Enzyme, pyridoxal 5-phosphate (75 μM), L-aspartate (150 μmoles) and potassium phosphate buffer (0·2M, pH 7·0) were incubated in silica cells of 1 cm. light-path for 10 min. at 20° to give the reactivated enzyme, before the addition of α-oxoglutarate (75 μmoles) to start the reaction. The final volume was 3 ml. The increase in $E_{280\ m\mu}$ was measured in a Unicam SP. 500 spectrophotometer, readings being taken at 15 sec. intervals for 3 min. The relation between initial velocity and enzyme concentration departed from linearity at high concentrations of enzyme; consequently all assays were performed with lower concentrations of enzyme in the linear range. Under these conditions, a unit of enzyme activity is defined as the amount of enzyme that will cause a rise in $E_{280\ m\mu}$ of 0·01/min. Specific activity is defined as the number of units/mg. of protein. The validity of the assay was established by correlating the changes in $E_{280\ m\mu}$ with the appearance of the expected amino and keto acids on paper chromatograms.

Purification procedure

Fresh cauliflower heads were defoliated and placed in a cold-room at 3–4° for 1 hr. before being ground. All subsequent steps were performed in a cold-room. The top 0·5–1·0 cm. of florets were removed with a knife and homogenized in 200 g. lots in 250 ml. of chilled phosphate buffer (0·05M, pH 7·0) for 1 min. in a bottom-drive glass blender (Ato-Mix). The debris was strained off with a cloth and the solution returned to the blender with a further 200 g. of florets. After the second homogenizing, the solution was strained as before and spun in a Servall Super-Speed Centrifuge at 31 000 *g* for 10 min. The supernatant solutions were pooled to form the crude extract. The average specific activity of six crude extracts was 3·4. In each large-scale preparation 20–24 cauliflower heads were used, yielding a fresh weight of florets in the range 3–4 kg.

Step 1: *first ammonium sulphate fractionation.* Powdered ammonium sulphate (28 g.) was added with constant stirring to each 100 ml. of crude extract. After 15 min. the precipitate was removed by centrifuging at 20 000 *g* for 10 min. A further 14 g. of ammonium sulphate was added to the supernatant solution; the precipitate was centrifuged down after 15 min. and resuspended in a small volume of phosphate buffer (0·2M, pH 7·0). The resuspended fraction was dialysed in a $\frac{1}{4}$ in. Visking dialysis tube for 5 hr. against a flow of 5 l. of phosphate buffer (0·05M, pH 7·0). The dialysed material was cleared by centrifuging.

Step 2: *second ammonium sulphate fractionation.* An equal volume of a solution of ammonium sulphate (490 g./l.) in phosphate buffer (0·05M, pH adjusted to 6·0 with aq. ammonia soln.) was slowly added to the cleared dialysed fraction. After 15 min. the precipitated protein was removed by centrifuging and discarded. A further 0·4 vol. of ammonium sulphate was added to the supernatant solution; the precipitated protein was collected after 15 min. and resuspended in 10–20 ml. of phosphate buffer (0·2M, pH 7·0). The supernatant solution was treated with a further 0·6 vol. of ammonium sulphate, and the precipitated protein resuspended as above.

Step 3: *treatment with calcium phosphate gel.* The fraction from step 2 which exhibited the highest specific activity was dialysed for 5 hr. against a running stream of 5 l. of dipotassium hydrogen phosphate (2 mM). The dialysed enzyme was diluted to 25–50 ml. with chilled water. Calcium phosphate gel (8·3 mg./ml.) was added in successive 5 ml. portions, the gel being centrifuged down 15 min. after each addition. After 10–15 ml. of gel had been added, the specific activity of the supernatant solution

had reached a maximum. The product of step 3 was a clear, pale-yellow liquid.

Step 4: *chromatography on diethylaminoethylcellulose.* The solution from step 3 was added to the DEAE-cellulose column in portions; the yellow pigment was adsorbed at the top of the column, where it remained during elution. Phosphate buffer (0·2 M, pH 8·0) was dripped into a litre mixing flask containing dipotassium hydrogen phosphate (2 mM), mixing being accomplished by a magnetic stirrer. The resulting solution was forced through the column by a head of liquid (1–2 ft. was sufficient) and 10 ml. fractions were collected. The activity was eluted by this treatment in 3–4 hr. when about 500 ml. of eluate had been collected. The elution pattern of the best preparation is shown in Fig. 1; the two peaks of activity found in this case were not always observed. The fractions of the highest specific activity were pooled and dispensed into tubes; the enzyme was stored at −17°, where it was stable for at least 6 months.

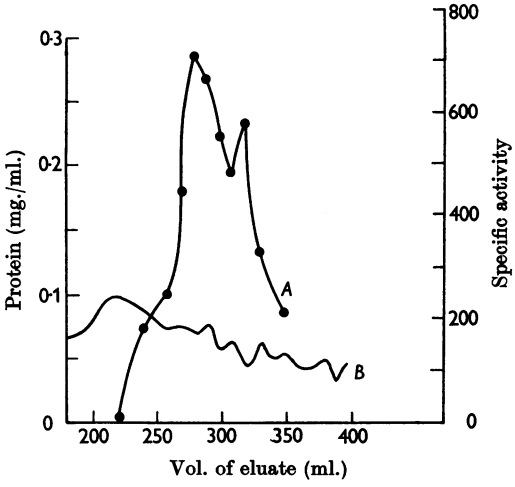

Fig. 1. Elution pattern of glutamic–oxaloacetic transaminase from DEAE-cellulose column. Protein from step 3 (60·4 mg., specific activity 55·6) was placed on top of the column; gradient elution was carried out by raising the molarity and pH of phosphate buffer forced through the column. Fractions of 10 ml. were collected. Samples (0·1 to 0·5 ml.) were incubated for 10 min. with L-aspartate (150 μmoles) and pyridoxal 5-phosphate (75 μM) in phosphate buffer (0·2 M, pH 7·0) at 20° in 1-cm. silica cells. α-Oxoglutarate (75 μmoles) was added to bring the volume to 3 ml., and the rise in $E_{280\ m\mu}$ measured for 3 min. *A*, Specific activity; *B*, concentration of protein.

RESULTS

Purification

Table 1 shows the details of the best preparation obtained. The highest purification achieved was 250-fold. In the more usual case, the peak fractions showed a purification in the range 100- to 200-fold. One possible explanation of the occasional appearance of two peaks of activity in the column eluates is that proteolytic activity present in the crude extract had removed a portion of the enzyme molecule which is not essential for its activity; a similar phenomenon has been reported for papain (Hill & Smith, 1956). The degree of activation of purified preparations by pyridoxal 5-phosphate was the same as that produced by the first ammonium sulphate precipitation; the maximum stimulation observed was 300%.

Specificity

Reactions catalysed by crude extract. The occurrence of glutamic–pyruvic transaminase and glutamic decarboxylase in crude extracts was easily shown by the paper chromatography of the amino acids involved. Attempts to demonstrate transaminations between several other amino acids and α-oxoglutarate were unsuccessful. The use of more concentrated crude extracts, or of dialysed ammonium sulphate precipitates, led to the appearance after incubation of many ninhydrin-positive spots on the chromatogram, presumably amino acids and peptides formed by proteolysis. The

Table 1. *Purification of cauliflower glutamic–oxaloacetic transaminase*

Enzyme activity was assayed by measuring the rise in $E_{280\ m\mu}$ when aspartate, α-oxoglutarate and reactivated enzyme were mixed in 1-cm. silica cells. A unit of activity is the amount of enzyme producing a change in E of 0·01/min. A fresh weight of cauliflower florets of 3·1 kg. was used as the starting material.

Fraction	Vol. (ml.)	Total activity (units)	Protein (mg./ml.)	Specific activity (units/mg.)	Recovery (%)	Purification factor
Crude extract	2550	86 700	13·4	2·54	100	1·0
First $(NH_4)_2SO_4$ fraction	200	26 400	10·8	12·2	30·4	4·8
Second $(NH_4)_2SO_4$ fraction	22	9 900	13	34·6	11·4	13·6
Gel-treated fraction	84	3 360	0·72	55·6	3·9	22
Peak fractions from cellulose column	10	360	0·062	580	0·4	228
	10	400	0·068	588	0·5	232
	10	260	0·04	650	0·3	254
	10	100	0·03	333	0·1	131
Pooled peak fractions	100	2 900	0·05	568	3·3	224

Table 2. *Amino acid–oxaloacetate transaminations catalysed by crude extracts*

A dialysed crude extract of cauliflower florets was incubated with pyridoxal 5-phosphate (0·1 mM) for 10 min. at 20°. Samples (0·25 ml.) were mixed with an amino acid and oxaloacetate (3 μmoles) in 3 ml. of phosphate buffer (0·2 M, pH 7·0) at 20° in 1-cm. silica cells.

Amino acid	Concn. of amino acid (mM)	Decrease in $E_{280\ m\mu}$/10 min.
D-Alanine	25	0
L-Alanine	25	0
α-Aminobutyric acid	33	0
γ-Aminobutyric acid	33	0
L-Asparagine	33	0
L-Cysteic acid	33	0·027
L-Cysteinesulphinic acid	10	0·026
L-Cysteine	33	0
D-Glutamate	10	0
L-Glutamate	10	0·118
L-Glutamine	33	0
L-Serine	33	0
No amino acid	—	0

spectrophotometric assay of oxaloacetate was used to determine whether the crude extract would catalyse the disappearance of this compound when an amino acid was added. Table 2 shows that the only amino acids of those tried which transaminated with oxaloacetate were L-glutamic acid, L-cysteic acid and L-cysteinesulphinic acid.

Preliminary indications of the substrate specificity of glutamic–oxaloacetic transaminase were obtained by observing the effects of added amino acids on the initial velocity of the aspartate–α-oxoglutarate reaction catalysed by a dialysed crude extract; a decrease in this velocity would be expected if the added amino acid can occupy the aspartate site on the enzyme. The concentration of aspartic acid in the assay cell was lowered to 10 mM to ensure that a small inhibitory effect would not be overlooked. Table 3 shows that the only inhibitory compounds of those tried were cysteic acid and compounds containing a sulphydryl group. The lack of inhibition by most of the amino acids tried was taken as presumptive evidence for the high substrate specificity of the transaminase.

Inhibition of transaminases by compounds containing a sulphydryl group has been noted by other workers (cf. du Vigneaud, Kuchinskas & Horvath, 1957; Kenney, 1959). du Vigneaud *et al.* interpret this effect as due to the formation of a thiazolidine ring compound between the added reagent and the cofactor, pyridoxal 5-phosphate.

The cauliflower transaminase was also inhibited 50% by incubating the crude extract with *p*-chloromercuribenzoate (0·5 mM) for 30 min. at 37° before assay, but was not inhibited by a similar incubation with iodoacetate (5 mM). The activity in the crude extract was not affected by addition of ethylenediaminetetra-acetic acid (EDTA, 60 mM), or after incubation for 30 min. at 37° with indol-3-ylacetic acid (100–1 μM) or gibberellic acid (1·5 mM).

Reactions catalysed by purified enzyme. A 67-fold purified preparation contained no glutamic–pyruvic-transaminase or glutamic-decarboxylase activity, and did not catalyse any detectable transamination between α-aminobutyric acid, γ-aminobutyric acid, β-aminoglutaric acid, cysteine, methionine, arginine, leucine, lysine, tryptophan, tyrosine and α-oxoglutarate. The purified preparation was found to catalyse the following reactions:

(1) Glutamate + oxaloacetate ⇌ aspartate + α-oxoglutarate

(2) [^{14}C]Glutamate + α-oxoglutarate ⇌ α-oxo[^{14}C]glutarate + glutamate

(3) γ-Hydroxyglutamate + {α-oxoglutarate / oxaloacetate} ⇌ {glutamate + γ-hydroxy-α-oxoglutarate / aspartate}

(4) γ-Methyleneglutamate + {α-oxoglutarate / oxaloacetate} ⇌ {glutamate + γ-methylene-α-oxoglutarate / aspartate}

(5) β-Hydroxyaspartate + {α-oxoglutarate / oxaloacetate} ⇌ {glutamate + dihydroxyfumarate / aspartate}

(6) Cysteate + {α-oxoglutarate / oxaloacetate} ⇌ {glutamate + β-sulphonylpyruvate / aspartate}

(7) Cysteinesulphinate + {α-oxoglutarate / oxaloacetate} ⇌ {glutamate + β-sulphinylpyruvate / aspartate}

Reaction (1) was found to be specific for the L-forms of the amino acids. The exchange of the carbon chains [reaction (2)] was demonstrated by incubating 100 units of the purified enzyme with L-[^{14}C]glutamic acid (1 μmole, $2{\cdot}9 \times 10^5$ counts/min.) and unlabelled α-oxoglutarate (10 μmoles) in a final volume of 2·8 ml. for 4 hr. at 37°. The incubation mixture was then placed on a column (1 cm. × 8 cm.) of Dowex-50 (H^+ form), and the column washed with water. The first 15 ml. of eluate was collected, and 100 μl. samples were chromatographed on Whatman no. 1 paper in butan-1-ol–formic acid–water (55:5:20, by vol.);

Table 3. *Substrate specificity of cauliflower glutamic–oxaloacetic transaminase*

Dialysed crude extract (6·1 units) was mixed with L-aspartate (30 μmoles), α-oxoglutarate (75 μmoles) and the added compound in 3 ml. of phosphate buffer (0·2M, pH 7·0) at 20° in 1-cm. silica cells. The rise in $E_{280\ m\mu}$ was measured for 3 min.

Compound added	Concn. of added compound (mM)	Inhibition of aspartate–α-oxoglutarate activity (%)
L-Alanine	150	0
D-Alanine	150	0
β-Alanine	150	0
L-Arginine	300	0
L-Asparagine	150	0
L-Cysteic acid	150	24
L-Cysteine	150	73
Glutathione	150	69
Glycine	150	0
Glycylglycine	150	0
L-Histidine	150	0
DL-Homocysteine	100	52
DL-Leucine	150	0
L-Lysine	75	0
DL-Methionine	100	0
DL-Serine	150	0
Thioglycollate	37	32

subsequent radioautography revealed a radioactive area in the α-oxoglutarate position. Reactions (3), (4) and (5) were demonstrated in the forward direction by paper chromatography of the amino acids. Reaction (5), where α-oxoglutarate was the acceptor, was also demonstrated by the assay of dihydroxyfumarate at 290 mμ (Kun, 1956). The initial rates for the reactions between α-oxoglutarate (25 mM) and aspartate (8 mM) or hydroxyaspartate (8·3 mM) in phosphate buffer (0·2M, pH 7·5) at 20° were in the ratio 1250 to 1·0. It is therefore apparent that the substitution of a β-hydrogen atom of aspartate by a hydroxyl group considerably reduces the reactivity of the molecule as a substrate for the transaminase. Incubation of the purified enzyme with dihydroxyfumarate and either glutamate or aspartate failed to lead to the production of detectable amounts of hydroxyaspartate on a paper chromatogram. Addition of EDTA (1·0 mM) and Mg^{2+} ions (1·0 mM), which was reported by Kun (1956) to catalyse the interconversion of the enol and keto forms of dihydroxyfumarate, to a mixture of glutamate, dihydroxyfumarate and enzyme, failed to increase the rate of decrease of $E_{290\ m\mu}$ above that of the control.

Table 4. *Activity ratios with sulphur amino acids during purification of glutamic–oxaloacetic transaminase*

Enzyme assays were performed in 3 ml. of phosphate buffer (0·2M, pH 7·5) at 20° in 1-cm. silica cells; the enzyme was incubated with pyridoxal 5-phosphate (0·1 mM) for 10 min. before assay. The units of aspartate–α-oxoglutarate activity were measured as in Fig. 1. The other activities were assayed by mixing L-cysteic acid (100 μmoles) or L-cysteinesulphinic acid (30 μmoles) with oxaloacetic acid (3 μmoles), and measuring the decrease in $E_{280\ m\mu}$; the units of activity are expressed as $10^2 \times$ decrease in E/min.

Fraction	*A* Aspartate–α-oxoglutarate (units of activity)	*B* Cysteic acid–oxaloacetic acid (units of activity)	Specific activity of glutamic–oxaloacetic transaminase	*A*/*B*
$(NH_4)_2SO_4$ fraction	4·6	2·0	1·4	2·3
Crude extract	3·3	1·0	3·9	3·3
$(NH_4)_2SO_4$ fraction	12·7	3·9	7·9	3·3
$(NH_4)_2SO_4$ fraction	5·3	2·0	8·7	2·7
$(NH_4)_2SO_4$ fraction	10·2	3·3	13·5	3·1
$(NH_4)_2SO_4$ fraction	3·1	1·0	13·8	3·1
Gel-treated fraction	5·2	1·9	28·0	2·7
$(NH_4)_2SO_4$ fraction	11·9	3·9	34·2	3·1
Fractions from cellulose column	3·3	0·92	94·0	3·6
	1·6	0·46	114·0	3·5

Fraction	*C* Aspartate–α-oxoglutarate (units of activity)	*D* Cysteine-sulphinic acid–oxaloacetic acid (units of activity)	Specific activity of glutamic–oxaloacetic transaminase	*C*/*D*
Crude extract	1·7	0·53	2·5	3·2
$(NH_4)_2SO_4$ fraction	4·5	1·8	5·7	2·5
$(NH_4)_2SO_4$ fraction	6·6	2·4	13·3	2·7
$(NH_4)_2SO_4$ fraction	4·9	2·3	31·4	2·1
Gel-treated fraction	2·0	1·0	55·6	2·0
Fractions from cellulose column	3·3	1·3	116·0	2·5
	5·0	2·1	333·0	2·4
	18·0	6·8	580·0	2·6
	20·0	7·0	588·0	2·8
	13·0	5·3	650·0	2·5

Reaction (5) could thus be demonstrated in the forward direction only.

Green *et al.* (1945) reported that oxomalonate underwent a slow transamination with glutamate in the presence of purified pig-heart transaminase. Oxomalonate (0·05–0·1M) strongly inhibited both the aspartate–α-oxoglutarate and glutamate–oxaloacetate reactions catalysed by crude, and purified, cauliflower transaminase. No detectable formation of aminomalonate or its decarboxylation product, glycine, was observed when oxomalonate was incubated with glutamate, or aspartate, and purified enzyme. A repetition of this experiment in borate buffer (pH 9·0), as used by Nagayama, Muramatsu & Shimura (1958) to demonstrate amino acid–oxomalonate transaminations in liver extracts, gave the same negative result. The use of [^{14}C]-glutamate, and subsequent isolation of α-oxoglutarate, indicated that a maximum of 1% transamination had occurred when 20 units of transaminase were incubated with glutamate (16 mM) and oxomalonate (21 mM) in phosphate buffer (0·2M, pH 7·5) at 30° for 4 hr. From these findings it seems possible that oxomalonate has an appreciable affinity for the active sites of the cauliflower enzyme, and hence acts as an inhibitor, but that it undergoes transamination at only a very slow rate.

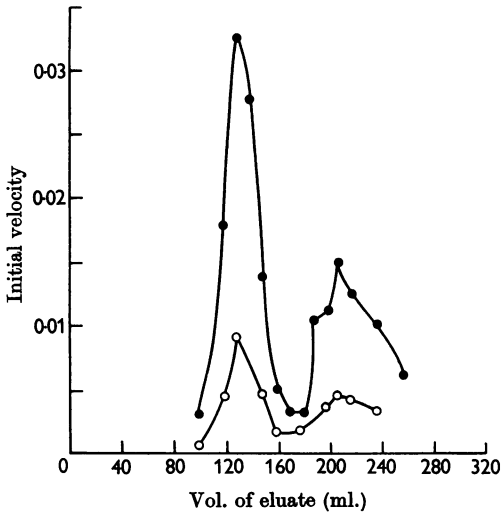

Fig. 2. Correlation of aspartate–α-oxoglutarate activity (●) with cysteate–oxaloacetate activity (○). The fractions eluted from a DEAE-cellulose column were assayed for the two activities. Aspartate–α-oxoglutarate activity was assayed in 0·1 ml. samples as in Fig. 1. Cysteate–oxaloacetate activity was assayed by measuring the decrease in $E_{280\,m\mu}$ when L-cysteate (100 μmoles) and oxaloacetate (3 μmoles) were added to 0·1 ml. samples of reactivated enzyme in a final volume of 3 ml. of phosphate buffer (0·2M, pH 7·0) at 20° in 1-cm. silica cells. Initial velocity equals the change in $E_{280\,m\mu}$/min.

Transamination of the sulphur amino acids. The reactions of cysteic acid and cysteinesulphinic acid with α-oxoglutaric acid were demonstrated by the chromatographic detection of glutamic acid. For the reactions involving oxaloacetate, the quantitative assay of oxaloacetate by the spectrophotometric method was combined with the chromatographic detection of aspartate. Evidence that one enzyme catalyses the reactions of aspartic acid with α-oxoglutarate, of cysteic acid with oxaloacetate, and of cysteinesulphinic acid with oxaloacetate was obtained by measuring the ratios of the initial rates of these reactions over a wide range of specific activity of the transaminase. Table 4 shows that these ratios were approximately constant throughout purification. Further evidence for the one-enzyme hypothesis as regards the cysteate–oxaloacetate reaction comes from the close similarity of the elution curves of the two activities from the DEAE-cellulose column (Fig. 2).

A further test of the hypothesis that glutamic–oxaloacetic transaminase catalyses the cysteinesulphinate–oxaloacetate reaction is derived from kinetic considerations. If the reaction velocity with one substrate is V_1, and that with the other substrate is V_2, then the velocity V_t when both substrates are present should be $V_1 + V_2$ if different enzymes act on the substrates. However, if a single enzyme is involved, then:

$$V_t = \frac{V_{max.1}\,S_1}{S_1 + K_{m1}(1 + [S_2/K_{m2}])} + \frac{V_{max.2}\,S_2}{S_2 + K_{m2}(1 + [S_1/K_{m1}])}$$

where $V_{max.}$ represents the maximum velocity, K_m the Michaelis constant and S the concentration of substrate (Thorn, 1949). This test was applied for the reactions between glutamate (S_1), cysteinesulphinate (S_2) and oxaloacetate. The results of

Table 5. *Interaction of substrates with cauliflower glutamic–oxaloacetic transaminase*

Glutamate–oxaloacetate and cysteinesulphinate–oxaloacetate activities were measured in 1-cm. silica cells in phosphate buffer (0·2M, pH 7·5) at 30° with a 224-fold purified enzyme preparation. V_1 and V_2 (expressed as the decrease in $E_{280\,m\mu}$/min.) are the initial velocities with glutamate (S_1) and cysteinesulphinate (S_2) respectively. The values of the apparent Michaelis constants and maximum velocities were obtained from Lineweaver–Burk double reciprocal plots (Davies & Ellis, 1961). V_t (obs.) is the velocity with mixed substrates; V_t (calc.) is the velocity obtained from the equation of Thorn (1949).

S_1 = 0·05M	S_2 = 0·05M
K_{m1} = 0·036M	K_{m2} = 0·062M
$V_{max.1}$ = 0·1	$V_{max.2}$ = 0·06
V_1 = 0·054	V_2 = 0·0268
V_t (calc.) = 0·058	
V_t (obs.) = 0·052	

this test show that V_t (obs.) is less than $V_1 + V_2$, and that V_t (cal.). for a single enzyme is in moderately good agreement with the observed velocity (Table 5).

Comparison with pig-heart transaminase

In view of the findings on the substrate specificity of the cauliflower transaminase, it was deemed of some interest to study the specificity of the same transaminase from another source. Samples of glutamic–oxaloacetic transaminase from pig heart, at differing stages of purity, were generously made available by Dr B. E. C. Banks. The preparation with the highest specific activity (61-fold purified) catalysed reactions (4), (6) and (7). Reactions (3) and (5) were not tested for. Ninhydrin-positive spots running with the R_F of glycine were found after incubation of the enzyme with oxomalonate and glutamate or aspartate or cysteate. The ratios of the initial rates of the reactions between aspartate and α-oxoglutarate, cysteate and oxaloacetate, and cysteinesulphinate and oxaloacetate, were measured with four samples of enzyme which differed in purity. The results shown in Table 6, indicating that these ratios were approximately constant, suggest that, as with the cauliflower enzyme, the transaminase from pig heart catalyses several reactions.

DISCUSSION

The results presented here indicate that glutamic–oxaloacetic transaminase from a higher plant source has a relatively high substrate specificity; there can now be no doubt that, as in animal tissues, glutamic–pyruvic and glutamic–oxaloacetic transaminases are separate enzymes. The purified enzyme would not catalyse the glutamic–pyruvic transamination, or transaminations involving several amino acids and either α-oxoglutarate or oxaloacetate; this finding suggests that more than one enzyme is responsible for the wide range of transamination reactions found in extracts of higher plants (Wilson *et al.* 1954).

Transaminations between γ-methyleneglutamic acid and oxaloacetate, α-oxoglutarate and pyruvate are catalysed by extracts from groundnut seedlings (Fowden & Done, 1953). The results of the present work suggest that the activity with α-oxoglutarate and oxaloacetate is due to the lack of specificity of the glutamic–oxaloacetic transaminase present in the extracts. It is possible that the activity with pyruvate is due to a similar lack of specificity of the glutamic–pyruvic transaminase present. Virtanen & Hietala (1955) demonstrated a transamination between α-oxoglutarate and γ-hydroxyglutamate in phlox extracts; the present results suggest, but do not prove, that the glutamic–oxaloacetic transaminase is responsible for this reaction. The demonstration that the purified preparations of transaminase will catalyse these reactions lends some support to the suggestion that one of the reasons for the occurrence of these non-protein amino acids is the lack of specificity of enzymes normally engaged in metabolizing the protein amino acids (Fowden, 1958). If this is so, then the problem why some plants only contain these acids revolves around the origin of the keto acid analogues and not around the origin of the amino group.

The conclusion drawn by Garcia-Hernandez & Kun (1957), that hydroxyaspartate is a poor substrate for animal glutamic–oxaloacetic transaminase, which catalyses only a very slow formation of hydroxyaspartate, has been confirmed for the plant enzyme except that for the latter no hydroxyaspartate formation could be detected at all. Hydroxyaspartic acid has not as yet been reported from plant tissues, but has been shown to occur in bound form from the extracellular material of *Azotobacter* (Virtanen & Saris, 1957), and has also been isolated from digests of casein (Sallach & Kornguth, 1959). Stafford (1957) has discussed the possible role of dihydroxyfumarate in plant metabolism, especially as regards its formation from tartrate.

One of the more interesting findings that has emerged from this study is the demonstration that the transaminase will catalyse reactions involving cysteic acid and cysteinesulphinic acid. Wilson *et al.* (1954) reported that crude extracts of several

Table 6. *Substrate specificity of pig-heart glutamic–oxaloacetic transaminase*

Enzyme assays were performed as described in Table 4 except that the temperature was 30°, 6 μmoles of oxaloacetate were used instead of 3, and 300 μmoles of cysteate were used instead of 100.

Purification factor	*A* Aspartate–α-oxoglutarate (units of activity)	*B* Cysteic acid–oxaloacetic acid (units of activity)	*C* Cysteine-sulphinic acid–oxaloacetic acid (units of activity)	Ratios *A*/*B*	Ratios *A*/*C*
1·0	24·5	4·2	24·0	5·84	1·02
16·6	6·25	1·0	6·0	6·25	1·04
47·7	5·5	0·9	4·4	6·11	1·25
61·2	3·0	0·7	2·8	4·29	1·07

plants catalyse the transfer of amino groups from cysteate to α-oxoglutarate. Cysteinesulphinic acid has been suggested as an intermediate in the oxidation of cysteine to sulphate in animal tissues; a system found in liver extracts catalyses a transamination between cysteinesulphinate and α-oxoglutarate to give glutamate and β-sulphinylpyruvate; this latter keto acid then decomposes to sulphite and pyruvate (Fromageot, Chatagner, Séjourné & Bergeret, 1952). The oxidation of cysteinesulphinate by extracts of rat-liver mitochondria is initiated by transamination with α-oxoglutarate and oxaloacetate (Singer & Kearney, 1956). The present findings with both the purified cauliflower enzyme and the purified pig-heart enzyme strongly indicate that it is the glutamic–oxaloacetic transaminase present in the extracts that catalyses these reactions; preliminary indications that this was so for the pig-heart enzyme were obtained by Green *et al.* (1945) for cysteate and by Singer & Kearney (1956) for cysteinesulphinate, but these workers used preparations of lower purity than those used here, and did not measure the ratios of activities throughout purification. It has been reported that aqueous extracts of oat leaves will catalyse a transamination between cysteinesulphinic acid and α-oxoglutaric acid; the enzyme involved is reported as being distinct from those involved in the transamination of cysteate and aspartate (Perez-Milan, Schliack & Fromageot 1959). However, no evidence is presented in this latter paper to establish the validity of the assay procedure in measuring the initial velocities of the various reactions, nor is the complication introduced by the presence of glutamic–pyruvic transaminase in the extracts considered. The available evidence does not therefore seriously contradict the conclusion of the present study that cysteinesulphinic acid is a substrate for glutamic–oxaloacetic transaminase. The values of the apparent Michaelis constants for the sulphur amino acids are reported in the next paper (Davies & Ellis, 1961). It may be noted here that the Michaelis constant for cysteinesulphinic acid is only about twice as great as that for glutamic acid, suggesting that if this sulphur amino acid occurs in plant tissues the reaction could have physiological importance. The transaminase has only a low affinity for cysteic acid, however, and was not saturated by the highest concentration employed (0·25 M). The smallest Michaelis constant of all those estimated was that of oxaloacetate in the glutamate–oxaloacetate reaction. This finding is consistent with the low concentration of oxaloacetate found in plant tissues compared with that of other keto acids (Krupka & Towers, 1958); to work efficiently *in vivo* the transaminase needs to possess a high affinity for oxaloacetate.

It is of some interest to speculate on the possibility that the transamination of cysteinesulphinic acid is involved in the incorporation of sulphur into organic combination. Singer & Kearney (1953) have suggested that a reversal of the desulphination of β-sulphinylpyruvic acid may be the step by which sulphur is attached to a carbon chain. Chapeville & Fromageot (1954) reported the synthesis of [^{35}S]cysteinesulphinic acid on incubation of [^{35}S]sulphite with glutamate, pyruvate and a rabbit-kidney extract. However, β-sulphinylpyruvate appears to be an unstable compound, readily decomposing to sulphite and pyruvate, and has not yet been isolated. The direct reversal of this breakdown is therefore unlikely on thermodynamic grounds, and, by analogy with the metabolism of oxaloacetate, it would seem more profitable to consider that the reversal may be accomplished by the combination of sulphite with phosphoenolpyruvate, rather than with pyruvate. Such a reaction would be analogous to the fixation of carbon dioxide into oxaloacetate via phosphoenolpyruvate. If this postulated reversal of desulphination can be substantiated, then transamination would seem to play a key role not only in carbohydrate and nitrogen metabolism, but in the metabolism of sulphur as well.

SUMMARY

1. Glutamic–oxaloacetic transaminase has been purified 250-fold from cauliflower florets; the purified enzyme is free from glutamic–pyruvic transaminase.

2. Purified preparations of the enzyme catalyse transaminations between γ-hydroxyglutamate, γ-methyleneglutamate, β-hydroxyaspartate, cysteate and cysteinesulphinate as amino-group donors, and both α-oxoglutarate and oxaloacetate as amino-group acceptors; oxomalonate is a good inhibitor but a poor substrate.

3. Kinetic data, and the constancy of ratios throughout purification, strongly suggest that one enzyme is involved in transaminations between glutamate, cysteate, cysteinesulphinate and oxaloacetate.

4. The substrate specificity of purified pig-heart transaminase is similar to that of the enzyme from cauliflower, except that oxomalonate is a better substrate for the pig-heart enzyme.

5. The possibility that the transamination of cysteinesulphinic acid may be involved in the pathway of sulphur reduction is discussed.

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Glutamic–Oxaloacetic Transaminase of Cauliflower

2. KINETICS AND MECHANISM OF ACTION

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The kinetics of glutamic–oxaloacetic transaminase have been investigated with crude preparations from pig heart (Nisonoff & Barnes, 1952) and maize-root tips (Cook, 1957). In both cases, the kinetic data were explained in terms of a ternary complex formed between the enzyme and its two substrates. Nisonoff & Barnes derived a rate law by a modification of the treatment introduced by Van Slyke & Cullen (1914). Their assumption of irreversible combination of enzyme with substrate could conceivably be valid for an irreversible reaction, but can hardly be justified for the readily reversible transamination. The general case of the mechanism proposed by Cook when treated by the steady-state assumption leads to a rate equation of quadratic form, but when treated by the equilibrium assumption leads to a rate equation which gives a linear Lineweaver–Burk plot (Haldane, 1930).

Both treatments are unsatisfactory in that they fail to recognize the participation of pyridoxal phosphate and pyridoxamine phosphate in transamination. On the basis of model experiments by Snell (1945), Schlenk & Fisher (1947) proposed a

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