Phytochemistry, 1965, Vol. 4, pp. 933 to 944. Pergamon Press Ltd. Printed in England

THE AMINO ACIDS OF SEEDS OF THE CUCURBITACEAE

PATRICIA M. DUNNILL and L. FOWDEN

University College, London

(*Received* 15 *April* 1965)

Abstract—In recent years several new amino acids have been isolated from members of the family Cucurbitaceae. Now their distribution in seeds of many genera has been determined and the new phytochemical information is examined against the existing classification of this family based mainly upon morphological criteria. The survey revealed that *m*-carboxyphenylalanine was a constituent of some members of this family, and the natural occurrence of N^4-methylasparagine is recorded for the first time. The presence of numerous other unidentified ninhydrin-reacting compounds was noted.

INTRODUCTION

FLAVONOIDS, alkaloids, terpenes and cyanogenetic compounds perhaps have attracted more attention than other types of substances in chemical taxonomic studies with plants. In each case the compounds fall into the loosely defined category of "secondary plant products". It is only recently that interest has been focused upon amino acids as another useful group of compounds in this context.[1] In addition to the basic twenty constituents that all plant species must synthesize, at least another hundred amino acids are now recorded occurring in plants.[2] These substances are certainly not universally distributed throughout the plant kingdom, although a few like γ-aminobutyric acid and α-aminoadipic acid occur very widely. More usually, the compounds have a restricted distribution; some show an infrequent, haphazard occurrence in isolated members of many families of plants, while others occur only as characteristic components of closely-allied groups of plants. Excepting a few dubious claims for their presence in plant proteins, the compounds only occur free or as γ-glutamyl peptides and it is convenient to refer to them as non-protein amino acids.

A group of such non-protein amino acids forms constituents of plants of the genus, *Lathyrus*, and Bell[3] has shown that about fifty species could be sub-divided into five main groups on the basis of associations of these amino acids within the seeds. The resulting chemical classification differed only slightly in detail from an earlier one based on morphological and cytological information.

Several of the non-protein amino acids were isolated first from members of the Cucurbitaceae,[2] and the present survey was undertaken to gain information concerning the generality of their occurrence within members of the family. Normally, seeds have been examined in preference to vegetative organs for their composition is less likely to be affected by variation in nutritional or environmental factors during plant growth. The results

[1] E. A. BELL and L. FOWDEN, in *Taxonomic Biochemistry and Serology* (Edited by C. A. LEONE), p. 203. Ronald Press, New York (1964).
[2] L. FOWDEN, *Ann. Rev. Biochem.* 33, 173 (1964).
[3] E. A. BELL, *Biochem. J.* 83, 225 (1962).

obtained are examined in relation to the classification of the family recently compiled by Jeffrey.[4,5]

RESULTS

The Nature of the Amino Acid Constituents

Each seed species was ground and extracted with 75% (v/v) ethanol and then the amino-acid fraction was separated from other aqueous–ethanol soluble constituents by absorption upon a cation-exchange resin. Individual amino acids were identified after separation on

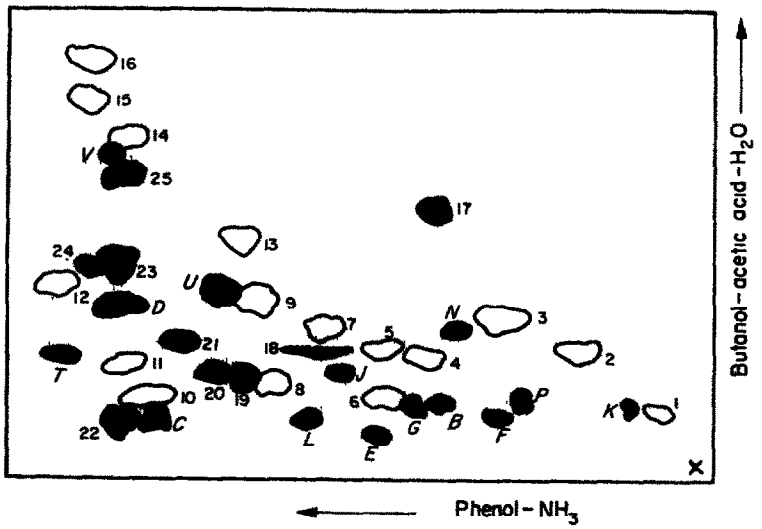
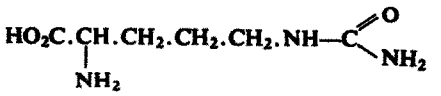


FIG. 1. DIAGRAMMATIC REPRESENTATION OF POSITIONS OF NON-PROTEIN AMINO ACIDS AND UNKNOWN NINHYDRIN-POSITIVE COMPOUNDS (solid spots) IN RELATION TO THE CHROMATOGRAPHIC PATTERN OF THE PROTEIN AMINO ACIDS (open spots).

Key to spots: 1, cysteic acid (from cyst(e)ine); 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 6, asparagine (brown); 7, threonine; 8, glutamine; 9, alanine; 10, arginine; 11, methionine sulphoxide (from methionine); 12, proline (yellow); 13, tyrosine; 14, valine; 15, phenylalanine; 16, leucines; 17, *m*-carboxyphenylalanine (IV); 18, γ-glutamyl-β-pyrazol-1-ylalanine (III); 19, N^4-hydroxyethylasparagine (VII) (brown); 20, citrulline (I); 21, N^4-methylasparagine (VIII) (brown); 22, cucurbitin (V); 23, β-pyrazol-1-ylalanine (II); 24, N^4-ethylasparagine (VI) (brown); 25, γ-aminobutyric acid; unknowns designated by letters *B*, *C* (green), *D*, *F*, *G*, *J*, *K*, *L*, *N*, *P* (yellow), *T* (brown), *U*, and *V* (brown).

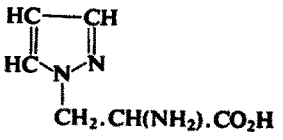
2-dimensional chromatograms prepared from each extract. Phenol–NH_3 was used as the first developing solvent and a butan-1-ol–acetic acid–water mixture as the second; the amino acids were located on the developed chromatograms using ninhydrin as the chromogenic reagent. Figure 1 is a diagramatic representation of the spots encountered in this survey and it will be seen that many remain uncharacterized. However, as far as possible the structures of compounds upon which emphasis has been placed in the tabulation of results are given below.

[4] C. JEFFREY, *Kew Bull.* **15**, 337 (1961).
[5] C. JEFFREY, *Kew Bull.* **17**, 473 (1964).

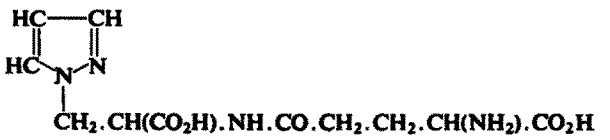


(I)

Citrulline (I). An α-amino-δ-ureido acid, it was first isolated by Wada[6] from watermelon (*Citrullus lanatus*). As an intermediate in the biosynthetic pathway leading from glutamic acid to arginine, it is presumably present in all plant species, yet often it is not detected by the usual 2-dimensional chromatographic procedures. In the cucurbitaceous seeds examined, very large differences in the concentration of citrulline existed between different species.



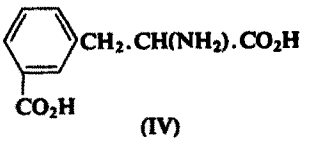
(II)



(III)

β-Pyrazol-1-ylalanine (II) and γ-glutamyl-β-pyrazol-1-ylalanine (III). The heterocyclic amino acid, first isolated from watermelon seed,[7] is isomeric with histidine, and is formed in cucumber seedlings by a condensation of pyrazole with serine,[8] catalysed by a synthetic enzyme possessing features reminiscent of tryptophan synthetase. The peptide[9] always occurs together with β-pyrazol-1-ylalanine, but invariably at a lower concentration. This co-existence presumably is dependent upon the action of a γ-glutamyl transferase, an enzyme previously observed in many plants and known to show only low specificity for the acceptor amino acid.[10]

The peptide always gives a spot of unusual shape, streaked (elongated) in the direction of phenol flow, yet very narrow along the second direction; this characteristic feature is useful for its identification.

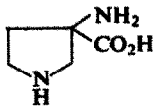


(IV)

m-*Carboxyphenylalanine (IV).* First isolated from *Iris tingitana* (Iridaceae)[11] and subsequently from *Reseda odorata* (Resedaceae) and *Lunaria annua* (Cruciferae),[12] *m*-carboxyphenylalanine was detected in many of the cucurbit seeds examined. Its identification was established by isolation from seed of *Ecballium elaterium* and comparison with authentic material.

Cucurbitin (3-aminopyrrolidine-3-carboxylic acid, V). This compound was isolated from the drug plant, *Cucurbita moschata*, by Chinese workers,[13] who claim it possesses anthelmintic

[6] M. WADA, *Biochem. Z.* **224**, 420 (1930).
[7] F. F. NOE and L. FOWDEN, *Biochem. J.* **77**, 543 (1960).
[8] P. M. DUNNILL and L. FOWDEN, *J. Exp. Botany* **14**, 237 (1963).
[9] P. M. DUNNILL and L. FOWDEN, *Biochem. J.* **86**, 388 (1963).
[10] J. F. THOMPSON, D. H. TURNER and R. K. GERING, *Phytochem.* **3**, 33 (1964).
[11] J. F. THOMPSON, C. J. MORRIS, S. ASEN and F. IRREVERRE, *J. Biol. Chem.* **236**, 1183 (1961).
[12] A. KJAER and P. O. LARSEN, *Acta Chem Scand.* **17**, 2393 (1963).
[13] S. FANG, L. LI, C. NIU and K. TSENG, *Sci. Sinica (Peking)* **10**, 845 (1961).



(V)

properties. Structurally, it is both an α-amino acid and a cyclic β-imino acid. Its occurrence in other *Cucurbita* species was detected in the present survey.

N^4-Ethylasparagine (VI), N^4-hydroxyethylasparagine (VII) and N^4-methylasparagine (VIII). These substituted amino acids react with ninhydrin to give brown spots (the colour

$$CH_3.CH_2.NH.CO.CH_2.CH(NH_2).CO_2H$$

(VI)

$$HO.CH_2.CH_2.NH.CO.CH_2.CH(NH_2).CO_2H$$

(VII)

$$CH_3.NH.CO.CH_2.CH(NH_2).CO_2H$$

(VIII)

is indistinguishable from that given by asparagine). N^4-Ethylasparagine and N^4-hydroxyethylasparagine were first isolated from vegetative parts of squirting cucumber (*Ecballium elaterium*)[14] and bryony (*Bryonia dioica*)[15] respectively. Radioisotopic studies with *Ecballium* seedlings indicate that both compounds are formed from asparagine,[16] presumably by exchanging the amide-N atom for a residue of either ethylamine of ethanolamine in a reaction comparable to that producing N^5-substituted glutamines in *Agaricus* species.[17]

N^4-Methylasparagine has been newly identified. It was observed as a brown spot on a chromatogram prepared from seed of *Corallocarpus epigaeus*. The spot was shown to be inseparable from chemically synthesized N^4-methylasparagine run on chromatograms developed in four solvents (phenol, butanol–acetic acid–water, butanol–NH_3, and ethyl acetate–pyridine–water). It yields aspartic acid and methylamine after hydrolysis with 2N-HCl for 16 hr at 100°. The hydrolysis products were identified by co-chromatography with authentic substances using phenol and butanol–acetic acid–water as solvents, while methylamine was also characterized as a volatile amine under alkaline conditions using the Conway microdiffusion techniques.

Amino Acid Distribution

The pattern of amino acid distribution throughout the family is shown in Table 1. The species are grouped following the classification of Jeffrey[4,5] which splits the family into two major sub-families, the Cucurbitoideae and Zanonioideae, consisting of eight and one tribes respectively, which may be split further into sub-tribes.

[14] D. O. GRAY and L. FOWDEN, *Nature* **189**, 401 (1961).
[15] L. FOWDEN, *Biochem. J.* **81**, 154 (1961).
[16] L. FOWDEN, in *Beiträge zur Biochemie und Physiologie von Naturstoffen* (Edited by D. GRÖGER, H. B. SCHRÖSTER and R. H. SCHÜTTE), Deutsche Akademie der Wissenschaft, Berlin (1965).
[17] H. J. GIGLIOTTI and B. LEVENBERG, *Biochim. Biophys. Acta* **81**, 618 (1964).
[18] E. J. CONWAY, *Micro-diffusion and Volumetric Error*, Crosby Lockwood, London (1939).

TABLE 1. THE NON-PROTEIN AMINO ACIDS AND UNKNOWN NINHYDRIN-POSITIVE COMPOUNDS OF SEEDS OF THE FAMILY, CUCURBITACEAE*

Species	Amino acid† II	III	VI, VII and VIII	I	IV	Unknowns (spot intensities in parentheses)
I. Sub-family Cucurbitoideae						
1. Tribe Joliffieae						
ii. Sub-tribe Thladianthinae						
Momordica cymbalaria	0	0	0	S	0	*D*(W), *J*(W)
M. calantha	0	0	0	S	0	*D*(W), *J*(W)
2. Tribe Benincaseae						
i. Sub-tribe Benincasinae						
Bryonia dioica	W	W	S‡(VI), M(VII)	S	M	*D*(T)
Ecballium elaterium	W	W	W(VI), W(VII)	M	M	*D*(T)
Acanthosicyos horridus	S‡	M	0	M	0	*D*(W), *B*(T), *T*(W), *L*(W)
Benincasa hispida	S‡	S	0	M	0	*D*(T)
Citrullus colocynthis	S‡	S	0	S	0	*D*(T)
Coccinia grandis	S‡	M	0	W	0	*D*(T), *L*(W), *J*(T)
C. hirtella	S‡	M	0	W	0	*D*(W)
Diplocyclos tenuis	S	M	0	T	0	
Lagenaria abyssinia	M	W	0	W	0	*J*(T)
L. sphaerica	M	W	0	M	0	
Ruthalicia eglandulosa	S	M	0	W	0	
Zombitsia cucorum	M	W	0	S‡	0	*D*(W)
Cogniauxia podolaena	0	0	0	T	M	
ii. Sub-tribe Luffinae						
Luffa acutangula	0	0	0	W	W	
L. cylindrica	0	0	0	W	S	
L. operculata	0	0	0	W	W	
3. Tribe Melothrieae						
ii. Sub-tribe Trochomeriinae						
Ctenolepis cerasiformis	S	M	0	M	0	*D*(T), *B*(T), *L*(T)
Dactyliandra welwitschii	S	M	0	W	0	*D*(W), *L*(T)
iii. Sub-tribe Melothriinae						
Apodanthera undulata	0	0	0	W	S‡	*L*(T), *J*(T), *K*(T)
Corallocarpus epigaeus	0	0	S(VIII)	M	M	*D*(T), *L*(T), *J*(T)
C. bainesii	0	0	0	W	M	*D*(T), *U*(S‡), *V*(M)
C. grevei	0	0	0	M	M	
Cucumis ficifolius	S	S	0	S	0	*L*(T), *K*(T)
C. melo	S	S	0	M	0	*L*(W), *K*(T), *N*(T)
C. sativus	S	S	0	W	0	*L*(T), *K*(T)
Kedrostis africana	M	W	0	M	S	*D*(T), *B*(W), *J*(T)
K. foetidissima	0	0	0	M	T	*D*(T)
K. elongata	0	0	W(VIII)	T	W	
Melothria japonica	W	W	0	T	W	*D*(S‡), *K*(T)
M. pendula	S‡	S	0	S	0	*D*(T), *P*(M), *L*(T)
M. scabra	S‡	S	0	W	0	*D*(T), *P*(W), *L*(T)
Mukia maderaspatana	M	W	0	M	0	*D*(T), *B*(T)

* See notes at foot of table, p. 938.

TABLE 1—*continued*

Species	Amino acid† II	III	VI, VII and VIII	I	IV	Unknowns (spot intensities in parentheses)
4. Tribe Cyclanthereae						
Cyclanthera explodens	0	0	0	W	S	*C*(M), *L*(T)
C. pedata	0	0	0	T	S	*C*(M), *L*(T)
Echinocystis wrightii	0	0	0	W	S‡	*C*(M), *L*(T)
Marah fabaceus	0	0	0	T	T	*B*(T), *G*(T), *K*(T)
M. macrocarpus	0	0	0	T	S	*J*(T)
M. oreganus	0	0	0	T	S‡	*B*(T), *G*(T), *J*(T)
5. Tribe Sicyoëae						
Sicyos angulatus	0	0	0	T	0	*D*(T)
6. Tribe Trichosantheae						
iii. Sub-tribe Trichosanthinae						
Peponium hirtellum	S‡	M	0	M	0	*D*(W)
P. mackenii	S‡	M	0	S	0	*D*(W)
Trichosanthes tricuspidata	0	0	0	W	S	
7. Tribe Cucurbiteae						
i. Sub-tribe Cucurbitinae						
Cucurbita maxima	0	0	0	W	W	*D*(W), *B*(T), *L*(T)
Roseanthus cucumerinus	0	0	0	T	T	
Sicana odorifera	0	0	0	W	S	*D*(T), *L*(T)
ii. Sub-tribe Abobrinae						
Cayaponia sp.	0	0	0	T	0	*L*(T)
II. Sub-family Zanonioideae						
1. Tribe Zanonieae						
i. Sub-tribe Fevilleinae						
Fevillea peruviana	0	0	0	T	0	
ii. Sub-tribe Zanoniinae						
Gerrardanthus grandiflorus	0	0	0	S	0	
Alsomitra macrocarpa	0	0	0	S	0	*J*(W), *L*(T)
iii. Sub-tribe Gomphogyninae						
Gynostemma pentaphyllum	0	0	0	S	0	*D*(S)

* Genera are grouped into sub-families, tribes and sub-tribes following Jeffrey.[5]

† Symbols denote relative strengths of chromatographic spots: 0, absent; T, trace; W, weak; M, medium; S, strong.

‡ Denotes that compound forms the predominant spot on the chromatogram.

(II)—β-pyrazol-1-ylalanine; (III)—γ-glutamyl-β-pyrazol-1-ylalanine; (VI)—N^4-ethylasparagine; (VII)—N^4-hydroxyethylasparagine; (VIII)—N^4-methylasparagine; (I)—citrulline; (IV)—*m*-carboxyphenylalanine

The genera forming sub-tribes generally possessed a comparable complement of non-protein amino acids present in similar relative concentrations. However some striking anomalies exist and are the subject of comment below. A distinct difference of composition between tribes or sub-tribes was frequently observed, but, in some instances, groups of genera clearly separated by morphological characters had very similar amino acid composition.

The largest single group of species examined were the genera forming the sub-tribe Benincasinae. This sub-tribe was recently redefined[5] and now includes two genera, *Acan-*

thosicyos and *Ecballium*, earlier separated as the sub-tribe Acanthosicyoinae. The members of the new sub-tribe are mainly of African origin, but *Ecballium* and *Bryonia* stand out as the only European species. With the exception of these two genera (and of *Cogniauxia*), the Benincasinae are characterized by a high content of β-pyrazol-1-ylalanine (II), which frequently forms the major component of the free amino-acid pool, and lesser amounts of γ-glutamyl-β-pyrazol-1-ylalanine (III). Citrulline (I) is also present, often in considerable quantity. In *Ecballium* and *Bryonia*, β-pyrazol-1-ylalanine and its peptide are present but only as minor constituents. More distinctive is that these two genera, alone amongst all the species examined, contain the two substituted asparagines, N^4-ethyl- and N^4-hydroxyethyl-asparagine (VI and VII); the presence of significant quantities of *m*-carboxyphenylalanine (IV) also places them apart from the other genera forming the sub-tribe. The geographical isolation of *Ecballium* and *Bryonia*, possibly since that Tertiary period, from the other genera is then manifested in an altered biogenetic capacity as a consequence of evolutionary change.

Zombitsia, a Madagascan genus described since Jeffrey's classification was compiled, has a composition in agreement with its inclusion in the Benincasinae and Jeffrey (Personal communication) has indicated that he would place it in this sub-tribe on morphological criteria. On chemical grounds, *Cogniauxia* remains an exceptional member of this sub-tribe. It more closely resembles *Luffa*, split off as the only member of the related sub-tribe, Luffinae, which together with the Benincasinae forms the tribe Benincaseae. It is noteworthy that *Cogniauxia* in its tendril and androecial characters appears to be the least specialized member of the Benincasinae; these considerations may similarly indicate that *Cogniauxia* forms a link between the rest of the sub-tribe and the Luffinae. Incidentally, Wright[19] describing a species of *Cogniauxia*, referred it to *Luffa* pending knowledge of its female flowers and fruits.

Three species of *Luffa* were examined (*L. acutangula*, *L. operculata*, and *L. cylindrica*): each had a similar composition although the former two species contained only minor amounts of *m*-carboxyphenylalanine (IV), whereas this aromatic amino acid was a major component of *L. cylindrica* seed. Jeffrey[4] points out that the structural features of the dry operculate fruits of *Luffa* species are approached elsewhere in the family only by members of the tribe Cyclanthereae, and so the sub-family Luffinae might be regarded as a link between the Benincasinae and Cyclanthereae. Undoubtedly the three *Luffa* species have amino acid compositions more closely allied to those of the genera, *Cyclanthera*, *Echinocystis* and *Marah*: none of these genera contain β-pyrazol-1-ylalanine (II) or its peptide (III) within their seeds, yet all synthesize *m*-carboxyphenylalanine. Chromatograms prepared from extracts of *C. pedata*, *C. explodens* and *E. wrightii* had an almost identical appearance and possessed an unusual green ninhydrin-reacting spot, in the area occupied by basic amino acids, which was not encountered in any other species examined.

The two members examined from the sub-tribe Trochomeriinae, could not be distinguished readily on the present chemical criteria from the African genera constituting the Benincasinae. This is also true of some genera of the Melothriinae, another sub-tribe of the Melothrieae. For instance, ten species of *Cucumis* and *Mukia maderaspatana* contained the β-pyrazol-1-ylalanine (II), γ-glutamyl-β-pyrazol-1-ylalanine (III), significant amounts of citrulline (I) and a minor "unknown *D*" complex associated with these other sub-tribes. However, viewed as a whole the members of the Melothriinae examined form one of the most diverse sub-tribes on chemical grounds. *Apodanthera undulata*, in which *m*-carboxyphenylalanine (IV) forms the major amino acid constituent, closely resembles the members of the Cyclanthereae, particularly *Marah macrocarpus* and *M. oreganus*, while substances like

[19] C. H. WRIGHT, in *Hooker's Icon. Pl.* **25**, plate 2490 (1896).

N^4-methylasparagine (VIII) (brown), *V* (possibly N^4-propyl- or N^4-isopropyl-asparagine, brown) and "unknown *Y*" (a lemon yellow-coloured spot), which have a very restricted distribution, strikingly separate certain species of *Corallocarpus* and *Kedrostis* and of *Melothria* from other members of the sub-tribe.

Morphologically, the Melothriinae are also insufficiently studied and again they exhibit diversity in a number of characters. For instance Marticorena[20] divides the sub-tribe into two pollen-morphologically differentiated groups. Of the genera we have studied *Cucumis* and *Mukia* fall into one group. The other group contains most of the species we have examined and it has been further divided into two, depending on the presence or absence of antherial hairs.

Many observers have encountered difficulties in circumscribing the closely-allied genera *Corallocarpus* and *Kedrostis*. Unfortunately, our analyses which have included three species described as *Corallocarpus* and three as *Kedrostis*, merely add to the uncertainty, for only two species (namely *C. epigaeus* and *K. elongata*) synthesized N^4-methylasparagine (VIII). The relationship between the two genera is stressed however by the fact the N^4-substituted asparagines occur very rarely elsewhere in the family. Morphologically, *Kedrostis* is still a rather heterogeneous genus which consists of at least four parts, so many more species must be studied chemically before a useful evaluation of the data can be made.

Peculiarities of amino acid composition were seen in all three species of *Melothria* examined. *M. pendula* and *M. scabra* seed each contained β-pyrazol-1-ylalanine (II) and the related peptide (III) as the most predominant constituents but a more conspicuous component gave a bright yellow spot "unknown *Y*" after treating the chromatograms with ninhydrin. This substance was presumed not to be an imino acid (negative isatin reaction) and although it occupied a position typical of acidic amino acids, it was not retained by an anion exchange column (Dowex-1). In contrast seed of *M. japonica* contained only minor amounts of β-pyrazol-1-ylalanine and peptide and no trace of "unknown *Y*": a predominant constituent was "unknown *D*" while a small amount of *m*-carboxyphenylalanine (IV) also was present. The entirely different appearance of chromatograms of the last species from those of *M. pendula* and *M. scabra* provides chemotaxonomic support for the view expressed by Jeffrey[4] that the genus *Melothria* be confined to New World species, and that certain species now defined as *Melothria*, of which the Asiatic *M. japonica* would be an example, be re-established as *Zehneria* species.

Two genera analysed from the sub-tribe Trichosanthinae, were *Peponium* and *Trichosanthes*. *P. hirtellum* and *P. mackenii* (African species) had closely-allied composition to the main group of African genera forming the Benincasinae (i.e. strong β-pyrazol-1-ylalanine and peptide, significant citrulline and a small amount of "unknown *D*"). *Trichosanthes* is by contrast an Asiatic genus and differs considerably in composition; indeed, the absence of β-pyrazol-1-ylalanine (II) and peptide (III) coupled with a high content of *m*-carboxyphenylalanine (IV) is more reminiscent of the genus, *Luffa*. *Peponium* is distinct from the other Trichosanthinae in the sexine structure of the pollen grains.[20]

Species grouped in the tribe Cucurbiteae possess few striking features of composition although *m*-carboxyphenylalanine is normally present. Analyses of seed of fourteen species of the genus *Cucurbita* are presented separately in Table 2 and are discussed in more detail below.

The sub-family Zanonioideae, as defined is very much smaller: it contains only one tribe split into four sub-tribes. Our very limited analyses of species of this sub-family did not

[20] C. MARTICORENA, *Grana Palynol.* 4, 78 (1963).

indicate any considerable differences of composition. In fact few of the compounds serving as useful chemical characters were present, and only *Gynostemma* accumulated such a component ("unknown *D*") in a marked amount.

Table 2 gives details of the non-protein amino-acid contents of the fourteen *Cucurbita* species examined. The seeds were kindly supplied by Dr. T. W. Whitaker (La Jolla, California) who also provided useful comments on their relationships. A common feature of all fourteen species not apparent from Table 2 was that β-pyrazol-1-ylalanine (II) and its peptide (III) were entirely absent. Variability of composition was seen between species in regard to the constituents listed in Table 2 (*m*-carboxyphenylalanine, cucurbitin (V) and unknowns *B*, *D* and *F*), although *F* was never present in more than minor quantities.

TABLE 2. THE NON-PROTEIN AMINO ACIDS AND UNKNOWN NINHYDRIN-POSITIVE COMPOUNDS OF SEEDS OF THE GENUS, *Cucurbita*

Species	Amino acid*				
	IV	V	*B*	*D*	*F*
Cucurbita lundelliana	0	T	M	W	0
C. martinezii	0	M	0	W	0
C. okeechobeenis	0	T	T	0	0
C. soraria	0	W	0	0	0
C. ficifolia	0	W	0	0	0
C. cylindrata	0	T	0	0	0
C. foetidissima	S	M	0	M	0
C. palmata	M	W	M	W	W
C. digitata	M	W	M	S	W
C. mixta	W	M	M	W	0
C. pepo	W	M	0	T	0
C. moschata	T	S†	0	W	0
C. maxima	W	S†	T	W	0
C. andreana	T	S	0	T	0

* Symbols denote relative strengths of chromatographic spots: 0, absent; T, trace; W, weak; M, medium; S, strong.

† Denotes that compound forms the predominant spot on the chromatogram.

(IV)—*m*-carboxyphenylalanine; (V)—cucurbitin (3-aminopyrrolidine-3-carboxylic acid).

The three annual mesophytic species (*C. lundelliana*, *C. martinezii*, and *C. okeechobeenis*) are listed first: in some respects their chemical relationship is not too close but none possessed *m*-carboxyphenylalanine (IV). *C. soraria* which has affinities with this group also lacks *m*-carboxyphenylalanine. *C. cylindrata* perhaps also resembles these mesophytes more closely biochemically that it does the xerophytes. This is also true for many morphological characters, although genetically it is most compatible with the xerophytic species.[21] It is possible to cross pairs of species within the group, *C. cylindrata*, *C. foetidissima*, *C. palmata* and *C. digitata*, the last two especially successfully. These two species possess almost identical seed composition in respect of non-protein amino acids while *C. cylindrata* and *C. foetidissima* exhibited obvious differences in the absence of *B* and *F*. The remaining species consist of a group of four cultivated annuals (*C. mixta*, *C. pepo*, *C. moschata* and *C. maxima*) together with *C. andreana*, which is a feral (or wild) species of *C. maxima*. The similarity of composition

[21] T. W. WHITAKER, *Evolution* **18**, 553 (1965).

within this group is distinctly seen; cucurbitin is either a marked or the most predominant constituent of each and definite differences of composition are observed only in relation to "unknown *B*". Of the wild mesophytes, *C. lundelliana* is genetically most compatible with the cultivated species,[21] and this species seems to show a closer chemical kinship to the latter group than do *C. martinezii* and *C. okeechobeenis*.

DISCUSSION

The overall picture emerging from the amino-acid analysis presented above is that useful support has been provided for many features of the current system, based on morphological characters, for classifying the genera forming the Cucurbitaceae. In most instances where an obvious discrepancy was shown to exist, some uncertainty remained concerning the morphological relationships between species or genera. Amino-acid data therefore provide an invaluable index whose use should be considered in any new attempt to re-define relationships, e.g. between species presently assigned to the genus *Melothria*, or perhaps within the confused group of *Corallocarpus* and *Kedrostis* species.

The amino acids which have been proved to have the greatest taxonomic usefulness so far are β-pyrazol-1-ylalanine (II) and its γ-glutamyl peptide (III), the various N^4-substituted asparagines (VI, VII and VIII) and *m*-carboxyphenylalanine (IV), together with unknown substances *D* and *Y*. If larger quantities of plant material become available efforts will be made to identify these two unknowns.

Species unable to synthesize β-pyrazol-1-ylalanine presumably lack the synthetase enzyme, described in several cucurbit seedlings,[8] which catalyse the condensation of pyrazole with serine. However, further differences may exist between the protein complex of β-pyrazol-1-ylalanine-producing and β-pyrazol-1-ylalanine-negative species because additional enzyme(s) are almost certainly required for the formation of pyrazole itself. The absence of β-pyrazol-1-ylalanine from a wide range of species then may have more than one cause, for the failure to synthesize any one of several required enzymes would manifest itself in the same way as far as the present analytical techniques are concerned. The synthesis of γ-glutamyl-β-pyrazol-1-ylalanine (III) has been observed to be invariably associated with that of the parent amino acid and is probably attributable to the action of a γ-glutamyl transferase which exhibits a relatively low specificity in regard to the acceptor amino acid.

Several hypotheses may be advanced to explain the production of the N^4-substituted asparagines by only a very few of the species studied. Unfortunately, the nature and properties of the enzyme effecting the final step in their synthesis remain uncertain, although the weight of evidence gained from isotopic tracer experiments[16] indicates that asparagine, and not aspartic acid, forms the immediate precursor. If one assumes that the substituted amides are formed by an exchange of the amido-NH_2 group of asparagines for one of methylamine, ethylamine or ethanolamine, then the presence (or absence) of suitable concentrations of these amines within the members of the Cucurbitaceae at the time of seed maturation could be a crucial factor controlling the synthesis of the N^4-substituted derivatives. It is unfortunate that the literature contains no detailed account of the distribution of simple amines within this family of plants. Alternatively, the absence of substituted asparagines from most members of the family may simply reflect the fact that the synthetic enzyme system has a very limited distribution. Even where the synthetic enzyme is present problems of specificity are again apparent for either N^4-ethyl- and N^4-hydroxyethyl-asparagines occur together or N^4-methyl-asparagine is present alone: the co-existence of all three has not been observed.

Conceivably two separate enzymes are involved and exhibit different specificities towards the amine substrate, but the fact that different mixtures of simple amines may be available in different groups of plants could provide an effective control over the type of product obtained by the action of a single enzyme. The last concept will be tested by supplying different simple amines to seedlings of *Ecballium* which produce additional quantities of N^4-ethyl- and N^4-hydroxyethyl-asparagines during their early phases of growth.

METHODS

Preparation of Amino Acid Fractions

Finely ground seed (1 g) was shaken with 75% (v/v) ethanol (25 ml) for one day. The supernatant extract obtained after centrifuging was applied to a small column (12 × 0·8 cm) of Zeokarb 225 (H^+ form in 75% (v/v) ethanol) to retain the amino acids; after thorough washing the amino acids were eluted from the column with aqueous ethanolic 2N-NH_3 (25 ml). After concentration, volumes equivalent to 0·25 g of the original seed were applied to sheets of Whatman 3 MM chromatographic grade filter paper.

Chromatography

Two-dimensional chromatograms were developed with 75% (w/w) phenol in the presence of NH_3 vapours, followed by a 1-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: unless otherwise stated on Fig. 1, the spots were blue-purple in colour. Two other solvents were used to confirm the identity of N^4-methylasparagine and *m*-carboxyphenylalanine: they were the upper phase of an ethyl acetate: pyridine: water (2:1:2, v/v) and butan-1-ol saturated with 3N-NH_3 solution.

Identification of Amino Acids

Generally, amino acids were identified by the characteristic features shown by their spots on 2-dimensional chromatograms. Several substances, e.g. the substituted asparagines, cucurbitin, and unknown *Y*, gave spots of unusual colour after ninhydrin treatment, while others moved to positions remote from the remaining amino acids. Since *m*-carboxyphenylalanine (IV) was previously not known to exist in any member of the Cucurbitaceae, an isolation from a typical species was undertaken to confirm its identity with an authentic sample. For similar reasons N^4-methylasparagine (VII) was synthesized chemically to provide material for comparative chromatographic studies.

(a) *Isolation of* m-*carboxyphenylalanine (IV) from* E. elaterium. Seed (4 kg) was extracted with 75% (v/v) ethanol and the amino acid fraction separated as above using a squat column containing 10 lb Zeokarb 225 (mesh 60–100). Ammonia was removed from the eluate by evaporation to dryness *in vacuo* below 50°.

The residue was dissolved in 750 ml water, decolourized with charcoal and adjusted to pH 6. The solution was applied at a rate of 180 ml/hr to a column of Dowex-1 × 10, acetate form (mesh 100–200, length 50 cm, diam. 2 cm); the column was washed thoroughly to remove neutral and basic amino acids and then the retained acidic amino acids were eluted with 0·25 N acetic acid (flow rate 60 ml/hr). Fractions (12 ml) were collected and analysis showed tube numbers 33–42 contained glutamic acid, numbers 45–69 aspartic acid and numbers 99–130 *m*-carboxyphenylalanine. The latter group of fractions were combined and after concentration yielded 0·26 g crystals on keeping at 0°. After recrystallization from

70% (v/v) ethanol, the material was shown to be identical with *m*-carboxyphenylalanine (provided by Dr. J. F. Thompson, Ithaca) by co-chromatography in the four solvent systems above and by paper electrophoresis at pH 1·9, 3·45 and 7·0 (buffers as Bell[3]). (Found C, 52·6; H, 5·8; N, 6·3. Calc. for $C_{10}H_{11}NO_4.H_2O$: C, 52·8; H, 5·7; N, 6·2%.) The isolate had $[M]_D^{20}+33·5°$ (*c* 1 in H_2O); authentic material had $[M]_D^{25}+35·5°$. Although Thompson *et al.*[11] did not report a monohydrate form, our material when recrystallized from aqueous ethanol gave an i.r. spectrum identical in every respect with that of *m*-carboxyphenylalanine isolated from *Iris*.

(b) *Synthesis of N^4-methylasparagine (VIII).* 4-Ethyl hydrogen L-aspartate (β-ethyl aspartate) was prepared by the method of Curtis and Koch,[22] modified as Fowden.[15] The product (4 g) was mixed with 33% (w/w) methylamine in dry ethanol (20 ml) and heated at 100° for 8 hr in a thick-walled ampoule. Ethanol and residual methylamine were removed by evaporation and the residue dissolved in water (100 ml). This was fractionated on a Dowex-50 × 8 column (mesh 100–200, 20 × 1·5 cm diam.) to remove traces of aspartic acid and unchanged β-ethyl aspartate. The fractions containing N^4-methylasparagine were combined and evaporated and the residue recrystallized from 80% (v/v) ethanol to give a pure product (2·6 g). (Found: C, 41·3; H, 6·8; N, 18·9. Calc. for $C_5H_{10}N_2O_3$: C, 41·1; H, 6·9; N, 19·2%.)

N^4-propyl-, N^4-isopropyl-, N^4-isobutyl- and N^4-isoamyl-asparagines were synthesized by a similar technique but were not purified from the crude mixture of reaction products.

Acknowledgements—The isolation of *m*-carboxyphenylalanine from *Ecballium* seeds was performed by Dr. Teresa M. Poole. We wish to thank Mr. C. Jeffrey (Royal Botanic Gardens, Kew) for his valuable comments and guidance during the preparation of this manuscript. He was also helpful in suggesting many experts who subsequently supplied us with seed material. Finally our thanks are due to Mrs. Jean Retallick for technical assistance.

[22] T. CURTIS and F. KOCH, *J. Prakt. Chem.* **38**, 473 (1888).