Phytochemistry, 1978, Vol 17, pp 107–112 Pergamon Press. Printed in England

SEPARATION OF THE FORMATION OF γ-CONICEINE AND ALIPHATIC AMINES FROM GOT ACTIVITY IN *CONIUM MACULATUM*

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(*Received* 1 *August* 1977)

Key Word Index—*Conium maculatum*; Umbelliferae; aminotransferase; γ-coniceine; aliphatic amines; alkaloid biogenesis; secondary metabolism; enzyme regulation.

Abstract—L-Alanine:5-keto-octanal aminotransferase has been separated from GOT and GPT activities by DEAE-cellulose chromatography. Two transaminase peaks A and B were observed for γ-coniceine formation and assay showed that these peaks also contained the amino acid:aldehyde aminotransferase (AAT) responsible for aliphatic amine biosynthesis. Kinetic studies showed that γ-coniceine formation with transaminase A had K_m for L-alanine and 5-keto-octanal of 27 mM and 1.6 mM respectively. The V_{max} was 1.3 nkats/mg protein and the activation energy was 3.0 kJ/mol. For transaminase B the K_m for L-alanine and 5-keto-octanal are 55 mM and 0.14 mM respectively with a V_{max} of 3.3 nkats/mg protein and an activation energy of 0.33 kJ/mol. Transaminase A and B had the same MW and have distinguishable pH_{max}. γ-Coniceine formation by transaminase A was inhibited uncompetitively by glyoxalate and competitively by pyruvate, whereas with transaminase B uncompetitive inhibition was also observed with glyoxalate; no inhibition was observed with pyruvate which at low concentration (0.25 M) showed slight stimulation of activity.

INTRODUCTION

Recent work by Hartmann *et al.* [1–3] has shown that aldehyde amination occurs through the activity of a normal amino acid-keto acid aminotransferase which also reacts with aldehydes. Their work suggests that the method of formation of aliphatic amines is widespread throughout the plant kingdom, and since there is no general correlation between the activity of the enzyme system and the occurrence of aliphatic amines, it is suggested that the presence of amines may therefore depend on the occurrence of the aldehydes [3].

Work on the biosynthesis of alkaloids in *Conium maculatum* [4] has shown that the first formed alkaloid γ-coniceine is polyketide derived via 5-keto-octanal and an aminotransferase which utilises L-alanine [5]. The expected intermediate, 5-amino-octanal which cyclises to γ-coniceine suggested [6] that the formation of this alkaloid could be a function of the aminotransferase involved in aliphatic amine formation, (AAT) and the recent work by Unger [7] with spinach AAT preparations would appear to confirm this. Recent work on the isolation of L-alanine 5-keto-octanal aminotransferase suggested that this aminotransferase was not a function of GOT activity as has been claimed by Unger and Hartmann for AAT activity in spinach [8]. In the present paper therefore the separation of γ-coniceine formation from GOT (glutamate-oxaloacetate) and GPT (glutamate-pyruvate aminotransferase) activity is described and its relationship with AAT activity investigated.

RESULTS

The transaminase responsible for γ-coniceine formation has been previously isolated and purified some 369-fold. These experiments suggested that L-alanine: 5-keto-octanal transaminase was completely separated from GPT which was not eluted from the DEAE-cellulose column with 0.1 M KCl, and that partial separation from GOT activity also occurred when a 0–0.4 M KCl was used [8].

The foregoing experiments utilised *C. maculatum* cv Bowles and since the phenolase content was high compared with the previously used cv Chelsea, DIECA 10^{-2} M was used routinely in the extracting buffer and this considerably improved the yield of γ-coniceine formed. The removal of phenolics by dialysis after $(NH_4)_2SO_4$ precipitation of enzyme activity was incomplete. It was therefore found advantageous to substitute a Sephacryl S-200 (2.5 × 90 cm) column for the batchwise use of calcium phosphate gel [8]. Complete separation of GOT and GPT activity from γ-coniceine formation was achieved by DEAE-cellulose chromatography, the transaminases other than GPT being eluted from the column with a 0–0.15 M KCl gradient. It was serendipitously discovered that increased elution of this column with standard buffer prior to the commencement of the KCl gradient separated L-alanine:5-keto-octanal aminotransferase into two protein peaks, hereafter referred to as transaminases A and B. However further improvement in the separation of γ-coniceine formation and GOT was not observed (Fig. 1). The foregoing experiments are therefore based on the simplified isolation of γ-coniceine formation given in Table 1.

γ-Coniceine formation and AAT activities

Monitoring of the eluate from the DEAE-cellulose column for AAT using L-alanine and *n*-hexanal as substrates, showed that AAT completely coincided with

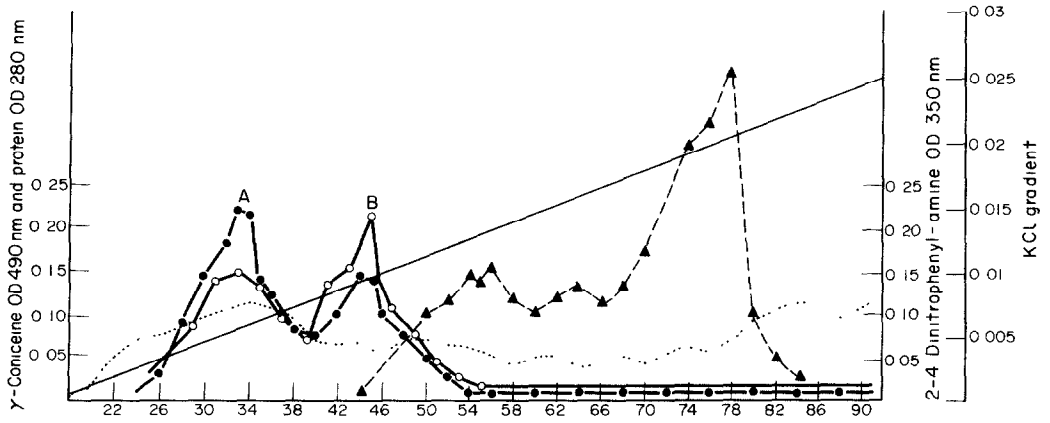

Fig. 1 Chromatography on DEAE-cellulose of a partially purified transaminase from *C. maculatum* leaves. γ-Coniceine formation ●——●; AAT O——O; GOT ▲— — —▲; Protein ·····.

Table 1. Summary of the separation of γ-coniceine formation and AAT from GOT and GPT activities

Fraction	Protein (mg)	Specific activity (nkats/mg protein) γ-Coniceine formation	AAT	GOT	GPT
1. Acetone powder extract	648	0.145	0.0016	6.0	266
2. 45–65% $(NH_4)_2SO_4$	112	0.77	0.029	31.6	1433
3. Sephacryl S-200 eluate	66	2.0	0.076	91.6	4433
4. DEAE-cellulose chromatography					
protein peak A (0.026 M KCl)	2.5	2.8	0.079	nil	nil
protein peak B (0.34 M KCl)	1.0	3.1	0.307	0.33	nil

The reaction mixtures and incubation at 35° for the assay of γ-coniceine formation, AAT, GOT and GPT were as given in the Experimental.

γ-coniceine formation and was therefore also separated from GOT activity. Whereas transaminases A and B showed similar activity in respect of γ-coniceine formation, transaminase B was more active than A in respect of AAT. However gel electrophoresis of transaminases A and B showed that each contained more than one protein. The relative amounts of A and B present appeared to be adversely affected by the action of phenolase and in initial preparations their relative occurrence was variable. However the routine inclusion of DIECA 10^{-2} M in the initial extracting buffer and the subsequent rapid removal of phenolic material made standard repeatable extractions of these enzymes possible.

The AAT activity of transaminases A and B

In view of the apparent general occurrence of AAT activity [1–3] and its activity in *C. maculatum* flowers [2], the AAT activity of transaminases A and B with a series of aldehydes and amino acids was investigated.

Amino acid specificity of transaminases A and B was investigated, using *n*-hexanal as NH_2-acceptor (Table 2). The results show that as with dog's mercury and spinach transaminase preparations [2, 8], L-alanine is the most efficient NH_2-donor with an observable difference in efficiency between A and B for this amino acid. The amino acids 4-aminobutyric and 6-aminohexanoic acid were found to be < 50% as efficient as L-alanine for transaminases A and B in *C. maculatum* and these results are similar to those obtained by Hartmann [2] and Unger [8].

Using a series of aldehydes and L-alanine as NH_2-donator the transaminases A and B from hemlock showed some interesting variations when compared with AAT activity in *M. perennis* and spinach [2, 8]. Both trans-

Table 2. The formation of aliphatic amines by transaminases A and B using *n*-hexanal as NH_2-acceptor

Amino-acid	2,4-Dinitrophenyl-amine derivative (nkats/mg protein) Transaminase A	Transaminase B
L-Alanine	0.079	0.307
3-Aminobutyric acid	0.001	0.008
6-Aminohexanoic acid	0.024	0.038
L-Glutamate acid	0.003	0.006

The reaction mixture contained 100 mM L-alanine, 10 mM *n*-hexanal, 1 mM pyridoxal phosphate enzyme, 0.25 ml (protein A = 430 μg, B = 250 μg) and 200 mM borate buffer pH 8.5 to a total vol. of 2.6 ml. Incubation was at 35° for 60 min. Aliphatic amines were estimated as 2,4 DNP-derivatives as given in the Experimental.

Table 3. The formation of aliphatic amines by transaminases A and B using L-alanine as NH_2-donor

Aldehyde	2,4 Dinitrophenyl-amine derivative (nkats/mg protein) Transaminase A	Transaminase B
n-Propanal	0.002	0.005
n-Butanal	0.003	0.005
2γ-Butanal	0.010	0.236
n-Hexanal	0.079	0.307
n-Octanal	0.060	0.123

The reaction mixture contained 100 mM L-alanine, 10 mM aldehyde, 1 mM pyridoxal phosphate, enzyme 0.25 ml (protein A = 430 μg B = 250 μg) and 200 mM borate buffer pH 8.5 to a total vol. of 2.6 ml. Incubation was at 35° for 60 min. Aliphatic amines were estimated as 2,4 DNP-derivatives as given in the Experimental.

aminases were most efficient in producing hexylamine, but unlike AAT in *M. perennis* and spinach, these transaminases showed high activity with 2^{γ} butanal which was 4-fold greater than that for *n*-butanal, and in the case of transaminase B, activity with 2^{γ} butanal was greater than activity with *n*-octanal (Table 3).

γ-Coniceine formation by transaminases A and B

pH. The formation of γ-coniceine by transaminases A and B showed some differences in activity with pH. In general, transaminase A had a broad pH_{max} 7.5–8.5, whereas transaminase B exhibited a sharp pH_{max} at 8.5 (Fig. 2).

Molecular weight. Using the techniques laid out in ref. [8], the transaminases A and B were found to have the same MW 56230.

NH_2-donator. Previous results [9] showed that amino acids other than L-alanine could act as NH_2-donator in the formation of γ-coniceine from 5-keto-octanal. However only 6-aminohexanoic acid was of any significance when compared with the activity with L-alanine. Transaminases A and B showed little variation in their activity with L-alanine, but there were significant differences with 4-aminobutyric and 6-aminohexanoic acid. Activity with 4-aminobutyric acid though low, was ten times greater with transaminase A than with transaminase B, whilst activity with 6-aminohexanoic acid was three times greater with transaminase B (45% of the activity with L-alanine) than with transaminase A (Table 4).

Kinetic data. A re-appraisal of the K_m, V_{max} and activation energy for γ-coniceine formation proved to be of particular interest since significant differences were observed for transaminases A and B (Table 5).

Table 4. The formation of γ-coniceine from 5-keto-octanal by transaminases A and B utilising amino acids other than L-alanine

	γ-Coniceine formed. Nkats/mg protein	
	Transaminase A	Transaminase B
L-Alanine	2.55	3.33
L-Glutamate	0.09	0.15
3-Aminobutyric acid	0.15	0.015
6-Aminohexanoic acid	0.51	1.53

The reaction mixture contained 200 mM amino acid; (20 mM glutamate) 4 mM. (Transaminase A) or 1 mM (Transaminase B) 5-keto-octanal, 1 mM pyridoxal phosphate, enzyme (100–160 μg) and 200 mM borate buffer pH 8.5 to a total vol. of 1.5 ml. Incubation was at 35° for 30 min.

Table 5. Kinetic data for γ-coniceine formation by transaminases A and B isolated by DEAE-cellulose chromatography

	Transaminase A	Transaminase B
K_m L-alanine	27 mM	55 mM
K_m 5-keto-octanal	1.6 mM	0.14 mM
V_{max}	1.26 nkats/mg protein	3.3 nkats/mg protein
Activation energy	30 kJ/mol	0.33 kJ/mol

The affinity for L-alanine for transaminase A is half that for transaminase B, whilst the affinity for 5-keto-octanal is ten times greater. Transaminase B showed significant inhibition of γ-coniceine formation at concentrations of 2 mM 5-keto-octanal and above, whereas this concentration was just adequate for maximum activity with transaminase A which did not show any significant inhibition with 10 mM concentrations of this aldehyde. Similar differences were found in V_{max} and activation energy in that transaminase B had a high V_{max} and low activation energy compared with transaminase A.

Inhibition of γ-coniceine by glyoxalate and pyruvate

Previous studies [9] showed that the oxoacids glyoxalate and pyruvate significantly inhibit γ-coniceine formation in hemlock. In the foregoing experiments both transaminase A and B were inhibited by 0.5 and 2.5 mM glyoxalate (Table 6) and plots of $1/V$ vs $1/S$ with 0.25 mM glyoxalate for transaminase A and 0.5 mM glyoxalate for transaminase B showed that this inhibition was uncompetitive (Figs 2 and 3). Furthermore the results in Table 6 showed that A was much more sensitive to glyoxalate inhibition than B.

Table 6. The effect of glyoxalate acid pyruvate on γ-coniceine formation by transamines A and B

		% Activity	
		Transaminase A	Transaminase B
Control		100	100
Glyoxalate	0.5 mM	32	74
	2.5 mM	14	50
Pyruvate	0.25 mM	—	130
	0.5 mM	73	100
	2.5 mM	40	100

The reaction mixture contained 200 mM L-alanine, 4 mM 5-keto-octanal (transaminase A); 1 mM 5-keto-octanal (transaminase B), 1 mM pyridoxal phosphate, enzyme (50–80 μg) and 200 mM borate buffer pH 8.5 and glyoxalate or pyruvate to a total vol. of 1.5 ml. Incubation was at 35° for 30 min. Enzyme and oxoacid were preincubated for 1 min at 25°.

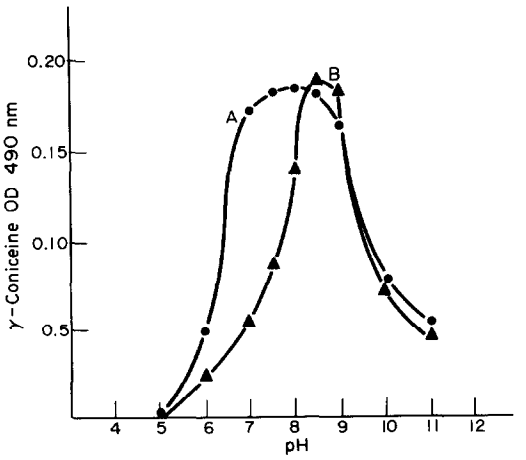

Fig. 2. The effect of pH on the formation of γ-coniceine by transamines A and B.

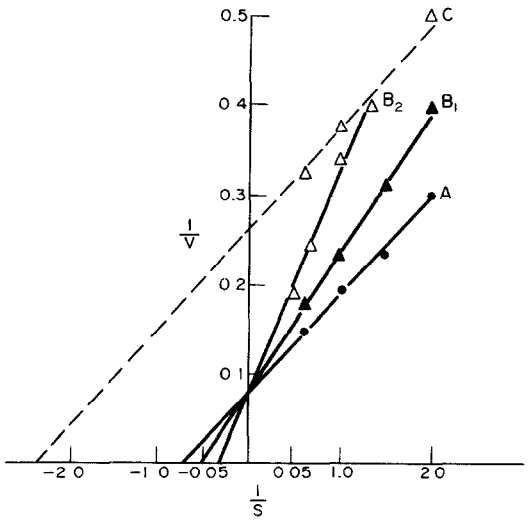

Fig. 3. The effect of pyruvate and glyoxalate on the formation of γ-coniceine by transaminase A. A, increasing concentrations of 5-ketooctanal; B_1, in the presence of 0.125 mM pyruvate; B_2, in the presence of 0.25 mM pyruvate, and C, in the presence of 0.25 mM glyoxalate.

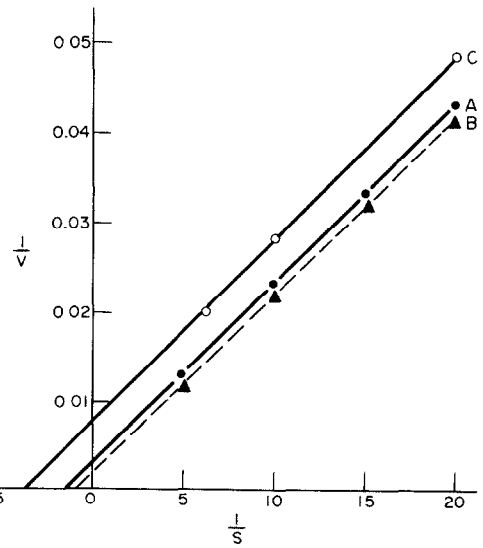

Fig. 4. The effect of pyruvate and glyoxalate on the formation of γ-coniceine by transaminase B. A, increasing concentrations of 5-ketooctanal; B, in the presence of 0.125 mM pyruvate and C, in the presence of 0.5 mM glyoxalate.

Pyruvate was found to have an inhibitory effect only on γ-coniceine formation by transaminase A and this inhibition was found to be competitive (Fig. 3). Low concentrations of pyruvate (0.25 mM) with transaminase B appeared to have a slight stimulatory effect and no inhibition was observed with 0.5 and 2.5 mM pyruvate (Table 6).

DISCUSSION

Isolation of the enzymes responsible for γ-coniceine formation by the methods given in the Experimental have shown that two enzymes transaminase A and B may be separated by DEAE-cellulose chromatography despite the severe losses in activity which occur during the procedure. The method completely separates γ-coniceine formation and AAT activity from GOT activity. It is therefore clear that these activities are not a function of GOT I in hemlock and it is likely that an alteration to the NaCl gradient in the spinach experiments of Unger and Hartmann [8] would produce similar results. Although AAT and γ-coniceine formation coincide the differences in activity between the transaminases A and B in respect of AAT (B is 4 × more active than A) but not γ-coniceine formation might suggest that different enzymes are responsible and could theoretically be separated, particularly since the transaminases A and B have been shown by electrophoresis to contain several proteins. Further it was also observed [9] that the behaviour of AAT and γ-coniceine formation in respect of inhibition by glyoxalate and pyruvate differed significantly in that γ-coniceine formation in hemlock shows lower inhibition with pyruvate and greater inhibition with glyoxalate than was found for AAT in spinach [3]. AAT in hemlock also shows some interesting differences between transaminase A and B in the utilisation of various aldehydes in amine formation, the most noticeable being the high activity with 2° butanal compared with *n*-butanal, a finding which significantly differs from the results obtained with dog's mercury and spinach transaminase preparations [3, 8].

The fact that two isozymes with the same MW and almost indistinguishable pH_{max} have been found in hemlock leaves which actively produce γ-coniceine and also exhibit AAT activity presents a novel situation for transaminases generally and at the same time sheds further light on the probable regulation of γ-coniceine formation. Previously [9] it has been suggested that γ coniceine formation is regulated by the occurrence of 5-keto-octanal and the researches on the formation of aliphatic amines [1, 3] have suggested that AAT occurs generally throughout the plant kingdom and that it is the occurrence of the appropriate aldehyde and not the enzyme which allows significant formation of aliphatic amines in some plant species. Indeed, Unger [7] has recently shown that spinach AAT will form γ-coniceine if presented with 5-keto-octanal. However, although he implies that spinach AAT is more efficient in γ-coniceine production than hemlock preparations, his comparison is made with a crude preparation [5] and comparison with a similarly purified enzyme from *C. maculatum* [9] shows quite clearly that the hemlock enzyme at 66-fold purification is ten times more efficient at the production of γ-coniceine than similar spinach preparations.

The possible role of the isozymes transaminase A and B in the regulation of γ-coniceine production by hemlock leaves has been revealed by a study of their kinetic characteristics. They differ significantly in that K_m values for their substrates, particularly for 5-keto-octanal as well as in their V_{max} and activation energy. Both isozymes exhibit high K_m for L-alanine (K_m for A 27 mM, for B 55 mM). Transaminase B has a low K_m for 5-keto-octanal (0.14 mM) and a relatively high rate at which it can convert this aldehyde into γ-coniceine with L-alanine as NH_3-donator. It is also found to be significantly inhibited by 2 mM 5-keto-octanal and is slightly stimulated by low (0.125 mM) concentrations of the other reaction product pyruvate. However transaminase A has a relatively high K_m for 5-keto-octanal (1.6 mM) and

converts it to γ-coniceine at a relatively slow rate compared with transaminase B. However this transaminase (A) is strongly inhibited by pyruvate which acts by competitive inhibition. It is perhaps significant that transaminase A only begins to show maximum efficiency when the concentration of 5-keto-octanal is at a level which significantly inhibits transaminase B. It is suggested therefore that the two isozymes possibly act in a concerted manner in the regulation of γ-coniceine formation. Since pyruvate is produced during the formation of γ-coniceine by L-alanine:5-keto-octanal aminotransferase, the competitive inhibition of transaminase A by pyruvate would appear to be an interesting case of feedback inhibition particularly since no inhibition of either transaminase A or B is observed with γ-coniceine, coniine or methylconiine. This type of metabolic control well documented for 1^{γ} metabolism [10, 11] is less well established as a control mechanism in 2^{γ} metabolism. However a number of examples are recorded and although in some cases the evidence is derived from physiological observations, control by gramine of gramine formation [12], the incorporation of acetate into 6-methylsalicylate by 6-methylsalicylate [13] and the control of dimethyllyltransferase by elymoclavine and agroclavine [14] are now reasonably well documented. Although both enzymes are uncompetitively inhibited by glyoxalate the role if any of this oxoacid in the regulation of these isozymes is unclear from the present data.

Since the existence of isozymes is genetically based it is possible that variations in the occurrence of transaminase A and B in different cultivators of *C. maculatum* may occur. It is also possible for the different isozymes to be present in different intracellular compartments. Both these facets of the existence of the isozymes transaminase A and B in *C. maculatum* require further investigation.

EXPERIMENTAL

Fr. young leaves of first year plants of *C. maculatum* cv Bowles and Wheathampsted, grown out of doors were harvested immediately before use and Me_2CO powders prepared as in [9].

Separation of transaminase activities. All procedures were carried out at 0–5°. (i) the Me_2CO powder was extracted with 10 vol. of 50 mM Tris pH 7.5 containing DTT 1 mM, EDTA 1 mM and DIECA 10 mM for 1 hr with constant gentle stirring. After removal of cell debris by filtration through cheesecloth, the remainder of the residue was removed by centrifugation at 38000 *g* for 10 min. (ii) Solid $(NH_4)_2SO_4$ was added slowly to 45% satn with constant stirring. The ppt. obtained after standing for 1 hr in the cold was removed by centrifugation at 38000 *g* for 10 min and discarded. The supernatant soln was then brought to 65% satn with solid $(NH_4)_2SO_4$ and allowed to stand for 1 hr. The protein which pptd between 45 to 65% $(NH_4)_2SO_4$ satn 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 and DIECA 10 mM. (iii) The $(NH_4)_2SO_4$ ppt. in soln was dialysed with stirring against the buffer containing DTT 1 mM and DIECA 10 mM for 2 hr. (iv) This soln after dialysis was layered on a Sephacryl S-200 column (2.5 × 90 cm) and transaminase activity estimated by γ-coinceine formation was eluted using 0.05 M Tris pH 7.5 buffer containing DTT 1 mM (standard buffer). (v) The Sephacryl S-200 eluate was dialysed 18 hr and then layered 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 ×4 the vol. of the enzyme soln the transaminases were eluted from the column with a linear gradient of 0–0.15 M KCl in standard buffer (300 ml). Fractions were collected in 2.5 ml aliquots which were monitored for transaminase activity using the enzyme assays as given below.

Protein determinations. The protein content of all enzyme preparations was determined using the method of ref. [15].

Determination of MW. Estimations of MW were carried out as described previously [9].

Enzyme assays. (i) Assay for pyruvate and oxaloacetate. The method previously described [9] using the assay technique of ref. [16] was employed in which the formation of the 2,4-dinitrophenylhydrazones of the ketoacids are measured spectrophotometrically in alkaline solution at 510 nm. (ii) Assay for the 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-keto-octanal (transaminase A); 1 mM 5-keto-octanal (transaminase B), 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 mins. Blanks contained no enzyme and controls boiled enzyme. γ-Coniceine was measured as described in ref. [9]. (iii) Assay for aliphatic amines (AAT). AAT activity was measured by the quantitative estimation of the amines. The reaction mixture contained 100 mM amino acid (L-alanine for the standard assay), 10 mM aldehyde (hexanal for the standard assay), 1 mM pyridoxal phosphate enzyme 0.5 ml and 200 mM borate buffer pH 8.5 to a total vol. of 2.6 ml. Incubation was at 35° for 60 min. The reaction was stopped with 0.5 ml 3% sulphosalicylic acid and the pptd protein removed by centrifugation. The quantitative estimation of the amines was carried out using the methods given in refs [1, 17] in which the amines are removed from the reaction mixture by alkaline steam distillation and reacted with 2,4-dinitrofluorobenzene to give the 2,4-ditrophenyl derivatives of the amines. 2,4,DNP-NH_3 formed was first removed by diazotisation and coupling with chromotropic acid using the method of ref. [18] and the 2,4,-DNP-amines were measured spectrophotometrically at 350 nm.

pH. This was investigated using the Na nitroprusside assay for γ-coniceine. After incubation 3% sulphosalicylic acid was added (0.5 ml) and the protein removed by centrifugation. The acid solns were extracted with 2 × 1.5 ml ether to remove 5-keto-octanal. The aq. acid solns were basified and the γ-coinceine extracted with 2 × 1 ml $CHCl_3$. After washing with H_2O (3 × 1 ml) the $CHCl_3$ was extracted with (1 + 0.5 ml) 1% HCl. To this 1% HCl soln of γ-coniceine was added 10% $NaCO_3$ (0.5 ml) and Na nitroprusside (1 ml). The colour which developed was estimated at 490 nm after 4 min.

The preparation of substrates and authentification of reaction products. For the γ-coinceine assay these were as indicated in ref. [9] and for the DNP-amine assay as given in refs [1, 17, 18].

Acknowledgements—The author wishes to acknowledge the excellent technical assistance of Mr. G. Abbey in connection with the aliphatic amine assays.

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