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THE SPECIFICITY OF AMINO ACID BIOSYNTHESIS IN THE CUCURBITACEAE

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Abstract—β-Pyrazol-l-ylalanine (βPA) is normally synthesized only by some members of the family Cucurbitaceae, the ultimate biosynthetic step being an enzymic condensation of pyrazole with serine. However, other plants and micro-organisms synthesize βPA when given pyrazole. Therefore the control of heterocyclic amino acid biosynthesis lies at the level of pyrazole synthesis, and the final condensation step is apparently catalyzed non-specifically by an enzyme present in plants of other families, as well as in cucurbits. The synthesis of a variety of other heterocyclic β-substituted alanines is probably catalyzed by the same enzyme. The formation of N^4-substituted asparagines by *Ecballium* and *Bryonia* seedlings occurs by a transferase reaction between asparagine and the appropriate primary amine. The specificity of the enzyme in relation to the amide and amine substrates was investigated.

INTRODUCTION

MEMBERS of the family Cucurbitaceae produce a number of non-protein amino acids and a distribution study of some of these compounds has indicated that they might provide supplementary chemotaxonomic criteria of value in instances where doubt remains concerning the assignment of species to particular genera or sub-tribes.[1] Several of these amino acids seem to be restricted to this plant family; these include β-pyrazol-1-ylalanine (Ia, βPA), its γ-glutamyl peptide, and N^4-ethyl-(IIa), N^4-hydroxyethyl-(IIb), and N^4-methyl-asparagine (IIc). βPA is produced by about half the cucurbit species examined earlier[1] and, when present, it is invariably accompanied by the peptide. The N^4-substituted asparagines show a much more restricted distribution; the ethyl and hydroxyethyl derivatives were recorded only in the general *Bryonia* and *Ecballium*, while the methyl derivative was present in certain *Kedrostis* and *Corallocarpus* species. A range of structurally-related, substituted glutamines occur in isolated members of other plant families: for instance, N^5-ethylglutamine (theanine) has been isolated from tea plants[2] and the mushroom *Xerocomos badius*,[3] and evidence recently has been provided that N^5-methylglutamine also occurs in tea.[4] In addition, N^5-isopropylglutamine has been obtained from *Lunaria annua*[5] while a series of aromatically-substituted glutamines occur in various species of *Agaricus* (see Ref. 6, for review).

Now the reasons underlying the restricted nature of the distribution of βPA and the substituted asparagines have been sought at the level of the biosynthetic enzymes. Earlier, the last step in βPA formation was shown to involve an enzymic condensation of pyrazole with serine[7] and the resemblance to one of biogenetic pathways leading to tryptophan was

[1] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 933 (1965).
[2] Y. SAKATO, *J. Agr. Chem. Soc. Japan* **23**, 262 (1950).
[3] J. CASIMIR, J. JADOT and M. RENARD, *Biochim. Biophys. Acta* **39**, 462 (1960).
[4] S. KONISHI and E. TAKAHASHI, *Plant Cell Physiol. (Tokyo)* **7**, 171 (1966).
[5] P. O. LARSEN, *Acta Chem. Scand.* **19**, 1071 (1965).
[6] L. FOWDEN, *Ann Rev. Biochem.* **33**, 173 (1964).
[7] P. M. DUNNILL and L. FOWDEN, *J. Exptl Botany* **14**, 237 (1963).

noted. However, this previous work did not indicate whether cucurbit plants producing βPA, in contrast to non-producers, possess an enzyme whose specific role is the catalysis of this final condensation or whether control of βPA biosynthesis is exerted at the earlier stage of pyrazole production. If only a limited range of species possess the ability to produce pyrazole, then βPA may be formed in these same species by the non-specific action of an enzyme whose normal function lies in basic intermediary metabolism. In establishing the correctness of this second possibility, the present experiments also have provided information concerning the range of heterocyclic substrates utilized by the enzyme.

There is no previous work concerned with the enzymic mechanism of N^4-substituted asparagine biosynthesis. Theanine is formed by a synthetase-type reaction in which glutamic acid and ethylamine are substrates in an ATP-requiring process[8] (cf. the synthesis of glutamine from glutamic acid, ammonia and ATP). In contrast, extracts of *Agaricus* catalyzed the formation of aromatically-substituted glutamines by a transferase-type reaction in which the amido-NH_2 group of glutamine is exchanged for a residue of the appropriate amino group-containing substrate.[9] After establishing that the N^4-substituted asparagines were synthesized by a transferase-type process, the amine substrate specificity of the enzyme was investigated.

RESULTS AND DISCUSSION

Studies Related to β-Pyrazol-1-ylalanine Synthesis

The view that some cucurbit species fail to elaborate βPA because they do not form the required precursor pyrazole was confirmed in experiments in which pyrazole was supplied to seedlings not normally containing βPA. Generally seeds were allowed to imbibe a 0·125% pyrazole solution for 24 hr at 25° and then the seedlings were grown for a further 2 or 3 days in moistened vermiculite. The species of the Cucurbitaceae used initially were *Cucurbita maxima*, *Luffa cylindrica* and *Sicyos angulatus*, which represent three different tribes of the family.[10] When the soluble amino acid pools of the seedlings were examined by routine two-dimensional chromatographic methods,[1] βPA was shown to be present in the extracts of each seedling at concentrations (100–200 μg/g fresh wt.) comparable to those encountered in βPA-producing species such as *Cucumis sativus* (cucumber).

The idea that an enzyme normally functioning in some basic cellular process was responsible for a non-specific conversion of pyrazole into βPA was further supported by showing that other species, such as *Phaseolus aureus* (a legume), *Chlorella vulgaris*, *Saccharomyces cerevisiae* and *Escherichia coli*, were all able to synthesize βPA if given pyrazole.

These findings are reminiscent of those of Massini,[11] who showed that a proportion of the herbicide amitrole (3-amino-1,2,4-triazole) absorbed by tomato plants was converted into 3-aminotriazol-1-ylalanine (Ib). When amitrole was supplied to cucumber or mung bean seedlings under the conditions described above using pyrazole, a compound chromatographically identical with aminotriazolylalanine was formed. A common feature of the biosynthesis of βPA and aminotriazolylalanine is the displacement of the only ring N-proton by an alanine residue, and it seems probable that both reactions are catalyzed by the same enzyme.

The possibility that a wider range of heterocyclic nitrogen compounds possessing an > NH

[8] K. SASAOKA, M. KITO and Y. ONISHI, *Japan J. Agr. Biol. Chem.* **29**, 984 (1965).
[9] H. J. GIGLIOTTI and B. LEVENBERG, *J. Biol. Chem.* **239**, 2274 (1964).
[10] C. JEFFREY, *Kew Bulletin* **15**, 337 (1961); *Kew Bulletin* **17**, 473 (1964).
[11] P. MASSINI, *Acta Botan. Neerl.* **12**, 64 (1963).

group may form similar β-substituted alanines was tested using seedlings, first of cucumber but subsequently of mung beans. Each heterocyclic compound listed in Table 1 was imbibed by mung bean seeds as a 0·125% solution (cf. above) and, after growth for 3 days, extracts of seedlings were examined chromatographically for the presence of additional amino acids. 4-Nitropyrazole and 1,2,4-triazole represented particularly good substrates leading to the production of amino acid Ic and Id respectively; 3-aminopyrazole, 5-amino-1,2,3,4-tetrazole and 1,2,4-triazol-3-ylalanine were converted less readily to their corresponding alanine derivatives (presumed structures Ie, If_1 or If_2, and Ig respectively). The relative amounts of the amino acids synthesized from the various heterocyclic substrates by mung bean seedlings were compared by measuring the incorporation of radioactivity into each from [3-^{14}C] serine, supplied together with the heterocyclics, and the values are reported in Table 1. [3-^{14}C] Serine, rather than [U-^{14}C] serine, was chosen so that the distribution of radioactivity over a large number of compounds would be minimized; in fact ^{14}C-label became concentrated in

TABLE 1. GROWTH INHIBITION AND AMINO ACID FORMATION IN *Phaseolus aureus* SEEDLINGS SUPPLIED VARIOUS HETEROCYCLIC NITROGEN COMPOUNDS AND ^{14}C-SERINE

Compounds converted to amino acids			Compounds not converted to amino acids	
Compound*	Growth inhibition	Amino acid formed†	Compound	Growth inhibition
Pyrazole	Stimulatory	100	Adenine	None
4-Nitropyrazole	Stimulatory	136	Adenosine	None
3-Aminopyrazole	Slight	n.d.	3,4-Dihydroxypyridine	Strong
1,2,4-Triazole	None	105	Orcinol	Stimulatory
3-Amino-1,2,4-triazole	Strong	38	Piperidine	Moderate
1,2,4-Triazol-3-ylalanine	Slight	n.d.	Uracil	None
5-Amino-1,2,3,4-tetrazole	Slight	40	Pyrimidine	None
Purine	Complete	n.d.	Pyrrole	Slight
Indole	Moderate	33	Pyrrolidine	Slight

* Seeds imbibed 0·125% solutions of the heterocyclic compounds containing 1 μc serine, see text.

† When ^{14}C-serine was supplied together with pyrazole, about 20% of the radioactivity became incorporated into βPA. The incorporation of serine-^{14}C into other heterocyclic amino acids is reported as a percentage of that entering βPA.

n.d. represents no quantitative determination.

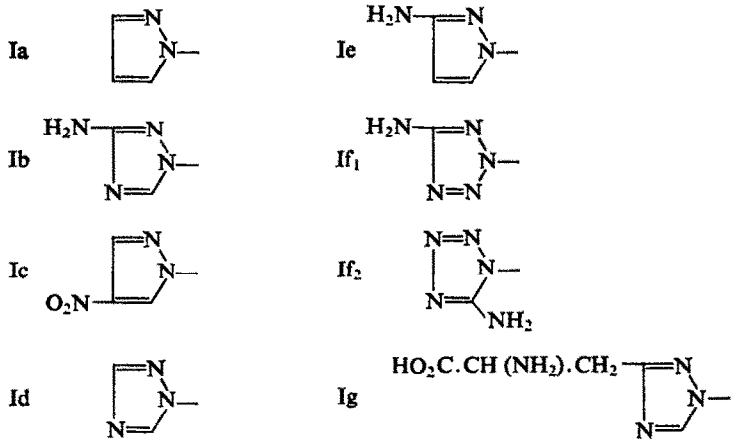
the particular heterocyclic amino acid under investigation and little radioactivity was present in other compounds forming the free amino acid pool of the seedlings.

No relationship existed between the growth inhibitory activity of the heterocyclic compound and the extent to which it was converted to an alanine derivative. For instance, although purine caused the highest degree of inhibition among the compounds tested, only slight formation of a new amino acid was observed (in fact two faint ninhydrin-positive, radioactive spots were formed by seedlings supplied purine plus ^{14}C-serine and these may represent isomeric compounds having the alanine residue attached at N-7 and N-9 respectively.)

In a single experiment using a cucumber seedling extract buffered at pH 6·7, the *in vitro* condensation reaction between ^{14}C-serine and a number of heterocyclic nitrogen compounds was observed over a 3-hr period. Pyrazole, 3-nitropyrazole, triazole, 3-aminotriazole and 5-aminotetrazole yielded small quantities of the corresponding ^{14}C-labelled β-substituted alanines; the relative amounts of radioactivity incorporated into each product followed a pattern similar to that established in the *in vivo* feeding experiments summarized in Table 1.

$$R.CH_2.CH(NH_2).CO_2H.$$
(I)

R is represented by:



STRUCTURE I.

$$R^1.NH.OC.CH_2.CH(NH_2).CO_2H.$$

R^1 is represented by:

IIa = $CH_3.CH_2$—
IIb = $HO.CH_2.CH_2$—
IIc = CH_3—

STRUCTURE II.

Pyrazole was shown[7] to undergo chemical condensation with serine under certain conditions which included an optimum pH of about 4·7 and an optimum temperature of approximately 60°. Similar chemical reactions between other heterocyclic compounds and serine have now been observed, and optimum pH values in the range 4·6–5·4 were determined for 4-nitropyrazole, 3-aminopyrazole and 5-aminotetrazole. These chemical reactions appear to proceed too slowly at 25° to account for the *in vivo* synthesis of the β-substituted alanines. They are also pyridoxal phosphate dependent, requiring a concentration greatly in excess of its normal intra-cellular level. For example, in a mixture containing 8 mg aminotetrazole, 4 mg serine and 1 mg aluminium sulphate per ml, aminotetrazolylalanine production was only detected during 20 hr at 60° when pyridoxal phosphate was present in quantities greater than 1 mg. Under these circumstances, two ninhydrin-positive products (separable by chromatography in the butanol-acetic acid-water solvent) are produced and are assumed to represent the structures If_1 and If_2. In contrast only one aminotetrazolylalanine product was obtained when aminotetrazole was supplied to seedlings. This fact is perhaps the strongest piece of evidence for the enzymic nature of amino acid synthesis *in vivo*.

5-Aminotetrazolylalanine was isolated from a large batch of mung bean seedlings that had been treated with aminotetrazole. The isolate was shown to be identical chromatographically with the low R_f product of the chemical condensation. The material had $\lambda_{max} = 245$ nm ($\epsilon = 2820$ in 0·1 N NaOH): by analogy with u.v. absorption maxima observed for 1-alkyl-5-aminotetrazoles ($\lambda_{max} = 222$ nm) and 2-methyl-5-aminotetrazole ($\lambda_{max} = 241$ nm),[12]

[12] D. B. MURPHY and J. P. PICARD, *J. Org. Chem.* **19**, 1807 (1954); R. A. HENRY, W. G. FINNEGAN and E. LIEBER, *J. Am. Chem. Soc.* **76**, 2894 (1954).

it is suggested that the compound from mung bean seedlings was 5-aminotetrazol-2-ylalanine (If_1). Therefore the two ring substituents, i.e. the amino group and the alanine residue, probably bear the same spatial relationship to each other as in the aminotriazole derivative characterized earlier.

The positions at which these newer heterocyclic amino acids are found on a two-dimensional chromatogram developed in phenol-NH_3 followed by butanol-acetic acid-water are shown in Fig. 1: the spots of new compounds have been superimposed upon a chromatogram prepared from an extract of cucumber seedlings.

An outstanding problem concerns the nature of the enzyme effecting the various condensation reactions. The close structural similarity existing between the various heterocyclic precursors suggests that they might all act as substrates for the same condensing enzyme. However, no model compounds possessing the same type of N-alanine C linkage are found among the normal protein amino acids of cells: the reaction between indole and serine to give tryptophan perhaps represents the closest analogy. Therefore it would be of interest to know if tryptophan synthetase can effect the synthesis of the heterocyclic alanine derivatives, but our attempts to obtain a purified enzyme following the procedure of Nair and Vaidyanathan[13] were unsuccessful. An alternative, indirect solution to this question might be obtained by determining whether bacterial mutants lacking tryptophan synthetase also lack the ability to form βPA from pyrazole.

Another unanswered question concerns the nature of the biogenetic pathway leading to pyrazole. Natural products containing linked N atoms as in pyrazole are relatively few, so again there are no model reactions upon which a speculative pathway to pyrazole may be based. Cyclization linking the two nitrogens of a diaminopropane-type precursor, followed by dehydrogenation, is one obvious possibility: α,γ-diaminobutyric acid, which is naturally-occurring, could furnish diaminopropane by decarboxylation. Whatever pathway is finally established, presumably at least one of the implicated enzymes will be found to be restricted to those cucurbit species that normally contain βPA. However, the present studies have provided clear evidence that plants may effect some economy in the number of different enzymes they must elaborate solely for the biosynthesis of a particular secondary product. The possible wider implication of similar concepts in the field of secondary product biosynthesis will be awaited with interest.

Biosynthetic Studies with N^4*-Substituted Asparagines*

Experiments with intact seedlings. N^4-Ethyl- and N^4-hydroxyethyl-asparagine are actively synthesized during the early growth of seedlings of *Ecballium elaterium* (squirting cucumber). Therefore it should be possible to distinguish between the use of a synthetase (equation (1)) or a transferase (equation (2)) type reaction for the synthesis of N^4-substituted asparagines by comparing the efficiency with which ^{14}C-aspartic acid and ^{14}C-asparagine are utilized as precursors by *Ecballium* seedlings.

$$R.NH_2 + asp. + ATP \rightarrow asp.NH.R + ADP + Pi \quad (1)$$

$$R.NH_2 + asp.NH_2 \rightarrow asp.NH.R + NH_3 \quad (2)$$

In the present experiments the procedure of supplying $H^{14}CN$ to young seedlings has been adopted as an effective way of producing ^{14}C-labelled asparagine within the tissues. All seedling species tested metabolize ^{14}C-cyanide by incorporating it first into β-cyanoalanine which then is converted into either asparagine or γ-glutamyl-β-cyanoalanine (or a mixture of

[13] P. M. Nair and C. S. Vaidyanathan, *Arch. Biochem. Biophys.* **104**, 405 (1964).

the two).[14, 15] When seedlings of *Ecballium* (6–10 days old) were allowed to assimilate $H^{14}CN$ over a 24-hr period, the relative amounts of radioactivity entering asparagine and the peptide varied considerably in different experiments but asparagine always acquired the larger amount. In one early experiment reported previously,[15] radioactivity in asparagine represented 24 per cent, and that in the peptide 19 per cent, of the total activity of the soluble amino acids extracted from the seedlings. The remaining activity was found mainly in N^4-ethyl- and N^4-hydroxyethyl-asparagine (about 40 and 10 per cent respectively of the total activity), while only 1 per cent was present in aspartic acid. The specific activity of asparagine was approximately double that of either of the N^4-substituted asparagines and about ten times that of aspartic acid. Unless one assumes considerable non-equilibration between sub-cellular metabolic pools, aspartic acid could not have constituted the direct precursor of the substituted asparagines. This conclusion was supported by the observation that [U-^{14}C] aspartic acid, when supplied to *Ecballium* seedlings through their roots during a 24-hr period, gave rise to a number of labelled amino acids including weakly-labelled asparagine, but under these circumstances no radioactivity was detected in either N^4-ethyl- or N^4-hydroxyethyl-asparagine.

The amines required in reactions 1 or 2 for the synthesis of N^4-ethyl- or N^4-hydroxyethyl-asparagine, i.e. ethylamine and ethanolamine respectively, were shown to be normal constituents of *Ecballium* seedlings. Ethanolamine was detected on two-dimensional chromatograms prepared from seedling extracts, while ethylamine formed the only volatile amine present in the distillate obtained by heating macerated seedlings with magnesium oxide and sodium chloride.[16] Each amine was present at a concentration of about 10 μg/g fresh wt. seedlings. The range of N^4-substituted asparagines present in a particular species then may be dependent upon the nature of the endogenous amine pool. This idea was tested experimentally by growing 6-day-old seedlings for a further 2 days in solutions of various amines. N^4-Methyl-, *n*-propyl-, *iso*propyl-, *iso*butyl- and *iso*amyl- asparagines were detected as new brown (ninhydrin) spots on chromatograms (positions as in Fig. 2) prepared from extracts of seedlings receiving the corresponding amines. Secondary amines, e.g. dimethylamine and *N*-methyl-ethanolamine, did not give rise to new substituted asparagines, suggesting that a primary amino group is essential for enzymic synthesis.

The rate of N^4-ethylasparagine synthesis was reduced when other amines were available as enzyme substrates. This is clearly shown by the data included in Table 2. Four different primary amines (methyl, *n*-propyl, *iso*butyl and *iso*amyl) were supplied to separate batches of *Ecballium* seedlings for a 24-hr period; then the groups of seedlings were exposed simultaneously to $H^{14}CN$ for a period of 12 hr. Subsequent analysis indicated the distribution of radioactivity within the major labelled compounds constituting the soluble amino acid pool. Generally, these seedlings converted a smaller proportion of labelled asparagine into N^4-substituted asparagines (cf. earlier experiment), but in all cases the supply of an additional amine to plants reduced the amount of ^{14}C-label entering N^4-ethylasparagine by about 50 per cent. The various N^4-substituted asparagines synthesized in response to the amine supplied were always labelled more heavily than the normal N^4-hydroxyethylasparagine.

Similar experiments were performed with seedlings of *Bryonia dioica* and again exogenously-supplied amines were shown to reduce the amount of radioactivity incorporated into N^4-ethylasparagine under conditions of $H^{14}CN$ feeding.

[14] S. BLUMENTHAL-GOLDSCHMIDT, G. W. BUTLER and E. E. CONN, *Nature* **197**, 718 (1963).
[15] L. FOWDEN and E. A. BELL, *Nature* **206**, 110 (1965).
[16] M. RICHARDSON, *Phytochem.* **5**, 23 (1966).

TABLE 2. RADIOACTIVITY INCORPORATED INTO VARIOUS AMINO ACIDS OF *Ecballium* SEEDLINGS ASSIMILATING $H^{14}CN$ AFTER PRE-TREATMENT WITH DIFFERENT PRIMARY AMINES

Amino acid or amide	Radioactivity present after treatment with amine below*				
	None	Methyl	Propyl	*Iso*butyl	*Iso*amyl
Aspartic acid	5·7	4·8	8·9	7·0	5·9
γ-Glutamyl-β-cyanoalanine	12·1	15·1	11·9	12·3	14·7
Asparagine	68·6	67·3	66·4	69·0	68·7
N^4-ethylasparagine	10·9	5·6	5·9	5·4	5·5
N^4-methylasparagine	–	3·1	–	–	–
N^4-propylasparagine	–	–	4·4	–	–
N^4-*iso*butylasparagine	–	–	–	3·6	–
N^4-*iso*amylasparagine	–	–	–	–	2·1

* Radioactivity present in individual compounds is expressed as a percentage of total activity of the free amino acid fraction.

Although asparagine constitutes the major radioactive compound obtained after $H^{14}CN$ assimilation by *Cucumis sativus* and *Citrullus vulgaris* seedlings (either normal or amine-treated), no radioactivity could be detected on chromatograms in positions corresponding to N^4-substituted asparagines. This observation suggests that the crucial factor controlling the distribution of N^4-substituted asparagines within the Cucurbitaceae is the possession, by a relatively few genera, of a specific biosynthetic enzyme: the availability of amines as precursors is a factor affecting only the type of substituted asparagine produced.

An experiment performed with *Lunaria annua* seedlings indicated that the enzyme responsible for the synthesis of N^5-substituted glutamines exhibits loose specificity towards the amine substrate. The only N^5- substituted glutamine reported in these plants is the *iso*propyl derivative, but when ^{14}C-ethylamine was supplied to seedlings, radioactive N^5-ethyl-glutamine (theanine) was detected in extracts of plants analyzed 48 hr later.

Enzymic studies. Conditions similar to those used to demonstrate the enzymic synthesis of theanine by tea seedling extracts were adopted in initial experiments. *Ecballium* seedlings (grown for 8 days at 20°) were macerated in a grinding medium buffered at pH 7·5 containing Mg^{2+} and mercaptoethanol. The ability of crude homogenates, and of two ammonium sulphate fractions (0–33 and 33–60 per cent saturation), to catalyze N^4-ethylasparagine formation was measured. Aspartic acid and asparagine were supplied as alternative substrates, with and without addition of ATP, and ethylamine was added in a ^{14}C-labelled form. Synthesis was measured by assaying the incorporation of radioactivity into N^4-ethylasparagine separated chromatographically from other compounds of the reaction mixture. The data in Table 3 show clearly that an active enzyme is associated with the 33–60% ammonium sulphate fraction. Asparagine rather than aspartic acid serves as the substrate and ATP is not required for synthesis. Therefore synthesis proceeds by a transferase-type reaction 2.

Under the reaction conditions indicated in Table 2 using asparagine as a substrate, the rate of reaction at 30° is constant for approximately 90 min, and N^4-ethylasparagine synthesis shows an optimum pH in the range 7·5–8·0.

The intact seedling experiments described above indicate that other amines should be acceptable as substrates for the enzyme. This proposition has not been tested directly, but evidence showing that the presence in reaction mixtures of other primary amines inhibits the synthesis of ^{14}C-labelled N^4-ethylasparagine (see Table 4) suggests their substrate role for

the enzyme in competition with ^{14}C-ethylamine. Propylamine and *iso*butylamine show a greater affinity for the enzyme than methylamine and ammonia: similar relative affinities were determined for the different amines in respect to the theanine-synthesizing enzyme.

TABLE 3. FORMATION OF RADIOACTIVE N^4-ETHYLASPARAGINE CATALYZED BY PREPARATIONS FROM *Ecballium* SEEDLINGS

Enzyme preparation	N^4-Ethylasparagine formed from amino acid or amide substrate*	
	Aspartic acid	Asparagine
Crude homogenate	1·16	2·06
0–33% amm. sulph. fraction	0·24	0·23
33–60% amm. sulph. fraction	0·29	5·47

* Values expressed as mμc N^4-ethylasparagine formed during 1·5 hr at 30°. Initial activity present as ^{14}C-ethylamine in each mixture was 620 mμc.

TABLE 4. THE EFFECT OF ADDITIONAL AMINES UPON THE CONVERSION OF ^{14}C-ETHYLAMINE INTO RADIOACTIVE *N*-ETHYLASPARAGINE BY AN *Ecballium* PREPARATION

Amine added	N^4-ethylasparagine formation*	
	mμc	Relative to control = 100
None	12·80	100
Methylamine	5·50	43
Propylamine	0·90	7·0
*iso*Butylamine	1·07	8·4
Ammonia	5·25	41

A 33–60% satd. ammonium sulphate ppt. protein fraction was used as a source of enzyme. Amines (1 μmole) were added to the normal reaction mixtures (see METHODS), which contained 0·08 μmoles of ^{14}C-ethylamine (620 mμc).

* Values are given for synthesis during 1·5 hr at 30°.

Although asparagine is obviously the substrate involved in the synthesis of N^4-ethylasparagine, the role of amide substrate in reaction 2 can be filled by various substituted asparagines. For example, ^{14}C-labelled N^4-ethylasparagine is synthesized enzymically from ^{14}C-ethylamine and any of the following substituted amides: N^4-methyl-, ethyl-, *n*-propyl-, and hydroxyethyl-asparagine. Table 5 shows that all of the substituted asparagines form better substrates for the enzyme than asparagine itself, and that the transferase reaction proceeds most rapidly when one ethylamine residue is exchanged for another. Neither glutamine nor theanine could serve as a substrate for the 33–60% ammonium enzyme fraction.

The ability of substituted asparagines to participate in the transferase reaction explains an observation made by Gray,[17] that although [*ethyl*-^{14}C] ethylasparagine normally undergoes little degradation when supplied to *Ecballium* plants, ^{14}C-label becomes widely distributed among other cell constituents when a primary amine is provided together with the labelled material.

[17] D. O. GRAY, Unpublished observations.

TABLE 5. THE USE OF DIFFERENT SUBSTITUTED AMIDES AS SUBSTRATES FOR RADIOACTIVE N^4-ETHYLASPARAGINE FORMATION FROM ^{14}C-ETHYLAMINE BY AN *Ecballium* PREPARATION

Amide substrate	mμc	N^4-ethylasparagine formation* Relative to asparagine = 100
Asparagine	3·47	100
N^4-methylasparagine	6·25	183
N^4-ethylasparagine	12·6	364
N^4-propylasparagine	9·09	262
N^4-hydroxyethylasparagine	10·3	296

A 33–60% satd. ammonium sulphate ppt. protein fraction was used as a source of enzyme.
* Values are given for synthesis during 1·5 hr at 30°.

A similar active enzyme fraction could be isolated from *Bryonia dioica* seedlings: again asparagine, and not aspartic acid, served as a substrate for N^4-ethylasparagine production. However, when the same procedure was applied to seedlings of *Lunaria annua* the ability to synthesize substituted amides was found to be confined to glutamine derivatives. In this case the enzymic mechanism of N^5-ethylglutamine synthesis (using ^{14}C-ethylamine as precursors) was identical with that described for tea seedlings, i.e. a synthetase-type reaction operated and required glutamic acid and ATP as substrates.

The transferase enzyme responsible for N^4-ethylasparagine synthesis could not be detected in *Lagenaria leucantha*, a genus placed in the same sub-tribe of the Cucurbitaceae as *Ecballium* and *Bryonia*. Since *Lagenaria* seedlings synthesize asparagine during growth, there is again seemingly no connexion between the transferase enzyme studied here and the enzyme responsible for asparagine formation.

METHODS

Chemicals and Radiochemicals

The heterocyclic compounds listed in Table 1 were commercial products except the following which were received as gifts: 3-aminopyrazole (Ciba Research Laboratories, Basle); 1,2,4-aminotriazol-3-ylalanine (Dr. F. Schneider, Tubingen); and 3,4-dihydroxypyridine (Dr. M. P. Hegarty, Brisbane). 4-Nitropyrazole was prepared from pyrazole following the nitration procedure of Finar and Hurlock.[18]

[3-^{14}C] Serine was obtained from the Radiochemical Centre, Amersham. [2-^{14}C] Acetonitrile was hydrogenated in the presence of Adams PtO catalyst to yield [2-^{14}C] ethylamine: the labelled ethylamine was purified from traces of other radioactive compounds by chromatography using the butanol-acetic acid-water solvent system.

Paper Chromatography

Two-dimensional chromatograms were developed with 75% (w/w) phenol in the presence of NH_3 vapour, followed by a one-phase butan-1-ol: acetic acid: water mixture (90:10:29, v/v). Spots were revealed using 0·1% ninhydrin in ethanol as the chromogenic reagent.

Occasionally, the upper phase of a *tert*-amyl alcohol: acetic acid: water mixture (10:1:10, v/v) was used for one dimensional separations.

Formation of Heterocyclic Amino Acids by Seedlings

Cucumber or mung bean seeds were allowed to imbibe a 0·125% solution of the heterocyclic nitrogen compound for 24 hr at 25°, after which they were grown in moist vermiculite for a further 2 days at 25°. When treatments included the supply of [3-^{14}C] serine (1 μc, 6 μg), the number of seeds was restricted to six. The serine was dissolved in a solution of the appropriate heterocyclic compound, the volume being such that it was completely absorbed by the seedlings during the 24-hr imbibition period.

[18] I. L. FINAR and R. J. HURLOCK, *J. Chem. Soc.* 3024 (1957).

Seedlings were extracted with 75% (v/v) ethanol and the amino acid fractions were separated from the extracts using small Zeokarb 225 cation-exchange resin columns.[1] After separation on two-dimensional chromatograms, radioactivity present in the various heterocyclic amino acids was determined by surface scanning of papers using a Geiger system (counting efficiency about 3 per cent).

Large Scale Preparation of 5-Aminotetrazol-2-ylalanine

Mung bean seeds (5 kg) were soaked in 7 l. of 0·2% 5-aminotetrazole solution buffered with 0·02 M phosphate at pH 5·8. After 24 hr, the seedlings were planted in vermiculite and grown for a further 3 days. The seedlings were harvested, frozen at −20°, and then minced. The press juice (collected by pressing at 6000 lb/in^2) was adjusted to pH 4·5 and warmed to 60° for 15 min. to coagulate most of the protein. The cleared filtrate was applied to a column of Zeokarb 215 (H^+ form, length 100 cm, dia. 8 cm) to separate the cationic fraction containing the amino acids, and then the column was eluted with N-NH_3. Fractions (500 ml) were collected and numbers 20–28 containing the bulk of the amino acids were pooled. After concentration, these fractions were applied to a Dowex-50 × 8 column (H^+ form, length 75 cm, dia. 3·6 cm): elution was now with 0·25 N-ammonium formate (pH 4·5) and 50 ml fractions were collected. 5-Aminotetrazol-2-ylalanine was present in fractions 1–24 together with aspartic acid, glutamic acid, serine, threonine and asparagine. These fractions were pooled and stored at 4° when the heterocyclic amino acid crystallized out. Further crystals were obtained after concentration and eventually 1·4 g of recrystallized product was obtained. (Found: C, 28·4; H, 4·7; N, 48·4; $C_4H_8N_6O_2$ requires C, 28·0; H, 4·6; N, 48·8%). The isolated compound had the following u.v. absorption characteristics: in 0·1 N-HCl, λ_{max}=250 nm, ϵ=2430; in 0·1 N-NaOH, λ_{max}=245 nm, ϵ=2820.

Preparation of N^4-Substituted Asparagines

4-Ethyl hydrogen aspartate was prepared by the method of Curtis and Koch,[19] as modified by Fowden.[20] The solid ester was then mixed with a 33% (w/v) solution of the appropriate amine in dry ethanol and heated at 100° for 8 hr in an ampoule. The N^4-substituted asparagines formed were purified as previously described for the methyl derivative.[1]

Amine Determination in Seedlings

Seedlings (10 g) were macerated in 10–15 ml water and the slurry was transferred to a small distillation flask together with MgO (3 g) and NaCl (1 g). The contents were distilled, and the distillate collected in N-HCl (5 ml). After approximately 10 ml of distillate had been collected, the contents of the receiver were evaporated to dryness. Portions of the residue were applied to one-dimensional chromatograms developed in the butanol-acetic acid-water and the *tert*-butanol-acetic acid-water mixtures. Semi-quantitative determination of the amines present in the distillate were made by comparing the size and intensity of the spots (developed with ninhydrin) against a series of amine standards separated on the same paper.

Amine and Cyanide Treatment of Seedlings

Normally seedlings of 6–10 days old were used. The seedlings were grown in moistened vermiculite and, when amine-treated batches were required, seedlings were transferred to vermiculite wetted with a 0·01 M-amine solution, buffered at pH 5·8 with 0·02 M-phosphate, 24 hr before commencing cyanide feeding. Each treatment usually consisted of six seedlings. During exposure to cyanide the seedlings were maintained in the light in a closed glass vessel (volume 1 l.): $H^{14}CN$ (25 μc, 18 μg) was generated within the vessel by the addition of N-H_2SO_4 to a $Na^{14}CN$ solution. Groups of seedlings used in the experiments reported in Table 2 were exposed simultaneously to the ^{14}C-cyanide treatment.

At the end of the cyanide assimilation period, the seedlings were extracted with 75% (v/v) ethanol, and the extracts treated as above for assay of radioactivity in amino acids separated by two-dimensional paper chromatography.

Enzymic Synthesis of N^4-Ethylasparagine

Ecballium seedlings, 10 g (or occasionally seedlings of other genera), were macerated with an equal amount of a grinding medium containing 0·05 M-potassium phosphate (pH 7·5), 0·01 M-$MgCl_2$ and 0·01 M-mercaptoethanol. The supernatant obtained after centrifuging at 25,000 *g* for 15 min provided the crude homogenate. Protein fractions were obtained from the supernatant by saturating to 33%, and then to 60% ammonium sulphate. The protein fractions were redissolved in small volumes (about 2 ml) of grinding medium, and the solutions were dialyzed against two changes of grinding medium with stirring for 3 hr to remove residual ammonium sulphate. All operations were performed at about 4°.

[19] T. CURTIS and F. KOCH, *J. Prakt. Chem.* **38**, 473 (1888).
[20] L. FOWDEN, *Biochem. J.* **81**, 154 (1961).

Reaction mixtures contained enzyme preparation (50 μl), amide substrate (2·5 μmole), ATP if present (1 μmole), $MgCl_2$ (2·5 μmole), mercaptoethanol (2·5 μmole), and ^{14}C-ethylamine (0·64 μc, 3·6 μg) in a final volume of 0·1 ml. N^4-Ethylasparagine synthesis was allowed to proceed at 30° for 1·5 hr when reaction was terminated by the addition of two volumes of ethanol. Carrier N^4-ethylasparagine (50 μg) was added and then precipitated protein was removed by centrifuging. The supernatant solutions were evaporated to dryness several times by addition of ethanolic ammonia solution to remove unchanged volatile ^{14}C-ethylamine. The residues were applied to paper chromatograms developed for 20 hr in butanol-acetic acid-water, and radioactivity present in the separated N^4-ethylasparagine was assayed using a Packard Radiochromatogram Scanner.

The technique used to study the synthesis of N^5-substituted glutamines by *Lunaria* was similar, except that carrier theanine was added at the end of the incubation period.

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