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PURIFICATION AND PROPERTIES OF L-ALANINE:5-KETOOCTANAL AMINOTRANSFERASE FROM *CONIUM MACULATUM*

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Key Word Index—*Conium maculatum*; Umbelliferae; L-alanine; 5-ketooctanol aminotransferase; enzymology; secondary metabolism; alkaloid biogenesis.

Abstract—L-Alanine:5-ketooctanal aminotransferase has been isolated from leaves of *Conium maculatum* and purified 369-fold. The enzyme MW 56200 catalyses the formation of the alkaloid γ-coniceine and shows optimum activity at pH 8.5. The K_m and V_{max} for L-alanine were 27 mM and 3.3 nkats./mg protein respectively, and for 5-ketooctanal were found to be 0.14 mM and 3.3 nkats/mg protein. The activation energy calculated from both V_{max} and V_{init} values was 2 kJ/mol. The enzyme was inhibited by Na semicarbazide, hydroxylamine, isoniazid, iodoacetic acid, *p*CMB and KCN and showed enhancement of activity with pyridoxal phosphate after precipitation with $(NH_4)_2SO_4$. A reduction in activity was observed with additions of 2-ketoglutarate pyruvate and glyoxalate at concentrations of 0.5 and 2.5 mM, but the activity was not affected by additions of the reaction product γ-coniceine *or* the subsequently formed alkaloids coniine and methylconiine.

INTRODUCTION

In recent years plant aminotransferase (EC 2.6.1) have been studied in some detail. However, in general, they have been incompletely described in regard to their amino acid specificities. Some partially purified preparations are highly specific for particular amino acids, while other preparations appear to utilise a variety of amino acids [1–5]. Aminotransferases have also been implicated in the formation of amines in numerous higher plants with the amino groups of L-alanine transferred to a range of aldehydes from methanal to octanal [6].

The involvement of aminotransferases in the formation of many alkaloids has long been assumed and cell free preparations have been isolated from a number of plants including the alkaloid containing *Lupinus angustifolia* and *Papaver somniferum*. The cell free extracts utilise appropriate amino acids in the formation of necessary intermediates of alkaloid biogenesis [7, 8]. The presence of an aminotransferase in *Conium maculatum* which utilises 5-ketooctanal in the formation of the first formed alkaloid γ-coniceine 2-propyl Δ^1-piperidine) has been established [9]. In this paper the partial purification of L-alanine:5-ketooctanal aminotransferase is described and kinetic data and properties have been determined which allow conclusions to be drawn on the role of the enzyme in the formation of γ-coniceine and the subsequently formed alkaloids coniine (2-propyl-piperidine) methylconiine (*N*-methyl-2-propyl-piperidine), conhydrinone (2(1-hydroxypropyl) piperidine) and conhydrin (2(1-oxopropyl)piperidine).

RESULTS

The occurrence of aminotransferases in the vegetative parts of *C. maculatum* which utilise a variety of amino acids and oxoacids were investigated. Using the Reitman and Frankel assay [13] and enzyme solutions from acetone powders, a direct comparison of activity was made with the activity of the enzyme responsible for the conversion of 5-ketooctanal to γ-coniceine. Table 1 shows that L-alanine acts as an amino donor for 2-ketoglutarate, glyoxalate and oxaloacetate as well as 5-ketooctanal. To investigate the specificity of the aminotrans-

Table 1. Aminotransferases in *Conium maculatum*

Aminotransferase	Activity U/mg protein →	Activity U/mg protein ←
Aspartate/2-ketoglutarate ⇌ Oxaloacetate/glutamate	40	384
L-Alanine/2-ketoglutarate ⇌ Pyruvate/glutamate	34	14
L-Alanine/glyoxalate ⇌ Pyruvate/glycine	102	no activity
L-Alanine/oxaloacetate ⇌ Pyruvate/aspartate	40	no activity
Glycine/2-ketoglutarate ⇌ Glyoxalate/glutamate	no activity	no activity
Glycine/oxaloacetate ⇌ Glyoxalate/aspartate	26	—
L-Alanine/5-ketooctanal ⇌ Pyruvate/γ-coniceine	10	no activity

The Reitman and Frankel assay [12] was used. The reaction mixture contained M substrate (0.1 ml), 10 mM keto acid (0.1 ml) enzyme step 3 (0.01–0.1 ml) and 50 mM Tris buffer pH 8 to a total vol. of 1 ml. Incubation was at 35° for 5 min (30 min for 5-ketooctanal). Controls contained boiled enzyme.

ferase responsible for the conversion of 5-ketooctanal to γ-coniceine, a purification procedure for this aminotransferase system was devised.

Purification of L-*alanine*:*5-ketooctanal aminotransferase*

Table 2 shows details of the best preparation obtained. The highest purification achieved was 369-fold with an average purification for the various preparations of 340-fold. Details of the steps in the purification are given in the Experimental. The use of protamine sulphate gave no great advantage and with some preparations this step was omitted. The use of $(NH_4)_2SO_4$, although not greatly improving the purification served to concentrate the protein and thereby reduce the volume. Although the use of calcium phosphate gel usually only gave a 2.5-fold increase in purification, the protein removed at this stage improved the speed at which the active protein could be eluted from the DEAE cellulose column in the subsequent step and therefore helped to reduce losses in activity. The use of two DEAE cellulose columns (steps 5 and 6) in sequence with, on each occasion, rapid removal of the active protein facilitated by the use of Tris buffer rather than the borate buffer used in the assay, also helped to reduce the losses in activity which could be obtained at this stage. These two steps gave the best increase in purification.

The inclusion of DTT 1 mM and EDTA 1 mM in the initial extracting buffer were beneficial. The addition of 15% glycerol as used by some workers [10] appeared to have little effect on the maintenance of activity during the extraction procedure or on storage. An increase in salt concentration by the inclusion of 0.1 M KCl in the buffer during elution of the enzyme from Sephadex G200 (step 7) helped to maintain activity. Storage of enzyme solutions at −20° showed an average loss of 50% of their activity over 2–3 weeks which was improved to 20% if the total salt concentration was increased to 0.15 with KCl. The best results on storage, particularly with the more highly purified enzyme solutions (step 5 onwards), were obtained using storage in liquid N_2 although levels of activity at step 7 in thawed enzyme solutions were variable as the enzyme was highly labile at low protein concentrations. Some advantage was obtained if BSA (1 mg/1 ml) was added to step 7 as the material was eluted from the G200 column. During the purification procedure the aminotransferase activity exhibited when L-alanine is presented with 2-ketoglutarate was considerably reduced and with all preparations was absent from step 6. Furthermore GOT activity was considerably reduced at step 7.

The purest enzyme preparation (step 7) exhibited one major and one minor band of protein when examined by standard polyacrylamide gel electrophoresis [11, 12]. In some preparations the minor protein band was almost undetectable and concentration of the protein at this stage invariably produced irreversible precipitation with loss of activity. Gel electrophoresis of the concentrated samples compared with the original protein sample suggested that L-alanine-5-ketooctanal aminotransferase activity resided in the major protein band.

Products of the reaction and assay requirements

Production of γ-coniceine as a result of aminotransferase activity in the presence of L-alanine and 5-ketooctanal was verified by PC and TLC as described in the Experimental. The assay for the measurement of γ-coniceine was used routinely except for the determination of pH and studies of alkaloid concentration on activity. The development of a red colour over 3–5 min with alkaline Na nitroprusside in the presence of γ-coniceine is dependent on the final pH of the solution and was therefore unsuitable for the determination of pH maxima. Since the Reitman and Frankel assay [13] for the estimation of pyruvate gave equivalent results [9], this assay was used for all determinations of pH maxima. It was found that 5-ketooctanal eventually produced a red colour with alkaline Na nitroprusside. However this reaction was slow compared with that with γ-coniceine, but made it essential to (a) include 5-ketooctanal in the blanks and (b) avoid too high a concentration: hence the use of 4 mM concentrations of 5-ketooctanal in the assay mix. 5-Ketooctanal at concentrations > 6 mM showed some inhibition of the reaction as did concentrations of L-alanine > 250 mM. Under these conditions repeatable results could be obtained providing that samples were assayed immediately on completion of the incubation period.

As Hartmann *et al.* had found with their amino acid:aldehyde transferase (AAT) activity in borate buffer was twice that in Tris buffer [14], and a comparison of activity of L-alanine:5-ketooctanal transferase activity showed that borate buffer was 2.5 times that in Tris buffer. Borate buffer was therefore used routinely in all assays. As the enzyme additions in all assays were of 0.1 ml or less it was found that no inhibition due to Tris buffer was observable and therefore in routine assays no dialysis of samples against borate was made.

Enzyme solutions at all steps in the purification procedure showed aminotransferase activity without the addition of pyridoxal phosphate, and after prolonged dialysis (2 days) of enzyme solutions from steps 3 and 4

Table 2. Summary of the purification of L-alanines:5-ketooctanal aminotransferase

Step	Fraction	Total protein mg	Total activity nkats	Specific activity nkats/mg protein	Yield %	Purification
1	Acetone powder extract	8160	53.8	0.007		
2	Protamine sulphate supernate	2990	46.1	0.016	85	2·1
3	45–65% $(NH_4)_2SO_4$ precipitate	1100	37.8	0.033	83	5.2
4	Calcium phosphate supernate	348	30.5	0.087	79	12
5	DEAE cellulose column I	39	18.3	0.476	52	66
6	DEAE cellulose column II	15	14.3	0.953	78	132
7	Sephadex G200 gel eluate	3.5	9.3	2.666	65	369

The γ-coniceine assay was used. The reaction mixtures contained protein (0.1 ml) M, L-alanine (0.2 ml) 4 mM, 5-ketooctanal (0.1 ml), pyridoxal phosphate 1 mM and 200 mM borate buffer pH 8.5 to a total vol. of 1.5 ml. Incubation at 35° for 30 min.

little alteration in activity was observed. However, the addition of pyridoxal phosphate (1 mM) enhanced aminotransferase activity 2-fold with all steps of the purification procedure other than the acetone powder extract (Table 5). Pyridoxal phosphate (1 mM) was therefore routinely included in the assay mixture. Experiments in which the enzyme was pre-incubated for different periods with pyridoxal phosphate prior to the initiation of the reaction with 5-ketooctanal, showed that a pre-incubation period of 5 min was adequate to give maximum activation.

A series of experiments conducted at pH 5, 8 and 11 using the Reitmann and Frankel assay gave no indication of the formation of L-alanine when pyruvate and γ-coniceine were incubated with enzyme solutions from step 4.

Michaelis constants for L-alanine and 5-ketooctanal

The effect of varying substrate concentrations on the rate of reaction was examined. Each substrate was varied in the presence of a saturating concentration of the other. This allowed each substrate to be determined as a pseudo-first order reaction at initial velocity and the data fitted into the straight line form of the Michaelis–Menten equation. To investigate the probable mechanism of the reaction, the initial reaction velocities were plotted versus varying concentrations of one substrate at two fixed concentrations of the second substrate. A number of determinations were made using different samples of partially purified enzyme 15–100 μg (steps 6 and 7). In all cases the Lineweaver–Burk plots were linear and parallel. As described by Alberty [15] this would suggest a reaction involving successive binding of substrates (binary mechanism) [16]. The apparent K_m values obtained from these results were 27 mM for L-alanine and 0.14 mM for 5-ketooctanal. The apparent V_{max} calculated from assays using step 7 enzyme were 3.3 nkats/mg protein with varying concentrations of 5-ketooctanal and 3.3 nkats/mg protein with varying concentrations of L-alanine.

Reaction rate and enzyme concentration, time pH

The formation of γ-coniceine was linear for at least 40 min using the assay conditions given in the experimental with up to 100 μg of step 7 enzyme solution.

The pH was determined using phosphate–citrate borate, Tris (20) and borate buffers. The optimum pH in these buffers was 8.5 with half maximal activity at pH 5 and 11.

The reaction was stable up to 60 min up to 35° but showed some loss of activity at 40° after 20 min. The reaction rate was measured at different temperatures and by means of an Arrhenius plot using both V_{max} and V_{init} values an activation energy of 2 kJ/mol was calculated.

Specificity of L-alanine as the amino group donor and the effect of oxoacids or increased alkaloid concentration on γ-coniceine formation

The ability of various amino acids to replace L-alanine as amino donor to 5-ketooctanal was investigated. Preliminary experiments relied on 6–12 hr incubation at 35° and the detection of γ-coniceine by chromatographic procedures. Previous work had shown that only the L-isomer of alanine was active [9]. The following amino acids were investigated: L-aspartate, D,L-glycine, L-phenyl alanine, L-glutamate, D,L-valine, L-serine, L-glutamine, L-lysine, L-leucine, L-isoleucine, 3-aminobutyric acid and 6-amino hexanoic acid. Of these amino acids, serine, glutamic, 3-aminobutyric and 6-amino hexanoic acids showed traces of activity with step 6 enzyme. Table 3 shows this activity was detectable after 90 min using the standard assay procedure for γ-coniceine.

The inclusion of concentrations of 2-ketoglutarate, pyruvate, oxaloacetate and glyoxalate (0.5 and 2.5 mM) (ratio of 5-ketooctanal : oxoacid 10 : 1 and 2 : 1) in assay mixtures with steps 6 and 7 (Table 4) showed that some inhibition of the formation of γ-coniceine was observed, particularly with glyoxalate and pyruvate, where inhibition was 89 and 33% respectively at 2.5 mM. However, the formation of γ-coniceine was unaffected by the inclusion of 1 and 0.1 mM concentrations of γ-coniceine, coniine and methylconiine.

Inhibitor studies

It has previously been shown that some aminotransferases are stimulated by cations [4, 17] although the role of metallic ions in enzymic transamination has not been established. Experiments using the chlorides of Ca^{2+}, Mg^{2+} and Mn^{2+} at final concentrations of 2 mM showed no stimulation of the production of γ-coniceine using standard conditions.

The effect of a series of inhibitors which react with carbonyl groups, and particularly with pyridoxal phosphate, together with reagents which react with SH groups was observed. Hydroxylamine, KCN, and isoniazid at final concentrations of 1 mM were the most effective inhibitors. In the case of hydroxylamine and semicarb-

Table 3. The formation of γ-coniceine from 5-ketooctanal utilizing amino acids other than L-alanine

Amino acid substrate	γ-Coniceine formed per mg of protein	
	nmol/30 min	nmol/90 min
L-Alanine	140	—
D-Alanine	0	—
L-Glutamic acid	negl.	10
L-Serine	negl.	4.6
3-Aminobutyric acid	7.6	23
6-Aminohexanoic acid	18.5	68

The standard assay was used with amino acid concentrations of 120 mM, except for L-glutamic acid where a concentration of 60 mM was used. Enzyme consisted of 0.5 mg of step 6 protein.

Table 4. The effect of oxoacids on L-alanine : 5-ketooctanal amino transferase

Oxacids	Concentration mM	% Activity
nil		100
Glyoxalate	2.5	11
	0.5	41
Pyruvate	2.5	66
	0.5	100
Oxaloacetate	2.5	82
	0.5	100
α-Ketoglutarate	2.5	61
	0.5	74

The standard assay was used except that the concentration of 5-ketooctanal was 5 mM. The addition of enzyme was 15 μg of step 7 protein.

Table 5. The effect of pyridoxal phosphate and common aminotransferase inhibitors on the formation of γ-coniceine from 5-ketooctanal

Treatment		% Activity
Complete system		100
+ Pyridoxal phosphate	0.1 mM	180
	1.0 mM	390
+ Semicarbazide	0.1 mM	75
	1.0 mM	61
+ Semicarbazide	1.0 mM + pyridoxal phosphate 1.0 mM	85
+ Hydroxylamine	0.1 mM	40
	1.0 mM	14
+ Hydroxylamine	1.0 mM + pyridoxal phosphate 1.0 mM	70
+ Isoniazid	0.1 mM	82
	1.0 mM	20
+ Iodoacetic acid	0.1 mM	46
	1.0 mM	41
+ *p*CMB	0.1 mM	58
	1.0 mM	49
+ KCN	0.1 mM	44
	1.0 mM	18

The reaction mixture contained M L-alanine (0.2 ml), 4 mM 5-ketooctanal (0.1 ml) enzyme step 6 (0.1 to 0.2 mg protein) and 200 mM borate buffer pH 8.5 to a total vol. of 1.5 ml. The enzyme was pre-incubated for 10 min with the inhibitors and pyridoxal phosphate at 25°. The reaction was initiated with 5-ketooctanal and incubation was at 35° for 30 min.

azide, the inclusion of pyridoxal phosphate (1 mM) largely restored activity. Effective inhibition was also observed with iodoacetate and pCMB (Table 5).

Determination of MW

Using the procedure developed by Andrews [18] and a column of Sephadex G200, the average MW of L-alanine : 5-ketooctanal amino transferase was 56200.

DISCUSSION

L-Alanine : 5-ketooctanal aminotransferase which catalyses the formation of γ-coniceine was purified 369-fold from acetone powders of young leaves. The enzyme was highly labile and readily precipitated from solution when attempts were made to concentrate highly purified enzyme solutions.

The fact that the reverse reaction could not be detected probably results from the formation of 5-aminooctanal which immediately cyclises to γ-coniceine. It is likely that the latter step occurs non-enzymatically and irreversibly such that the formation of γ-coniceine is unlikely to be regulated by the activity of the reverse reaction.

The high K_m for L-alanine led to a search without success for an alternative amino acid group donor. Previous work [8] indicated that only L-alanine was utilized and that use of DL-alanine slightly reduced activity [9] suggesting that the D-isomer had a slightly inhibitory effect. Although the levels of L-alanine in the tissues of *C. maculatum* are not known, the high K_m suggests that high levels of this substrate are necessary for the formation of γ-coniceine to take place despite the low K_m for 5-ketooctanal. This factor together with the inhibitory effect of pyruvate could be of prime importance therefore in the regulation of the rate at which γ-coniceine is formed. Table 1 indicates very active involvement of L-alanine in aminotransferases involving other oxoacids and this suggests considerable competition for the available L-alanine. Alkaloid production in hemlock may therefore be determined by the ability of the plant to produce 5-ketooctanal and a high concentration of L-alanine during the active growth period. Unlike many aminotransferases which utilize either oxoacids or aldehydes as the amino group acceptor the enzyme responsible for γ-coniceine formation showed marked preference for L-alanine. The AAT systems investigated by Hartmann *et al.* [6] in *Mercurialis perennis* and in Spinach [19] showed that with propanal and pentanal respectively, significant activity was also observed with 3-aminobutyric acid and 5-aminocaproic acid. Activity with these two amino acids and 5-ketooctanal also occurred to a small extent with hemlock preparations and the formation of γ-coniceine was also inhibited by the oxoacids pyruvate 33% and glyoxalate 89%. This suggests that the formation of γ-coniceine may well utilize the group of enzymes involved in the formation of aliphatic amines. However Hartmann [6] found that with AAT systems pyruvate was a more effective inhibitor than that observed for γ-coniceine formation. In all hemlock enzyme preparations of steps 6 and 7 enzyme all GPT activity was lost, although some GOT activity remained, indicating that GPT activity and γ-coniceine formation involved separate proteins. However, the extent to which γ-coniceine formation is associated with AAT and GOT activity is at present unclear. The recent work of Unger and Hartmann [19] suggesting that AAT activity may be a minor activity of an ubiquitous transaminase GOT-1, requires further clarification. The high activity of L-alanine : glyoxalate amino transferase in hemlock and the strong inhibitory effect of glyoxalate on γ-coniceine formation may well be significant in this respect. Other transaminases from a variety of sources [19–22] have not shown amino acid specificity and γ-coniceine formation appears to be similar in this respect.

L-alanine : 5-ketooctanal aminotransferase also shows some similarities in respect of pH maxima and the requirement for pyridoxal phosphate. The pH optimum at 8.5 is similar to that for pea aminotransferase [21], and AAT activity in *M. perennis* and spinach [6, 19], glyoxalate transferase, (pH 8.2) from wheat [23], glutamate : aspartate aminotransferase (pH 8–8.5) from wheat germ [1] and ornithine transaminase (pH 8) from squash [10].

The essentiality of pyridoxal phosphate for the enzyme was difficult to demonstrate as has often been found with plant transaminases [1, 2, 4, 20]. Prolonged dialysis did not remove pyridoxal phosphate from the enzyme completely. However, pyridoxal phosphate at a concentration of 1 mM increased enzyme activity 2-fold with all stages in the purification beyond step 2. Substances known to react with aldehydes (e.g. semicarbazide, hydroxylamine and iproniazid) showed strong inhibition of activity, though recovery of activity was observed by the subsequent addition of pyridoxal phosphate to solutions containing hydroxylamine and semicarbazide.

Evidence for the involvement of SH groups in the mechanism of aminotransferase is variable and unlike transaminase from wheat germ [1] and animals [24], L-alanine : 5-ketooctanal aminotransferase was inhibited by iodoacetate and *p*CMB as is ornithine transaminase

[10] and in keeping with the latter was also found to be stabilized by the addition of dithiothreitol to the standard buffer.

The MW (56200) is low compared with that of the multispecific aminotransferase from bush bean seedlings [5] or the mammalian aspartate aminotransferase [25] (91000–110000). It is, however, similar to ornithine aminotransferase [16] (43000).

The kinetic results suggested a binary mechanism for L-alanine : 5-ketooctanal aminotransferase and this is in keeping with data for other plant and animal aminotransferases [4, 26, 27].

The characteristics of L-alanine : 5-ketooctanal aminotransferase in relation to other aminotransferases and its possible regulation by availability of L-alanine are significant in respect of its role in the biogenesis of the *Conium* alkaloids, since the product γ-coniceine is the first formed alkaloid [28, 29]. γ-Coniceine is subsequently either reduced to coniine which is later methylated, or γ-coniceine forms conhydrin via conhydrinone [29]. In most instances the former pathway is the major route of alkaloid formation, but in certain circumstances the latter pathway can become equally dominant. Further studies on the isolated γ-coniceine reductase [30] and *S*-adenosylmethionine : coniine methyltransferase [31] together with the L-alanine : 5-ketooctanal aminotransferase may shed further light on the significance of enzyme regulation in secondary metabolism and in alkaloid biogenesis in particular.

EXPERIMENTAL

Fresh young leaves of first year plants of *C. maculatum* cv Chelsea, grown out of doors were harvested immediately before use.

Preparation of Me_2CO powers. Pre-cooled young leaves from which the petiole had been removed were homogenized at top speed in a blender with Me_2CO at −20°. 5 successive vol. of Me_2CO were used and the resulting powder after filtration to remove excess Me_2CO was vacuum dried at room temp. This material could be stored for 2 to 3 months without significant loss of activity.

Enzyme purification. All procedures were carried out at 0–5°. (1) The Me_2CO powder was extracted with 10 vol. of 50 mM Tris pH 7.5 containing DTT 1 M and EDTA 1 mM for 1 hr with constant gentle stirring. The residue was removed by centrifugation at 38000 *g* for 10 min. (2) The vol. of the supernatant soln was measured and a vol. of 1% protamine sulphate soln equal to 50% of the supernatant was added slowly with stirring. The resulting dark brown ppt. was removed by centrifugation and discarded. (3) Solid $(NH_4)_2SO_4$ was added slowly to 45% saturation with constant stirring. The ppt. obtained after stirring 20 min in the cold was removed by centrifugation at 38000 *g* for 10 min and discarded. The supernatant soln was then brought to 65% saturation with solid $(NH_4)_2SO_4$. The protein which pptd between 45 to 65% $(NH_4)_2SO_4$ saturation was collected by centrifugation at 38000 *g* for 10 min and dissolved in a minimum of 50 mM Tris buffer pH 7.5 containing DTT 1 mM (standard buffer). (4) The $(NH_4)_2SO_4$ ppt. in soln was dialysed with stirring for 4 hr against standard buffer. To this soln was added 1 ml of Ca phosphate gel (B.D.H. 3% w/v suspension in H_2O) for every 120 mg of protein. The suspension was gently stirred for 5 min and the gel rapidly removed with a low speed bench centrifuge. The resulting soln was dialysed 18 hr against standard buffer. (5) The enzyme soln after dialysis was put on a DEAE-cellulose column (1.6 × 30 cm) which was equilibrated with standard buffer and after washing the column with a vol. of standard buffer equivalent to × 3 the vol. of the enzyme soln, the active protein eluted from the column with standard buffer containing 0.1 M KCl. The active fractions were pooled and dialysed 18 hr against standard buffer. (6) The enzyme soln from the DEAE-column I (1.6 × 30 cm) after dialysis was placed on a second DEAE–cellulose column and eluted from the column with a linear gradient of 0–0.3 M KCl in standard buffer. The fractions were collected in 3 ml aliquots and those containing the enzyme pooled. Preliminary expts showed the active protein to be eluted with a salt concn of 0.04 M. (7) The enzyme soln from the DEAE–cellulose column II were put on a Sephadex G200 (1.6 × 55 cm) column which had been previously equilibrated with standard buffer containing 0.1 M KCl. The active fractions were then collected and pooled.

Protein determinations. The protein content of all enzyme preparations was determined using the method of ref. [32].

Electrophoresis. Polyacrylamide gels were prepared in glass columns 0.7 × 8.5 cm and the procedure used was that of ref. [11] using Tris–glycine buffer. Electrophoresis was carried out using 4mA per column for about 1.5 hr at 2°. Bromophenol blue was used as a marker and protein stained with 0.25% w/v naphthol blue black in 8% HOAc. Gels were destained with 8% HOAc.

Determination of MW. Estimations of MW of the purified aminotransferase were carried out using gel filtration [18]. A column of Sephadex G200 (1.6 × 55 cm) was prepared using 0.05 M Tris buffer pH 7.5 containing 0.1 M KCl and calibrated using the following proteins of known MW—cytochrome *c* (horse heart) MW 12400, ovalbumen (chick) MW 45000, BSA MW 67000 and γ-globulin (human) MW 180000. The void vol., V_0 was determined with β-dextran. The ratio of V_e/V_0 where V_e = elution vol. for each standard was plotted against log MW and the MW of the transaminase calculated from the calibration curve obtained.

Enzyme assays. (i) Assay for pyruvate and other oxoacids. The method of ref. [13] was employed in which the formation of the 2 : 4-dinitrophenylhydrazones of the ketoacids are measured spectrophotometrically in alkaline solution at 510 nm. The reaction mixture, unless specifically stated, contained M substrate (0.2 ml) 10 mM keto acid (0.1 ml), enzyme (0.01–0.1 ml) and 50 mM Tris (borate) buffer to a total vol. of 1 ml. The reaction time varied from 5–10 min at 35° depending on the oxoacid under investigation. Periods of 30 min were required with 5-ketooctanal for adequate measurements of activity to be made. Control samples were (a) minus ketoacid, (b) with boiled enzyme (i) with ketoacid (ii) minus ketoacid. Enzyme activity only occurred with the complete mixture. 1 μmol 5-ketooctanal had A_{510} equivalent to 0.190. The other oxoacids gave values as given in ref. [13]. (ii) Assay for formation of γ-coniceine. The presence of γ-coniceine was determined by its colour reaction in alkaline soln with Na nitroprusside. The reaction mixture contained 200 mM L-alanine. 4 mM 5-ketooctanal, 1 mM pyridoxal phosphate, enzyme (0.05–0.2 ml) and 200 mM borate buffer pH 8.5 to a total vol. of 1.5 ml. Incubation was at 35° for 30 min. Blanks contained no enzyme and controls boiled enzyme. γ-Coniceine was measured by the addition of 10% Na_2CO_3 (0.5 ml), followed by 1% Na nitroprusside (1 ml). The *A* for maximum colour development was measured after 3–5 min at 490 nm. Enzyme activity for γ-coniceine formation was measured in nkats sp. act. = nkats/mg of protein.

Preparation of substrates and authentication of reaction products. (i) 5-ketooctanal was prepared as described previously using a variation of the method of ref. [33]. 1-Propylcyclopentene was converted to the ozonide which was reduced with Zn/HOAc to give 5-ketooctanal [9]. IR, GLC and the formation of the disemicarbazide mp 184–185° showed this material to be identical with that prepared by the method of ref. [33]. (ii) γ-Coniceine was authenticated by chromatography using a pure reference sample. Two systems were used (a) PC using the solvent system of ref. [34] and (b) TLC using Si gel G and $CHCl_3$–MeOH–NH_4OH (45 : 25 : 4). Dragendorff's reagent and alkaline Na nitroprusside were used for visualization. The reaction products in expts with amino acids and oxoacids assayed by the Reitman and Frankel method were further checked by PC for the detection of the appropriate amino acids using standard solvent systems [35].

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