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PARTIAL PURIFICATION AND PROPERTIES OF WILLARDIINE AND ISOWILLARDIINE SYNTHASE ACTIVITY FROM *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; willardiine; isowillardiine; uracilylalanines; pyrimidine amino acids; alanylation.

Abstract—The enzymic activity responsible for synthesis of willardiine and isowillardiine in pea seedlings has been extracted and partially purified. Fresh tissue, pulverized in liquid-N_2, was extracted in a phosphate buffer (pH 7) and subjected to fractional precipitation with ammonium sulphate. After desalting on Sephadex G-25 and concentration by ultrafiltration, the fraction containing the activity was chromatographed sequentially on DEAE-Sepharose CL-6B, DEAE-cellulose (DE 52) and Sephadex G-200. Electrophoretic separation in polyacrylamide gels was also used. A 120-fold purification was achieved but at no stage was there any indication of a separation of willardiine synthase activity from that of isowillardiine synthase. Both activities paralleled one another when the enzymic preparation was progressively denatured by subjecting it to gradually increasing temperatures. Similarly, ageing at 4° and at −196° resulted in a parallel loss of activity. Both synthase activities were maximal at 7.8–7.9 and the pH optimum curves were of closely similar shape. From the results described, it is concluded that a single enzyme of relatively low MW (*ca* 50 000) is responsible for the synthesis of both uracilylalanines. Studies of the alanylation of uracil using a pyridoxal-metal ion model-enzyme system are described.

INTRODUCTION

Two isomeric uracilylalanines of plant origin have been identified. The first of these, obtained from seeds of *Acacia willardiana*, was identified as β-(2,4-dihydroxypyrimidin-1-yl)alanine and named willardiine [1]. It was later found to occur in various other species of *Acacia* and in *Mimosa asperata* [2]. Later, the occurrence of a second uracilylalanine in plants was reported [2]. It was concurrently and independently characterized by Brown and Mangat [3] and Lambein and Van Parijs [4] as an isomer of willardiine, *viz.* β-(2,4-dihydroxypyrimidin-3-yl)alanine. Although the substance was itself unknown at the time, this structure had been earlier described as isowillardiine [5]. Its biosynthesis was investigated in this laboratory and it was shown to arise from a preformed uracil molecule by an enzyme-catalysed condensation with *O*-acetylserine [6]. The enzyme was found to require pyridoxal 5-phosphate for its activity [6]. Willardiine was shown to be formed by a similar mechanism [6]. Subsequent work demonstrated the occurrence of willardiine- and isowillardiine-synthesizing activity in *Lathyrus, Albizia, Leucaena* and *Fagus* [7]. These later studies also included preliminary observations on the properties of the enzyme systems involved. The work reported here concerns the partial purification and more detailed investigation of the enzyme mechanism involved in willardiine and isowillardiine synthesis and in particular sought to answer the question as to whether willardiine synthase and isowillardiine synthase are separate entities or a single enzyme.

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RESULTS

Partial purification of willardiine/isowillardiine synthase

A crude enzymic extract exhibiting willardiine and isowillardiine synthase activity was obtained from pea seedlings as described in the Experimental. This extract was fractionated by precipitation with ammonium sulphate; all the synthase activity precipitated in the 35–60% saturation fraction. Suspended in 3 M ammonium sulphate, the precipitated activity could be stored in a liquid N_2 Dewar for up to 8 weeks. During this time, there was about 30% loss of activity in respect of both willardiine and isowillardiine synthase activities. Storage beyond 8 weeks resulted in a rapid decline in both activities. For examination or assay of the activities obtained by fractional precipitation, the fraction was freed from ammonium sulphate by passage through a column of Sephadex G-25 and then concentrated by ultrafiltration under N_2 (for details, see Experimental).

In an attempt to separate willardiine and isowillardiine synthases, the crude extract was chromatographed on a column of DEAE-Sepharose CL-6B using a linear elution gradient of 5–200 mM phosphate buffer (pH 7.8). Details of the procedure are given in the Experimental. The elution diagram showed six protein peaks. The willardiine and isowillardiine synthase activity was confined to the last of these, eluting between 177–297 ml in the elution sequence. The fractions were pooled and concentrated as before. As can be seen from Table 1, this was a successful method in separating the synthase activities from other proteins but not in resolving the two synthase activities from one another. Also, there was a substantial fall in total activity recovered.

The possibility of further purification of the preparation by anion-exchange chromatography on a column

Table 1. Partial purification of willardiine and isowillardiine synthase(s)

Step	Synthase activities							
	Willardiine				Isowillardiine			
	Total activity (enzyme units)*	Specific activity units/mg protein	Fold purification	Recovery %	Total activity (enzyme units)*	Specific activity units/mg protein	Fold purification	Recovery %
$(NH_2)_4SO_4$ 36–60% satn. fraction†	986	8.3	1	100	8394	70.9	1	100
DEAE-Sepharose CL-6B	313	34.7	4.2	32	2774	308.3	4.3	33
DEAE-cellulose	107.9	38.6	4.7	11	1063	379.7	5.3	12.7
Sephadex G-200	75.5	1006	121.2	7.6	492	7563	106.7	5.9

*Enzyme units are dpm/min (incorporation of [2-^{14}C]uracil into willardiine or isowillardiine). Each assay incubation contained 1 μCi of [2-^{14}C]uracil (sp. act. 50 mCi/mmol).

†Desalted on Sephadex G-25.

of DEAE-cellulose was investigated using a linear elution gradient of 5–200 mM phosphate buffer (pH 7); details of the procedure are given in the Experimental. Monitoring at 280 nm, five peaks were seen in the elution sequence. One of these, eluting between 50 ml and 65 ml, contained all the willardiine and isowillardiine synthase activity. The fractions constituting this peak were pooled, and concentrated by ultrafiltration to 2 ml. Although a significant purification was achieved, the total recovery of both willardiine and isowillardiine synthase activity was down to 11–12% at the end of this step (Table 1).

Still aiming to resolve the willardiine and isowillardiine synthase activities from one another, fractionation of the crude enzyme preparation by gel-filtration chromatography on a column of Sephadex G-200 was examined. Isocratic elution with 5 mM phosphate buffer (pH 7.8) was used. Two main protein peaks were obtained but neither contained synthase activity. Fractions 66–74 ml, which eluted immediately after the first peak, were found to contain the enzymic activity but again there was no separation of willardiine synthase from isowillardiine synthase. Nevertheless, this step achieved a substantial increase in the overall purification of the combined synthase activities (Table 1).

The synthase fraction obtained from the DEAE-cellulose procedure was also examined by electrophoresis on polyacrylamide gel (3.5%). Samples (400 μl) containing 1.6 mg of protein were applied to the gels. After electrophoresis, as described in the Experimental, a series of slices (1 cm thick) were cut from the gel and eluted overnight in 0.5 ml portions of 5 mM phosphate buffer (pH 7.8) at 4°. Each eluate was separately assayed for protein content, and willardiine and isowillardiine synthase activities. All the enzymic activity was located in a single slice, representing a migration of 2–3 cm towards the cathode. Once again there was no indication of a separation of the two synthases from one another.

From the foregoing data, a scheme for the routine partial purification of the combined synthase activities was devised and is shown in Fig. 1. This scheme was used to obtain the combined activities for the further investigations described below.

Properties of the synthase preparation

Using the partially purified enzymic preparation, obtained by the procedure outlined in Fig. 1, further attempts were made to establish whether the willardiine and isowillardiine synthase activities are attributable to two separate enzymes or merely two facets of the catalytic activity of a single protein. Consideration was given first to the possibility that these activities are associated with different subunits of the same enzyme. This was tested by exposing the concentrated enzyme preparation to 8 M urea before and during gel-filtration chromatography on a column of Sephadex G-200. The column was eluted isocratically with 5 mM phosphate buffer (pH 7.8) to which had been added urea to a final concentration of 8 M. There was, however, no indication of the dissociation of the enzymic activity into separate catalytic subunits.

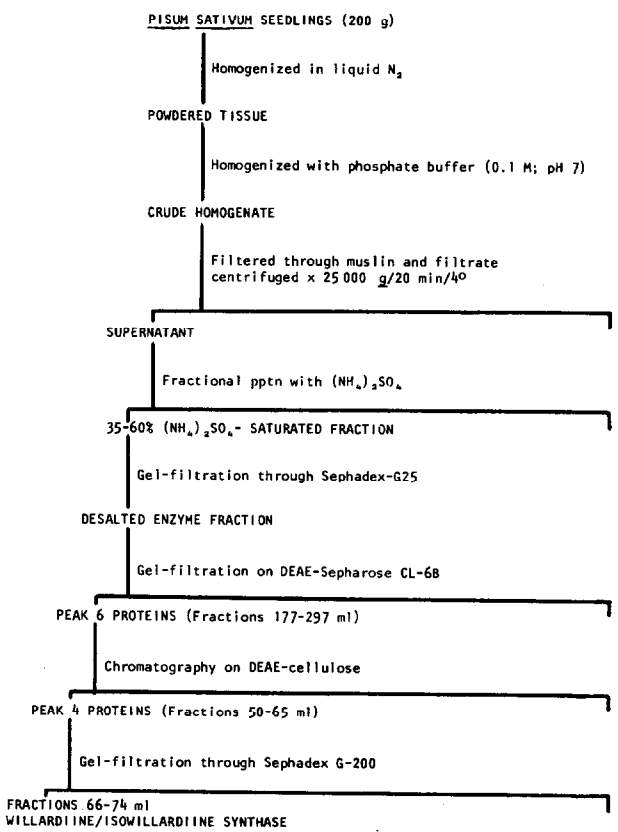

Fig. 1. Procedure adopted for the routine partial purification of willardiine–isowillardiine synthase.

Another approach to separating one synthase activity from the other was selective heat denaturation. Samples (0.5 ml) of the enzymic preparation were preincubated for 2 min at a range of temperatures from 4–90°. At the end of this 2 min preincubation, each sample was chilled in crushed ice for 5 min and then its willardiine and isowillardiine synthase activity was determined. The results showed a close similarity in behaviour between the two synthase activities with the ratio of willardiine to isowillardiine production remaining constant at 1 : 8 over the range of temperatures. Both synthase functions decreased slowly between 40 and 50° and rapidly between 50 and 60°; at 70° all activity had ceased. At no point was there any indication of one activity being more susceptible to heat denaturation than the other.

The effect of pH on the synthesis of both uracilylalanines was examined. A typical pH–enzymic activity curve was obtained, rising steeply from little detectable activity at pH 6.5 and as previously reported [7], attaining a maximum at pH 7.8–7.9. Above pH 8.0, a sharp fall was observed. However, Murakoshi *et al.* [7] have pointed out that it is not possible to assess synthase activity above pH 8.0 with any accuracy since under these conditions *O*-acetylserine undergoes a rapid *O*-acetyl to *N*-acetyl shift. In our study, the two synthase activities responded similarly to pH changes and showed a common pH optimum (pH 7.8–7.9). Tris–HCl buffers inhibited willardiine and isowillardiine formation equally (40% inhibition at pH 7.8). Under the conditions described, maximal synthase activity was obtained by incubating at 30°; temperatures above this resulted in significant reduction in activity. Similarly, extending incubation time beyond 90 min caused a rapid decline in activity probably due to accumulation of the end products willardiine and isowillardiine. These two compounds, at final concentrations of 30 mM, produced substantial inhibitions. With willardiine this amounted to 84% inhibition of willardiine synthesis and 96% inhibition of isowillardiine synthesis. Conversely, 30 mM isowillardiine produced 75% inhibition of willardiine synthesis and 93% inhibition of that of isowillardiine.

A variety of metal ions were examined for effect upon the synthase activity. At a final concentration of 50 μM, no significant effect was observed with Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Ga^{3+}, In^{3+}, MoO_4^-, or $B_4O_7^{2-}$. Similarly a variety of uracil analogues, at a final concentration of either 50 mM or 100 mM, were without effect on the enzymic activity. Among the compounds examined were uridine, thymine, cytosine, 3-methyluracil and 1,3-dimethyluracil. Strong inhibition (*ca* 90%) was, however, obtained with the thiol-antagonist *p*-chloromercuribenzoate at a final concentration of 1 μM; both synthase activities were affected to a similar extent. The inhibition was reversed by dithiothreitol at a final concentration of 1 mM.

Determination of the apparent MW of the willardiine/isowillardiine synthase was made using gel-filtration through Sephadex G-200. The results indicated an apparent MW of 50 000.

Preliminary kinetic experiments using Lineweaver–Burk plots gave a K_m of 10 mM uracil for willardiine synthesis and of 3.3 mM uracil for isowillardiine synthesis. In both cases excess *O*-acetylserine was present. These values are almost identical to those obtained earlier by Murakoshi *et al.* [7] with a crude synthase preparation. In the presence of excess *O*-acetylserine, the present study showed maximal rate of willardiine and isowillardine synthesis with a final concentration of uracil of 1 mM; higher concentrations (> 10 mM) cause marked inhibition. Examination of a wide range of structural analogues showed the synthases to have a specific substrate requirement for uracil and *O*-acetylserine. Amongst the compounds tested in this respect were all the common pyrimidine bases, ribosides and ribotides; 1-methyluracil, 3-methyluracil and 1,3-dimethyluracil; L-serine, 3-cyano-L-alanine, and *S*-methyl-L-cysteine. Whereas we confirmed earlier reports [6, 7] of the essential requirement by the synthase activities for pyridoxal 5-phosphate, concentrations of this coenzyme in excess of 0.1 mM were inhibitory. In the context of the specificity of the uracilylalanine synthase, further examination of a preparation of β-pyrazolyl-1-alanine synthase from cucumber seedlings confirmed the earlier finding [6] that whereas this latter enzyme could be demonstrated to catalyse the *N*-alanylation of pyrazole, it would not catalyse *N*-alanylation of uracil.

The effect of storage at 4° on the activity of the partially purified synthase preparation was examined. During the first 24 hr there was a 30% decline in the activity in respect of both willardiine and isowillardiine formation. This fell sharply during the next 24 hr until there was little activity left at the end of 48 hr. The rate of decay in activity for willardiine synthesis during storage at 4° closely paralleled that for isowillardiine synthesis. Storage in a liquid-N_2 Dewar vessel was more efficient than refrigeration at 4°. After 60 days, 68% of the original activity remained; again there was no difference in behaviour between the two synthase activities with both decaying at a parallel rate.

Alanylation of uracil by pyridoxal 'model enzyme' reactions

Metzler *et al.* [8] showed that pyridoxal-dependent enzyme reactions could be simulated by heating buffered solutions containing the substrate and the coenzyme in the presence of appropriate di- or trivalent metal ions. Amongst the reactions they studied was that between indole and serine to yield tryptophan. Subsequently, this reaction has been successfully used by others to produce heterocyclic 3-substituted alanines. Using this technique, Murakoshi *et al.* [7] demonstrated the synthesis of willardiine from uracil and serine (or *O*-acetylserine) in the presence of pyridoxal phosphate and gallium ions. However, they reported that formation of isowillardiine was negligible under their conditions. In the present study, these observations were extended in the hope that a better understanding of the non-enzymic model system would throw light upon the enzymic mechanism involved in the biosynthesis of the two isomeric uracilylalanines willardiine and isowillardiine. Using more vigorous conditions than those of ref. [7] (100°/30 min cf. 35°/2–3 hr) synthesis of willardiine was confirmed but in contrast to the earlier work at lower temperatures [7], significant amounts of isowillardiine were also shown to be formed. Of the Group IIIA elements examined for potential in catalysing these reactions at various pH, gallium ions were the most effective and indium ions were virtually devoid of activity; borate and aluminium ions were intermediate. The effects of varying pH of the reaction mixture are shown in Fig. 2. A bimodal curve was obtained for the synthesis of both willardiine and isowillardiine; maximal synthesis was at pH 8.

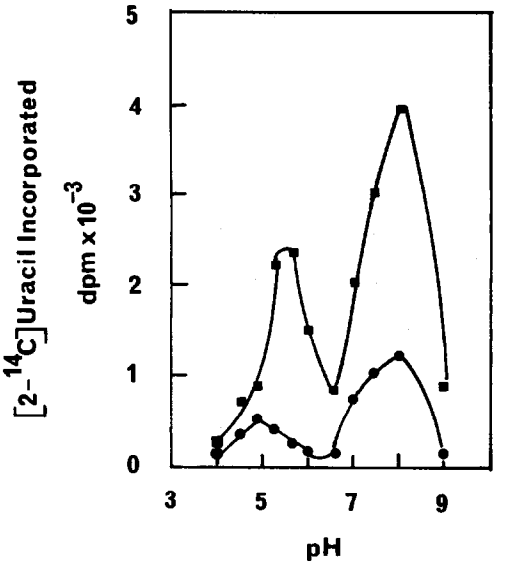

Fig. 2. Effect of pH on the non-enzymic synthesis of willardiine (■) and isowillardiine (●), from [2-^{14}C]uracil and *O*-acetylserine, by a pyridoxal model-enzyme system. The reaction mixture contained uracil (0.5 M), [2-^{14}C]uracil (1 μCi), *O*-acetylserine (60 mM), pyridoxal 5-phosphate (13 mM) and $Ga_2(SO_4)_3$ (5 mM), in 0.1 M K acetate buffer (pH 4–5.6) or 0.1 M Na/K phosphate buffer (pH 6.0–8.0). Tubes were kept at 100° for 30 min.

DISCUSSION

In the course of developing the procedure (Fig. 1) for partial purification of the uracilylalanine synthase activity, the latter was subjected to a variety of standard techniques for the resolution of mixtures of proteins. None of these purification steps gave any indication that willardiine and isowillardiine synthase activity is attributable to two separate enzymes. The possibility that the two alanylation reactions are ascribable to two discrete catalytic subunits of a single enzyme protein was also considered. However, after prolonged exposure of the enzymic activity to 8 M urea followed by gel-filtration chromatography in the presence of 8 M urea, no evidence was obtained for dissociation of the synthase into subunits. Attempts to eliminate one of the two enzymic activities by selective heat-denaturation were similarly ineffective. Both activities declined in parallel to one another as the preparation was exposed, before assay, to gradually increasing temperatures. The rate of denaturation during storage at 4° and at − 196° was also reflected equally in both synthase activities.

The pH optima studies gave results similar to those reported by Murakoshi *et al.* [7]. Both enzymic activities showed a maximum at pH 7.8–7.9 and the separate curves for willardiine and isowillardiine synthesis paralleled one another. Again, no indication was obtained from these results or from the kinetic data that two separate enzymes are involved in uracilylalanine synthesis. With respect to uracil, the K_m values for the synthase activity (10 mM for willardiine synthesis and 3.3 mM for isowillardiine synthesis) were similar to those reported earlier [7]. In the presence of excess *O*-acetylserine, rates of synthesis for both products were maximal with 1 mM uracil; marked substrate inhibition occurred with higher concentrations. It is of interest that in addition to this marked substrate inhibition, the synthesis of both uracilylalanines is subject to marked end-product inhibition. At 30 mM, both willardiine and isowillardiine caused 75–95% inhibition. Furthermore, although an essential coenzyme for this reaction, pyridoxal 5-phosphate at concentrations in excess of 0.1 mM were also inhibitory. This latter inhibition is probably due to competition for *O*-acetylserine between enzyme-bound pyridoxal phosphate and free coenzyme; under the prevailing experimental conditions, both the free and the bound forms of pyridoxal phosphate would readily condense with *O*-acetylserine to yield a Schiff's base.

From the results described, it is concluded that a single enzyme of relatively low MW (*ca* 50 000) is responsible for the synthesis of both willardiine and isowillardiine. This enzyme was shown to have a specific substrate requirement for uracil and *O*-acetylserine and could not be simulated by β-pyrazolylalanine synthase which catalyses the analogous *N*-alanylation of pyrazole.

Use of the pyridoxal–metal ion system [8] to model enzymic formation of the uracilylalanines worked best with gallium ions as previously reported by Murakoshi *et al.* [7]. However, in contrast to this earlier report, significant amounts of isowillardiine as well as willardiine were formed in our experiments (Fig. 2). Investigation of the effect of pH on this non-enzymic reaction yielded a bimodal curve (Fig. 2) showing that although pH 8 was optimal for both willardiine and isowillardiine synthesis, a significant amount of reactivity was exhibited at pH 5–6. However, unlike the enzymic system where the converse is true, willardiine was the predominant product under all conditions. The factors controlling the predominance of isowillardiine as a product of the enzymic alanylation of uracil, and the predominance of willardiine in the pyridoxal–metal ion system, remain to be elucidated. No clues in this respect are forthcoming from the organic chemistry of hydroxypyrimidines since the factors controlling orientation of the alkyl groups during the direct chemical methylation of uracil are equally obscure [9]. This, therefore, remains a problem for the future.

EXPERIMENTAL

Materials. Seedlings of *Pisum sativum* cv. Meteor were used throughout this work. Seeds were supplied by Sutton (Clause) U.K., Reading. They were surface-sterilized and germinated, and the seedlings grown, as previously described [6]. Where appropriate, routine chemicals were of analytical grade (Analar) purchased from BDH Ltd., Poole. Dithiothreitol, *p*-chloromercuribenzoic acid and pyridoxal 5-phosphate were obtained from Sigma London Chemical Co., Poole. Sephadex G-200, G-25 and DEAE-Sepharose CL-6B were from Pharmacia (GB) Ltd., Hounslow; DEAE-cellulose DE 52 (microgranular, preswollen) was from Whatman Ltd., Maidstone, Kent. $Ga_2(SO_4)_3$ was supplied by Fluorochem Ltd., Glossop; Polyclar AT and $In_2(SO_4)_3$ by BDH Ltd., Poole; and [2-^{14}C]uracil by Amersham International plc., Amersham.

Extraction of willardiine and isowillardiine synthase. A weighed sample (*ca* 200 g) of seedlings, from which the cotyledons had been removed, were dropped into a mortar containing liquid N_2 and immediately ground to a fine powder. As required, weighed portions of the powder were homogenized in an extraction medium consisting of cold phosphate buffer (0.1 M; pH 7) to which had been added Na ascorbate (final concentration 0.25 M)

and Polyclar AT (1.25 g/g fr. wt tissue). Before use, so as to ensure complete hydration of the Polyclar, the extraction medium was allowed to stand overnight, at 4° [10–13]. Coarse debris was removed from the homogenate by filtration through muslin. The filtrate was centrifuged at 25000 *g* for 20 min at 4° and the supernatant decanted for use as the 'crude enzyme preparation'.

Desalting and ultrafiltration procedures. Protein fractions, obtained from the crude enzyme preparation by fractional precipitation with $(NH_4)_2SO_4$, were desalted by passage, at 420 ml/hr, through a column (250 × 15 mm diameter) packed with Sephadex G-25. Where appropriate, protein preparations were concd by ultrafiltration. This was effected using an Amicon ultrafiltration cell fitted with a UM 10 membrane (Amicon Ltd., Gloucs.).

Anion-exchange and gel-filtration chromatography. Packings were prepared and columns were packed according to the manufacturers' recommendations. All columns were run at 4° and the A_{280} of eluates continuously monitored. Unless otherwise indicated, fractions (3 ml) were collected automatically.

The column used with DEAE-Sepharose CL-6B was 220 × 11 mm diameter. After equilibration, the sample (4 ml) was applied to the column and the column washed with 50 ml phosphate buffer (5 mM; pH 7.8). Elution was with a linear gradient of 5–200 mM phosphate buffer, pH 7.8; total volume 500 ml; flow rate 60 ml/hr. For anion-exchange chromatography on Whatman DE 52 DEAE-cellulose, pre-swollen microgranular material was used in a column 120 × 8 mm diameter. After equilibration, a 4 ml sample was applied and the column eluted with a linear gradient of 5–200 mM phosphate buffer, pH 7; total vol. of gradient 250 ml. A flow rate of 60 ml/hr was used and 5 ml fractions collected automatically.

For gel-filtration chromatography using Sephadex G-200, a column 470 × 8 mm diameter was used. A 2 ml sample was applied and the column eluted with 5 mM phosphate buffer, pH 7.8, at a flow rate of 16 ml/hr. Fractions (2 ml) were collected automatically. For determination of apparent MW, the method used was essentially that of Leach and O'Shea [14]. The following reference proteins were used: cytochrome *c* (12500), bovine serum trypsin (24000), bovine serum albumin (67000), rabbit muscle aldolase (158000), beef liver catalase (240000) and ferritin (450000). A sample (2.5 ml) of the partially purified enzyme preparation was loaded onto a Sephadex G-200 column (470 × 8 mm diameter). Elution was with Na_2HPO_4:KH_2PO_4 buffer (5 mM, pH 7.8) at a flow rate of 16 ml/hr; 2 ml fractions were collected. Plotting V/V_0 (where V = elution volume and V_0 = void volume) vs log MW of reference proteins a straight line was obtained from which the MW of the synthase was estimated by the method of least squares [15].

Polyacrylamide gel electrophoresis. With minor modifications, the method of Davies [16] was used. Samples containing 200 μg of protein per 10 μl were applied; a current of 2 mA/tube was used. Gels were subsequently sliced into 1 cm segments, each segment was separately soaked overnight at 4° in 0.5 ml of phosphate buffer (pH 7.8) and then gently homogenized in a Potter–Elvehjem homogenizer. Each homogenate was centrifuged at 11400 *g* for 20 min at 4° and the supernatant taken for synthase assay. Protein concns were determined by the method of Lowry *et al.* [17]. Staining of protein on gels was effected with Coomasie Brilliant Blue R250 [18] followed by electrophoretic destaining.

Measurement of willardiine and isowillardiine synthase activity. The activity of the willardiine and isowillardiine synthase was assayed by determining the amount of radioactivity incorporated from [2-^{14}C]uracil into the two products. Incubations were at 30° for 90 min and the incubation mixture (final vol. 1 ml) contained 1 mM uracil, 1 μCi [2-^{14}C]uracil (sp. act. 50 mCi/mmol), 1 mM *O*-acetylserine, 0.1 mM pyridoxal 5-phosphate, 0.5 ml of enzyme preparation and 0.1 ml of 0.1 M phosphate buffer (pH 7.8). Where necessary, the final vol. of the incubate was adjusted to 1 ml with water. The reaction was terminated by adding 3 vols of spectroscopically pure ethanol. Precipitated protein was removed by centrifuging at 11500 *g* at 4°.

Willardiine and isowillardiine formation was measured by determining the amount of radioactivity incorporated into these amino acids. Routinely, a 2 ml sample of the aq. EtOH supernatant was chromatographed on Whatman 1 MM paper in butan-1-ol–HOAc–H_2O (12:3:5). The radioactive bands of willardiine and isowillardiine were detected using a radiochromatogram spark chamber (Birchover Instruments Ltd., Letchworth). These bands separated well from traces of other radioactive products and from unmetabolized [2-^{14}C]uracil. After eluting the radioactive willardiine and isowillardiine bands in 10 mM HCl, they were separately subjected to HV-electrophoresis on Whatman 3 MM paper. For this latter separation, a HCO_2H–HOAc buffer (pH 2) was used [19] in conjunction with an electrode potential of 3 kV (60 V/cm) for 1 hr. Again the bands were located by their radioactivity and eluted in 10 mM HCl. The radioactivity of these eluates was measured by scintillation spectrometry using a dioxane-based scintillant containing 2,5-diphenyloxazole, 1,4-bis(5-phenyloxazol-2-yl)benzene and naphthalene (NE250; Nuclear Enterprises Ltd., Edinburgh).

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