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THE ENZYMIC SYNTHESIS OF β-SUBSTITUTED ALANINES

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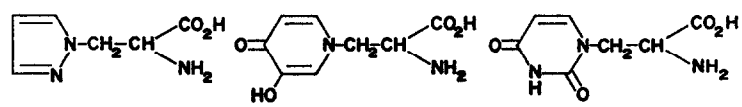
Abstract—An extract of *Leucaena* seedlings has been shown to catalyse an enzymic synthesis of mimosine from 3,4-dihydroxypyridine and *O*-acetylserine. Neither serine nor α,β-diaminopropionic acid served as direct substrates in the enzymic reaction A similar enzymic synthesis of β-pyrazol-1-ylalanine in watermelon seedling extracts was shown to be dependent upon pyrazole and *O*-acetylserine, and not serine. Some properties of the enzymes are described

INTRODUCTION

PLANTS produce a number of amino acids that may be regarded as heterocyclic β-substituted alanines. Tryptophan and histidine can be considered as members of this group, but more examples are found among the 'non-protein' amino acids synthesized in a more restricted manner by plants. β-Pyrazol-1-ylalanine (Ia, characteristic of many species from the family Cucurbitaceae), mimosine (Ib, *Mimosa* and *Leucaena* spp), willardiine (Ic, *Acacia willardiana*), lathyrine (Id, *Lathyrus* spp), and stizolobic acid (Ie, *Stizolobium hassjo*) are plant products that illustrate a range of heterocyclic ring structures (see Fowden[1] for a review of their chemistry and distribution)

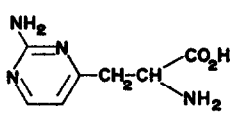
The common identity of these compounds as β-substituted alanines naturally leads to the idea that similar biogenetic pathways may be responsible for their formation in plants. Evidence so far available indicates that the C_3 side chain may originate from serine in a number of instances Certainly, serine provides the side chain required in tryptophan biosynthesis, whilst labelled precursor feeding experiments suggest that serine is rapidly



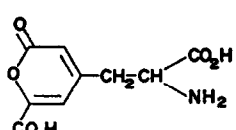
(Ia) β-Pyrazol-1-ylalanine

(Ib) Mimosine

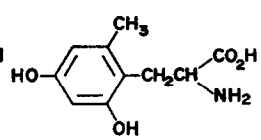
(Ic) Willardiine



(Id) Lathyrine



(Ie) Stizolobic acid



(If) Orcylalanine

[1] L FOWDEN, in *Progress in Phytochemistry* (edited by L REINHOLD and Y LIWSCHITZ), Vol 2, p 203, Interscience, London (1970)

incorporated into β-pyrazol-1-ylalanine by various cucurbit seedlings,[2] into mimosine in young plants of *Mimosa pudica*,[3] and into orcylalanine (If) in *Agrostemma githago*.[4] The enzymic synthesis of β-pyrazol-1-ylalanine from pyrazole and serine was shown to occur in crude extracts of cucumber seedlings.[5]

Quite recently, some revision of ideas concerning the role of serine in the biosynthesis of other amino acids, such as cysteine and *S*-methylcysteine, has occurred. It is now clearly established at the enzymic level that *O*-acetylserine, and not serine itself, is the substrate in reactions yielding these two sulphur-containing amino acids.[6,7] The demonstration of a serine transacetylase in plants by Smith and Thompson[8] has provided the necessary enzymic link between serine and the required *O*-acetyl derivative. On the basis of this new information, we have examined the possibility that certain of the β-substituted alanines are enzymically synthesized by condensation of an appropriate heterocyclic precursor with *O*-acetylserine. This paper presents a preliminary account of condensing enzymes present in seedlings of *Leucaena leucocephala* and watermelon that catalyse the synthesis of mimosine and β-pyrazol-1-ylalanine from dihydroxypyridine and pyrazole, respectively, and *O*-acetylserine.

RESULTS

The enzyme preparations used in the investigation were protein-containing extracts of seedlings, from which low molecular weight substances, i.e. substrates and coenzymes, had been removed by treatment with Sephadex G-25. The course of the enzymically-catalysed reactions was followed by determining the amount of radioactivity, from ^{14}C-labelled *O*-acetylserine provided as substrate, introduced into mimosine or β-pyrazol-1-ylalanine, respectively. The reaction products were separated from other components of the reaction mixtures either by paper chromatographic techniques, or by use of an automated amino acid analyser.

Enzymic Synthesis of Mimosine

The reaction now demonstrated to be responsible for mimosine biosynthesis is shown in equation (i).

Figure 1 established the presence of mimosine in a reaction mixture (vol. 0·4 ml) containing 3,4-dihydroxypyridine (10 μmoles), ^{14}C-*O*-acetylserine (5 μmoles, 0·5 μc) and a *Leucaena* enzyme preparation after a 30-min incubation period at 30°. The figure illustrates the profile of elution of amino acids from the analyser, together with an associated scan of radioactivity. Coincidence is clearly seen between peaks of ninhydrin-positive material and radioactivity eluting at a position (relative to an internal standard) corresponding with that of an authentic sample of mimosine. About 20% of the radioactivity supplied as ^{14}C-*O*-

[2] D. M. FRISCH, P. M. DUNNILL, A. SMITH and L. FOWDEN, *Phytochem* **6**, 921 (1967).
[3] H. P. TIWARI, W. R. PENROSE and I. D. SPENSER, *Phytochem* **6**, 1245 (1967).
[4] L. A. HADWIGER, H. G. FLOSS, J. R. STOKER and E. E. CONN, *Phytochem* **4**, 825 (1965).
[5] P. M. DUNNILL and L. FOWDEN, *J. Exptl. Bot.* **14**, 237 (1963).
[6] J. GIOVANELLI and S. H. MUDD, *Biochem. Biophys. Res. Comm.* **27**, 150 (1967).
[7] J. GIOVANELLI and S. H. MUDD, *Biochem. Biophys. Res. Comm.* **31**, 275 (1968).
[8] I. K. SMITH and J. F. THOMPSON, *Biochem. Biophys. Acta* **227**, 288 (1971).

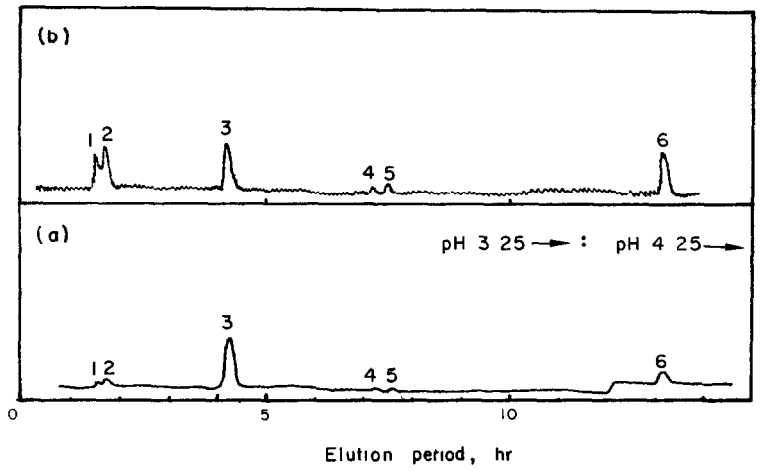


FIG 1 ILLUSTRATION OF THE PROFILE OF (a) AMINO ACIDS and (b) ASSOCIATED RADIOACTIVITY ELUTED FROM THE 150 cm COLUMN OF THE ANALYSER (SEE TEXT) AFTER APPLICATION OF A 30 min SAMPLE OF THE COMPLETE REACTION MIXTURE (WITH *O*-ACETYL-SERINE-3-^{14}C)

The peaks represent (1) and (2), unidentified compounds, (3) *O*-acetylserine, (4) glycine, (5) alanine and (6) mimosine

acetylserine was incorporated into mimosine in this experiment The scan of radioactivity showed that some ^{14}C still remained in *O*-acetylserine, but activity was also present in serine (and probably glycine) and at least two unidentified compounds A time-course study indicated that radioactivity incorporated into mimosine reached a maximum value between 15 and 30 min; presumably, ^{14}C-*O*-acetylserine became limiting after this time, whilst other enzymes in the incubation mixture, such as the mimosine C—N-lyase described earlier by Smith and Fowden,[9] gradually degraded the mimosine initially produced.

During short incubation periods, labelled mimosine was formed most rapidly at pH 7·9–8·0 Synthesis was not dependent upon added pyridoxal phosphate Mimosine was not found when 3,4-dihydroxypyridine was omitted from the reaction mixture, or when serine or *O*-phosphoserine replaced *O*-acetylserine. Use of a boiled *Leucaena* extract confirmed that chemical formation of mimosine did not occur from the substrates under the normal conditions of the reaction

Enzymic Synthesis of β-Pyrazol-1-ylalanine

In all experiments, ^{14}C-*O*-acetylserine was employed as substrate to allow β-pyrazol-1-ylalanine formation to be sensitively determined An early and crucial experiment established that the formation of β-pyrazol-1-ylalanine by a Sephadex-treated extract of watermelon seedlings was dependent upon *O*-acetylserine and pyrazole, suggesting the operation of equation (ii)

[9] I K SMITH and L FOWDEN, *J Exptl Bot* **17**, 750 (1966)

Neither serine nor *O*-phosphoserine could replace *O*-acetylserine as a substrate, similarly, no labelled β-pyrazol-1-ylalanine was formed when pyrazole was omitted from reaction mixtures.

The usual reaction mixture contained pyrazole (100 μmoles), *O*-acetylserine-3-^{14}C (5 μmoles, 0·6 μc) and enzyme (0·2 ml) in a final volume of 0·4 ml, pH 7 3 This pH represents a fairly sharp optimum for the condensation reaction When such mixtures were incubated at 30°, β-pyrazol-1-ylalanine production was linear for about 1 hr, when approximately 1·5 μmoles of β-pyrazol-1-ylalanine had been formed, i e 30% of the ^{14}C-*O*-acetylserine had been utilized Maximum rates of β-pyrazol-1-ylalanine synthesis were obtained with 100–150 μmoles pyrazole higher concentrations of pyrazole were less favourable for β-pyrazol-1-ylalanine synthesis Formation of β-pyrazol-1-ylalanine occurred from pyrazole and serine (5 μmoles) in the presence of the treated watermelon extract if acetyl CoA (5 μmoles) were also added, but the rate of reaction was only about 2% of that observed with pyrazole and *O*-acetylserine under similar conditions

The addition of pyridoxal phosphate to reaction mixtures caused neither stimulation nor inhibition of β-pyrazol-1-ylalanine formation However, when 3,4-dihydroxypyridine (50 μmoles) was added, β-pyrazol-1-ylalanine formation was reduced by about 40%, whilst a small amount of mimosine was formed therefore dihydroxypyridine may represent an alternative substrate for the condensing enzyme The enzyme exhibited reasonable stability when stored at 0° and, after 22 hr, exhibited about 75% of the activity associated with a freshly-prepared extract

DISCUSSION

Biosynthesis of mimosine and β-pyrazol-1-ylalanine by condensation reactions in which *O*-acetylserine provides the alanyl side-chain extend, to structurally more complex molecules, a type of reaction already established for the synthesis of cysteine and *S*-methylcysteine The necessary heterocyclic substrates have been identified earlier as constituents of the implicated plants, i e 3,4-dihydroxypyridine in *Leucaena*,[10] and pyrazole in cucurbits,[11] and an enzyme acetylating serine to yield *O*-acetylserine has been described in *Phaseolus vulgaris* [8] This enzyme is likely to be present universally in plants, with the role of activating the serine molecules necessary to provide the three-carbon chain of cysteine Presumably, such an acetyltransferase, together with acetyl CoA and the condensing enzyme now demonstrated, was present in the crude extract of cucumber seedlings previously used to demonstrate the enzymic synthesis of β-pyrazol-1-ylalanine from pyrazole and ^{14}C-serine [5] A number of the properties established for the condensing enzyme are closely similar to those that governed the synthesis of β-pyrazole-1-ylalanine in the earlier work for example, both systems had pH optima of 7 2–7·3, and synthesis was restricted by supra-optimal concentrations of pyrazole in both cases

Mimosine and β-pyrazol-1-ylalanine formation represent examples of biosyntheses in which an activated *O*-acetylserine molecule provides a three-carbon fragment required in the final product As in other reactions of this type, an initial labilization of the proton at the α-carbon atom probably occurs, coupled with elimination of the β-substituent (here acetate) and the formation of an enzyme-bound α-aminoacrylate moiety The enzyme-bound moiety then may be attached to various acceptor molecules, e g. 3,4-dihydroxypyridine or pyrazole, whose nature is defined by the specificity of the particular condensing enzyme

[10] M P HEGARTY, R D COURT and P M THORNE, *Austral J Agric Res* **15**, 168 (1964)
[11] F F. NOE and L FOWDEN, *Biochem J* **77**, 543 (1960)

Reactions of this type conceivably might be implicated in the biosynthesis of certain other non-protein amino acids, e g. willardiine from uracil and *O*-acetylserine, orcylalanine from orcinol (or orsellinic acid with a coupled decarboxylation[4]) and *O*-acetylserine, α,β-diamino-propionic acid from ammonia and *O*-acetylserine, and albizziine (α-amino-β-ureidopropionic acid) from urea and *O*-acetylserine However, preliminary tests made with Sephadex-treated extracts of appropriate seedlings (*Acacia dealbata*, *Agrostemma githago* and *L leucocephala*) have not given positive evidence for an intermediary role of ^{14}C-*O*-acetylserine in these instances In this context, young seedlings may not represent an ideal system for biosynthetic study since there are indications that the levels of certain of these non-protein amino acids decrease rapidly during the early stages of seed germination and growth Future experiments will have as their aim the purification of the enzymes catalysing mimosine and β-pyrazol-1-ylalanine formation from *O*-acetylserine, and the search for other enzymes catalysing reactions of this type in maturing, rather than germinating seeds.

EXPERIMENTAL

Labelled substrates *O*-Acetyl-L-serine-3-^{14}C was synthesized in our laboratory from L-serine-3-^{14}C purchased commercially The product was diluted with unlabelled *O*-acetyl-L-serine to give material of specific activity used in the different incubation mixtures

Plant materials L leucocephala seedlings had been grown in moistened vermiculite in the dark for 4 days at 30° and watermelon seedlings for 3 days at 30°. After harvest, the testas were removed and then the seedlings were cooled at 0° for 15–30 min before extraction

Enzyme preparations and reaction mixtures (a) *Leucaena* extracts for mimosine biosynthesis All operations were carried out at about 0° Seedlings were macerated in 0 1 M potassium phosphate buffer, pH 8 0 (1 ml/4 g seedlings) After expressing through fine muslin, the extract was centrifuged at 25,000 *g* for 20 min to obtain a clear supernatant The supernatant was applied to a column of Sephadex G-25 (fine) to obtain a protein-containing solution free from low mol wt substances 0 1 M phosphate buffer, of appropriate pH, was used for elution of the protein (enzyme) fraction The normal reaction mixture contained 3,4-dihydroxy-pyridine (10 μmoles), *O*-acetyl-L-serine or *O*-acetyl-L-serine-3-^{14}C (5 μmoles, 0 5 μc) and 0 2 ml enzyme preparation in a final volume of 0 4 ml (normally maintained at pH 7 7 by 0 1 M potassium phosphate buffer) Incubation was at 30° and reaction was stopped by addition of EtOH (3 vol) Precipitated protein was removed by centrifuging, and the residual supernatant was examined chromatographically for the presence of mimosine Occasionally, *O*-acetylserine was replaced by serine, *O*-phosphoserine, or α-β-diamino-propionic acid Pyridoxal phosphate (10–100 μg/ml) was added to certain reaction mixtures

(b) *Watermelon seedling* extracts for β-pyrazol-l-ylalanine biosynthesis Seedlings were macerated in 0 2 M potassium phosphate buffer, pH 7 5, containing 0 1% 2-mercaptoethanol (1 ml/2 g seedlings) The extract was treated essentially as above, the final enzyme protein fraction being eluted from Sephadex by 0 1 M potassium phosphate buffer, pH 7 3 Reaction mixtures normally contained pyrazole (100 μmoles), *O*-acetyl-L-serine or *O*-acetyl-L-serine-3-^{14}C (5 μmoles, 0 6 μc) and enzyme (0 2 ml) in a final volume of 0 4 ml, buffered at pH 7 3 by 0 1 M potassium phosphate In some mixtures, the amount of pyrazole was varied in the range 20–250 μmoles, whilst in others *O*-acetylserine was replaced by a mixture of serine (5 μmoles) and acetyl CoA (5 μmoles) Other specific alterations in the composition of the reaction mixtures are detailed in the Results Mixtures were incubated at 30° for appropriate periods and reaction was terminated by adding EtOH (3 vol) to precipitate protein β-Pyrazol-l-ylalanine present in the clear supernatant was determined chromatographically as below

Assay of mimosine and β-pyrazol-l-ylalanine formation Mimosine formation could be detected by paper chromatographic procedures, using ninhydrin or ferric chloride as chromogenic reagents it was shown to co-chromatograph with authentic material in the following solvent systems. (1) butan-l-ol–HOAc–H_2O (90 10 29, by vol), (2) phenol–EtOH–H_2O (3 1·1, by wt), (3) butan-l-ol-EtOH–H_2O (2 2 1, by vol), and (4) butan-l-ol-pyridine–H_2O (1 1 1, by vol) R_fs for mimosine determined for these solvents were 0 08, 0 14, 0 05 and 0 12 respectively, whilst *O*-acetylserine had the following R_fs 0 17, 0 47, 0 27 and 0 45, respectively Under the same conditions, serine moved at R_fs 0 08, 0 16, 0 14 and 0 27, so mimosine formation was established most conclusively by using solvents 3 and 4 Further confirmation of the identity of the reaction product as mimosine was obtained using an automatic amino acid analyser (Shibata model AA-500, Tokyo) Under standard operating conditions (150 cm column, 50°, 0 2 N sodium citrate buffers, pH 3 25 followed by 4 25, flow rate 0 5 ml/min), mimosine eluted from the column at about 13 2 hr, i e at a position close to an isoleucine reference peak Radioactivity associated with each amino acid peak was recorded by employing a Packard monitoring flow system, model 3002, coupled to ratemeter, model 282A For routine

radiochemical assay of mimosine formation, the amino acid analyser was employed with a smaller column (50 cm, 50°, 0 2 N sodium citrate buffer pH 4 25, 0 5 ml/min), which allowed rapid, quantitative determinations to be made (the mimosine peak now eluted at 108 min)

β-Pyrazol-l-ylalanine was eluted from the amino acid analyser under the standard conditions (see above 150 cm column procedure) at a position between peaks due to threonine and serine This nearness of β-pyrazol-l-ylalanine and serine peaks might jeopardize the accurate determination of radioactivity associated with β-pyrazol-l-ylalanine, since ^{14}C conceivably could enter serine from *O*-acetyl-serine-3-^{14}C However, β-pyrazol-l-ylalanine was well separated from *O*-acetyl-serine (and serine) when paper chromatograms were developed in solvent 1 above Radioactivity associated with each ninhydrin-positive substance on the chromatograms was determined using a gas-flow 4π radiochromatogram scanner (Aloka, Tokyo, model no PCS-2B) Quantitative determinations of β-pyrazol-l-ylalanine produced were also made using the cadmium-ninhydrin reagent and general method described by Atfield and Morris [12]

Acknowledgement—Our thanks are due to Miss Y Kunimoto for technical assistance in relation to quantitative determinations of amino acids

[12] G N ATFIELD and C J O R MORRIS, *Biochem J* **81,** 606 (1961)

Key Word Index—Leucaena leucocephala, Leguminosae, β-substituted alanines, mimosine, β-pyrazol-1-ylalanine, enzymic synthesis