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Dedicated to Prof. K. Mothes on the occasion of his seventieth birthday

A COMPARATIVE STUDY OF THE AMINO ACIDS AND PHENYLALANYL-tRNA SYNTHETASES OF *AESCULUS* SPP.

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Abstract—The distribution of two groups of structurally related, non-protein amino acids within the seeds of species of the genus *Aesculus* are recorded. Observed differences in amino acid composition allow the individual species to be assigned to five sub-generic groups that are identical in composition with the sections of *Aesculus* drawn up on the basis of morphological and geographical information. Species differences also were apparent when the kinetic properties of phenylalanyl-tRNA synthetases, isolated from species representing each of the five groups, were determined. The results of the amino acid survey, together with previous information relating to the composition of certain allied species, enable a re-assessment to be made of the degree of affinity existing between *Aesculus* and the allied genus, *Billia* (which together form the Hippocastanaceae), and the family Sapindaceae.

INTRODUCTION

PLANTS synthesize many types of amino acid in addition to the twenty compounds that form the basic constituents of protein molecules. A recent count[1] showed that nearly 200 such additional amino acids have been characterized as constituents of higher plants. As a group, they can be regarded as a further category of "secondary plant products", whose individual members show restricted distributions within species of the plant kingdom, some being characterized by infrequent, apparently haphazard occurrences, whilst others are produced by a relatively few closely allied species. Several publications have shown that a careful survey of the complement of such non-protein[2] amino acids in seeds can provide information useful in taxonomic studies, the chemical data proving valuable by strengthening, or slightly modifying, previous classifications reached on the basis of morphological or cytological criteria. Examples of this approach are seen in the publications of Bell and co-workers in relation to the genera *Lathyrus*[3] and *Vicia*,[4] of Turner and Harborne who surveyed the distribution of canavanine in the Papilionoideae,[5] and of Fowden and co-workers who directed attention to *Acacia* spp.[6] and to members of the family Cucurbitaceae.[7]

Knowledge concerning another group of structurally related amino acids has been assembled gradually as surveys have been made of plants forming the families Sapindaceae and Hippocastanaceae. The close affinity of these two families is widely recognized and, as late

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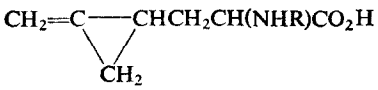
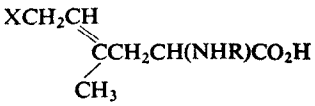
[1] L. FOWDEN, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, John Wiley, London, in press.
[2] L. FOWDEN, *Endeavour* **21**, 35 (1962).
[3] E. A. BELL, *Biochem. J.* **83**, 225 (1962).
[4] E. A. BELL and A. S. L. TIRIMANNA, *Biochem. J.* **97**, 104 (1965).
[5] B. L. TURNER and J. B. HARBORNE, *Phytochem.* **6**, 863 (1967).
[6] A. S. SENEVIRATNE and L. FOWDEN, *Phytochem.* **7**, 1039 (1968).
[7] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 933 (1965).

as 1926, Hutchinson[8] included the present Hippocastanaceae (consisting of the two genera, *Aesculus* and *Billia*) within the Sapindaceae. The amino acids can be divided into two groups, one based broadly on a branched C_7 skeleton, whilst the other group possess C_6 structures. Many of the individual compounds contain cyclopropyl residues.

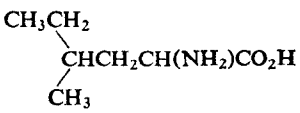
The first of these unusual amino acids were isolated from unripe fruits of Akee (*Blighia sapida*, family Sapindaceae): they were characterized as β-(methylenecyclopropyl)alanine (Ia, hypoglycin A) and its γ-glutamyl peptide (Ib, hypoglycin B).[9] Seed of *Aesculus californica* contains small amounts of the related β-methyl-β-(methylenecyclopropyl)alanine (IV),



(I) a: R = H
b: R = γ-glutamyl



(II) a: R = X = H
b: R = H; X = OH
c: R = γ-glutamyl; X = H



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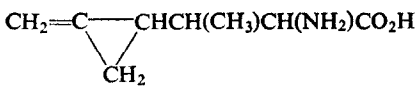

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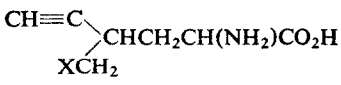
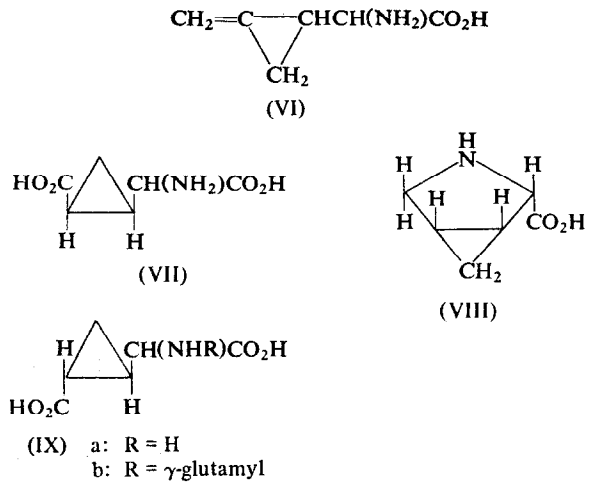


(V) a: X = H
b: X = OH

SCHEME 1. AMINO ACIDS BASED ON A C_7 STRUCTURE.

together with a high concentration of 2-amino-4-methylhex-4-enoic acid (IIa): other minor constituents of this seed are 2-amino-6-hydroxy-4-methylhex-4-enoic acid (IIb), the γ-glutamyl peptide (IIc) of 2-amino-4-methylhex-4-enoic acid, and 2-amino-4-methylhexanoic acid (III).[10] Two further amino acids possessing the same branched C_7 skeleton have been isolated from seed of *Euphoria longan* (Sapindaceae) and characterized as 2-amino-4-methylhex-5-ynoic acid (Va) and 2-amino-4-hydroxymethylhex-5-ynoic acid (Vb).[11]

[8] J. HUTCHINSON, *The Families of Flowering Plants. I. Dicotyledons*, p. 252, MacMillan, London (1926).
[9] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* **80** (1959).
[10] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[11] M-L. SUNG, L. FOWDEN, D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **8**, 1227 (1969).



SCHEME 2. AMINO ACIDS BASED ON A C_6 STRUCTURE.

The compounds forming the C_6 group all contain a cyclopropyl residue. α-(Methylenecyclopropyl)glycine (VI), the lower homologue of hypoglycin A, was isolated first from seed of *Litchi chinensis* (Sapindaceae).[12] Later, *cis*-α-(carboxycyclopropyl)glycine (VII) and *cis*-(*exo*)-3,4-methanoproline (VIII) were obtained from seed of *A. parviflora*.[13] These two compounds show a structural relationship identical with that existing between glutamic acid and proline, and VII would seem to be the natural biogenetic precursor of VIII. The diastereoisomeric form of VII, i.e. *trans*-α-(carboxycyclopropyl)glycine (IXa), is present in significant amounts in seed of *Blighia sapida*,[13] whilst its γ-glutamyl peptide (IXb) forms a minor component of the same seed.[14]

This paper describes the results of a detailed survey of the amino acids, especially those just described, occurring in seeds of species forming the genus *Aesculus*. The survey included ten of the total of thirteen spp. considered by Hardin[15,16] to constitute the genus. Recently, data also have become available concerning the amino acid composition of seed of *Billia hippocastanum*.[17] This new phytochemical information, when considered together with comparative kinetic values derived for phenylalanyl-tRNA synthetase enzymes from five spp. of *Aesculus*,[18] is interpreted as supporting the sub-generic divisions suggested by Hardin. However, the resulting pattern of amino acid distribution suggests that a re-appraisal of the status of the families Sapindaceae and Hippocastanaceae now may be desirable.

The last major revision of the genus *Aesculus* was made by Hardin,[15,16] who concluded that the genus consists of thirteen true spp., which he divided into five sections: Parryaneae, Aesculus, Calothyrsus, Pavia, and Macrothysus. The distribution of spp. between the five sections is shown in Table 1. However, a prevalence of hybrid forms exist, some of which represent important ornamental trees, e.g. *A. carnea* (*A. hippocastanum* × *A. pavia*). The species forming the section Pavia, *A. glabra*, *A. octandra*, *A. sylvatica*, and *A. pavia*, also

[12] D. O. GRAY and L. FOWDEN, *Biochem. J.* **82**, 385 (1962).
[13] L. FOWDEN and A. SMITH, *Phytochem.* **8**, 437 (1969).
[14] L. FOWDEN and A. SMITH, *Phytochem.* **8**, 1043 (1969).
[15] J. W. HARDIN, *Brittonia* **9**, 173 (1957).
[16] J. W. HARDIN, *Brittonia* **12**, 26 (1960).
[17] J. N. ELOFF and L. FOWDEN, *Phytochem.* **9**, 2423 (1970).
[18] J. W. ANDERSON and L. FOWDEN, *Biochem. J.* in press.

rather readily yield interspecific hybrids. Hardin[15] points to a large amount of natural variation within *A. glabra*, and attributes much of this to introgression from *A. pavia* and *A. octandra*. The section Parryaneae contains the single species, *A. parryi*, which is isolated geographically from all other *Aesculus* species being native in north-western Baja California, Mexico. Section Aesculus contains two spp., *A. hippocastanum* and *A. turbinata*, both now being widely cultivated trees. The former is endemic to the Balkans while the latter is native only in Japan. Calothyrsus contains five spp., of which all except *A. californica* are native in India and China: the four Asiatic spp. are themselves geographically separated.

TABLE 1. THE DISTRIBUTION OF SOME NON-PROTEIN AMINO ACIDS IN SEEDS OF *Aesculus* SPP. AND HYBRIDS*

Section and spp.	IIa	IIb	IIc	III	IV	VII	VIII	IXa	A	B	C
Parryaneae											
A. parryi	0	0	0	0	0	S	0	W	W	T	W
Aesculus											
A. hippocastunum	0	0	0	0	0	0	0	0	T	W	0
A. turbinata	0	0	0	0	0	0	0	0	T	W	0
(*A. carnea*)	0	0	0	0	0	T	0	0	T	W	0
Calothyrsus											
A. californica	S†	W	W	W	W	0	0	0	0	0	0
A. indica	0	0	W	W	0	0	0	0	T	W	0
Macrothyrsus											
A. parviflora	0	0	0	0	0	M	S†	T	W	W	0
Pavia											
A. glabra var. *glabra*	0	0	0	0	0	M	0	W	T	W	0
A. glabra var. *arguta*	0	0	0	0	0	M	0	M	W	W	0
A. octandra	0	0	0	0	0	W	0	W	W	W	0
A. sylvatica	0	0	0	0	0	M	0	W	M	T	0
A. pavia	0	0	0	0	0	T	0	T	T	W	0
(*A. hybrida*)	0	0	0	0	0	T	0	T	M	T	0
(*A. arnoldiana*)	0	0	0	0	0	T	0	T	M	W	0

Symbols denoting compounds, e.g. IIa, IIb, etc. are defined in Introduction.

* Symbols denote the relative strengths of chromatographic spots: S, strong; M, medium; W, weak; T, trace; 0, not detected.

† Denotes that the compounds form the predominant spot on the chromatograms.

Hybrid spp. are shown in parentheses.

A. chinensis may simply be a cultivated form of *A. wilsonii*.[16] Finally, Macrothyrsus represents another section with only a single genus, *A. parviflora*, which is endemic to Alabama and Georgia. A later-flowering and glabrescent form is infrequently found throughout the entire range of the spp. and has been designated as the forma *serotina*.

RESULTS AND DISCUSSION

Amino acid distribution studies were performed on cationic fractions prepared from aqueous-ethanolic extracts of seeds. Normally, an aliquot of the cationic fraction, equivalent to 0·5 g seed, was separated on two-dimensional paper chromatograms to reveal the individual amino acid constituents. Routinely, phenol–NH_3 was used as the first solvent and a butan-

1-ol–acetic acid–water mixture as the second. The location of amino acids I–IX on developed chromatograms is shown diagrammatically in Fig. 1: some other amino acids usually present in seed extracts are shown for reference.

The distribution of non-protein amino acids within the seed of *Aesculus* species is shown in Table 1. Normally, alanine and glutamic acid represented the principal free amino acids present in the seed extracts, but 2-amino-4-methylhex-4-enoic acid (IIa) and *cis*-3,4-methanoproline (VIII) were predominant in *A. californica* and *A. parviflora*, respectively. The amino

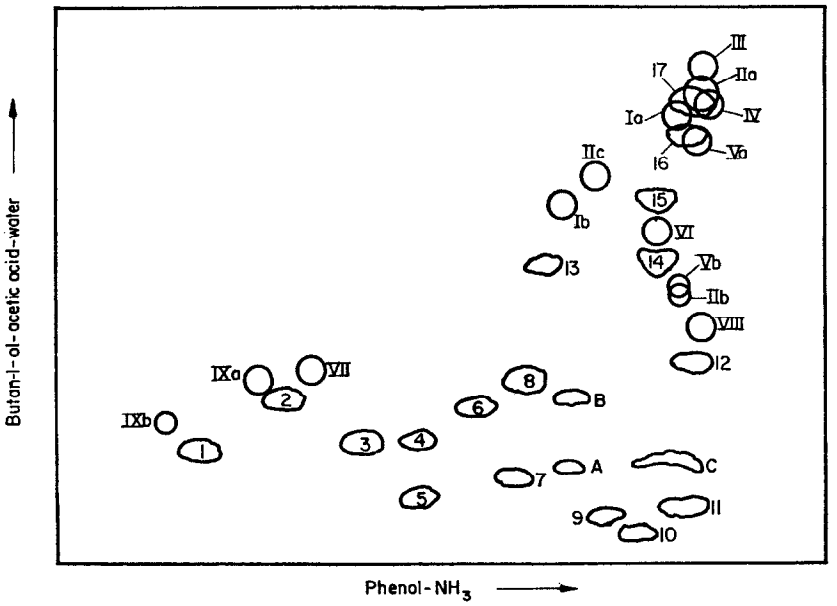


FIG. 1. DIAGRAMMATIC REPRESENTATION OF POSITIONS OF AMINO ACIDS I–IX ON A TWO-DIMENSIONAL CHROMATOGRAM IN RELATION TO COMMONLY OCCURRING COMPOUNDS.

Key to chromatographic spots: 1, aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, asparagine; 6, threonine; 7, glutamine; 8, alanine; 9, histidine; 10, lysine; 11, arginine; 12, proline; 13, tyrosine; 14, γ-aminobutyric acid; 15, valine; 16, phenylalanine; 17, leucine.

Ia, hypoglycin A; Ib, hypoglycin B; IIa, 2-amino-4-methylhex-4-enoic acid; IIb, 2-amino-6-hydroxy-4-methylhex-4-enoic acid; IIc, γ-glutamyl peptide of IIa; III, homoisoleucine; IV, β-methyl-β-(methylenecyclopropyl)alanine; Va, 2-amino-4-methylhex-5-ynoic acid; Vb, 2-amino-4-hydroxy-methylhex-5-ynoic acid; VI, α-(methylenecyclopropyl)glycine; VII, *cis*-α-(carboxycyclopropyl)-glycine; VIII, *cis*-3,4-methanoproline; IXa, *trans*-α-(carboxycyclopropyl)glycine; IXb, γ-glutamyl peptide of IXa.

acids designated A and B in Fig. 1 were present in low concentration in all species, with the possible exception of *A. californica*. A was probably citrulline, a compound isolated from seed of *Blighia sapida*,[19] but B remains uncharacterized.

Sharp differences of amino acid composition were found between species assigned to the different sub-generic sections by Hardin,[15,16] but an extremely close similarity of composition was apparent between those species collected together in the same section. Thus, the two spp. forming the section Aesculus have an almost identical but rather unremarkable composition and contained no representatives of the groups of C_6 and C_7 amino acids I–IX.

[19] L. FOWDEN and A. SMITH, unpublished results.

This feature allowed them to be distinguished from all other spp. All members of the section Pavia, some of which can form hybrids with *A. hippocastanum*, synthesize both the *cis*- and *trans*-diastereoisomers of α-(carboxycyclopropyl)glycine (VII and IXa). *A. parryi* (Parryaneae) exhibits a similar pattern of amino acid components to that characteristic of the section Pavia, but it can be distinguished by the presence of an additional amino acid C in seed extracts. C can be recognized readily because it invariably appears on chromatograms as a spot quite elongated in the direction of phenol solvent flow. *A. parviflora* (Macrothyrsus) can be identified immediately after inspection of the amino acid chromatogram obtained from its seed extract, for it alone of the *Aesculus* species examined produces *cis*-3,4-methanoproline (VIII), which appears as a brilliant yellow spot after treatment with ninhydrin. The spot has an almost luminous character which allows 3,4-methanoproline to be rather easily distinguished from the duller yellow spot of proline. The remaining section, Calothyrsus, has been studied incompletely, since seed of *A. assamica*, *A. chinensis* and *A. wilsonii* was not available. However, the other two species of this section, *A. californica* and *A. indica* again can be distinguished from all other species on the basis of amino acid composition of seeds. They do not synthesize the C_6 cyclopropyl amino acids that are characteristic of most other species, but they alone of the *Aesculus* species produce branched-chain C_7 amino acids. In contrast to *A. californica*, which elaborates five such compounds (IIa–IV) and accumulates IIa in very high concentration, only two of the compounds (IIc and III) were identified as seed components of *A. indica*. However, all five of these compounds may be presumed to be products (in part sequential) arising from a common initial biogenetic pathway. In the case of the section Calothyrsus, it then seems more important to seek evidence for the operation of a particular biogenetic pathway rather than for all individual products of the pathway. The same view was taken by Bell and co-workers during their study of the amino acids of legume genera.[3,4]

The hybrid form, *A. carnea*, generally resembles the spp. of the section Aesculus, to which one parent *A. hippocastanum* is assigned: however its seed does contain a trace amount of *cis*-α-(carboxycyclopropyl)glycine (VII), a compound characteristic of the other parent, *A. pavia* (section Pavia). *Trans*-α-(carboxycyclopropyl)glycine (IXa), another normal component of members of the section Pavia, could not be detected in the hybrid seed. Other hybrids examined included *A. hybrida* (*A. octandra* × *A. pavia*) and *A. arnoldiana* (*A. hybrida* × *A. glabra*): each hybrid contained the *cis*- and *trans*-α-(carboxycyclopropyl)glycines in their seed in amounts comparable with those present in the respective parent spp., which are all drawn from the section Pavia. Hardin[15] differentiates just two varieties of *A. glabra*, i.e. *A. glabra* var. *glabra* and *A. glabra* var. *arguta*; they overlap geographically in a narrow zone of Missouri and Arkansas, where they are rather difficult to distinguish. Apart from perhaps a slightly higher concentration of *trans*-α-(carboxycyclopropyl)glycine (IXa) present in the variety *arguta*, chromatograms prepared from the two varieties were almost indistinguishable. Similarly, no difference in composition was evident between *A. parviflora* and *A. parviflora* f. *serotina*.

The disparate geographical distribution of members of the section Calothyrsus (*A. californica* in California, and the other four spp. in South East Asia) reflects a possible subdivision of the section. Undoubtedly, the morphological interrelationships between species forming the Asiatic group are extremely close, whilst *A. californica* differs from the others in a number of subtle characters. The amino acid data reported in Table 1 tend to support this distinction, although analyses are available only for *A. californica* and *A. indica*. Differences, both qualitative and quantitative, are seen in the levels of the individual branched-chain

amino acids (IIa–IV) and in the content of A and B recorded for the two species; the differences are considerably more significant than those encountered between spp. forming the section Pavia. After a careful consideration of the interspecific relationships between the spp. assigned to the section Pavia, Hardin[15] realized that a case could be established for the creation of two sub-sections: *A. glabra* would form one of the sub-sections, whilst *A. octandra*, *A. sylvatica* and *A. glabra* would compose the second. However, he concluded that on balance the evidence was not sufficiently strong to warrant the introduction of two *named* sub-sections. The evidence now presented relating to the amino acid composition of these spp. provides no grounds for such a division for all spp. possess an essentially similar composition.

Hardin,[16] also gives his views on the origin of the *Aesculus* spp. in relation to their present distribution. He considered they had a centre of origin in Central or South America in a *Billia*-like ancestor: northward migration before and during the Tertiary period resulted in a wide and almost continuous distribution in the Tertiary forests which then linked Europe, Asia and N. America. The spp. forming the section Pavia probably were derived most recently from ancestral forms surviving in the Tertiary forests of the Appalachian region. Section Macrothyrsus could have arisen in the highlands of Mexico and then migrated to the eastern United States parallel with Pavia types. The part of the ancestral population that was isolated in the western part of the United States led to *A. parryi* and *A. californica*, and to the species now in the Old World. The ancestral forms present in the Old World presumably contained two elements; one eventually gave rise to the four Asiatic spp. of section Calothyrsus, whilst the other led to the present spp. of section Aesculus. Against this background, it is fascinating to note that an analysis of seed of *Billia hippocastanum*[17] showed the presence of hypoglycin A (Ia) and hypoglycin B (Ib) together with α-(methylenecyclopropyl)glycine (VI), i.e. representatives of the separate classes of C_6 and C_7 branched-chain amino acids occur together in this spp., although no example of the co-existence of these two types of amino acid is evident among the present spp. of the genus *Aesculus*. This could suggest that during evolution from a *Billia*-type ancestor, the principal features of one or other, but not both, of two ancient biogenetic pathways have been retained. The compounds representative of the C_6 and C_7 groups now present in *Aesculus* spp. show subtle structural differences from those of *Billia*, e.g. the exocyclic methylene-C of α-(methylenecyclopropyl)glycine in *Billia* occurs as a fully oxidized carboxyl-C in several *Aesculus* spp., whilst hypoglycin A (*Billia*) has been modified by the substitution of an additional β-methyl-C in *A. californica*. In both of these examples, the *Billia* compounds can be envisaged as possible biogenetic precursors of the *Aesculus* products; this would presuppose that the evolution of *Aesculus* spp. was associated with the production of additional enzymes whose function was to catalyse oxidation and C_1-transfer processes, respectively.

On the basis of morphological evidence, section Macrothyrsus is considered to be more closely related to section Calothyrsus, than to section Pavia,[16] although the present geographical distribution of species does not support this view. The amino acid data given in Table 1 also contradict this view, for whilst species assigned to the sections Pavia and Macrothyrsus all synthesize the C_6 group of compounds, those forming Calothyrsus do not; instead they produce compounds having the basic C_7 skeleton. *A. parviflora* (Macrothyrsus) obviously has evolved an enzyme complex, not present in any of the species of the Pavia section, which effects the synthesis of *cis*-3,4-methanoproline from *cis*-α-(carboxycyclopropyl)glycine.

The kinetic data derived as a result of a comparative study of the properties of phenylalanyl-tRNA synthetases also bear on the problem of inter-sectional relationships. Table 2 presents information concerning the substrate affinities and relative limiting velocities of the

ATP-[^{32}P] pyrophosphate exchange reaction measured for five enzyme preparations, i.e. phenylalanyl-tRNA synthetases purified from seed of a representative spp. of each of the five sections of the genus *Aesculus*. K_m values are given not only for the normal amino acid substrate phenylalanine, but also for 2-amino-4-methylhex-4-enoic acid (IIa) which behaves as an alternative (analogue) substrate. Clearly, the K_m values derived for phenylalanine and for IIa are in closer agreement for *A. parviflora* and *A. glabra*, than they are for *A. parviflora* and *A. californica*. The relative degree to which IIa is accepted as an alternative substrate at enzyme-saturating concentrations [see $(V_{IIa}/V_{Phe}) \times 100$ values, Table 2], is almost the same for enzymes prepared from *A. parviflora* and *A. glabra*, whereas the value derived for *A. californica* enzyme differs considerably. The more discriminatory nature of the phenylalanyl-tRNA synthetase from *A. californica* towards IIa extends to a range of other analogues possessing structural features akin to those found in the phenylalanine molecule. Whilst a fuller description of the biological significance of these findings is presented elsewhere,[18]

TABLE 2. KINETIC VALUES DERIVED FOR PHENYLALANYL-tRNA SYNTHETASE PREPARATIONS FROM VARIOUS *Aesculus* SPP.

	K_m ATP (mM)	K_m Phe (mM)	K_m IIa (mM)	k_m IIa/k_m Phe	$V_{IIa}/V_{Phe} \times 100$
Calothyrsus					
A. californica	0·33	0·105	3·19	30	30
Pavia					
A. glabra	0·36	0·049	1·78	36	90
Aesculus					
A. hippocastanum	0·28	0·031	1·18	38	100
Parryaneae					
A. parryi	0·38	0·019	0·89	47	100
Macrothyrsus					
A. parviflora	0·28	0·016	0·68	43	100

Abbreviations used: ATP, adenosine triphosphate; Phe, phenylalanine; IIa, 2-amino-4-methylhex-4-enoic acid. K_m, Michaelis constant; V, limiting velocity of phenylalanine-dependent ATP-[^{32}P]pyrophosphate exchange reaction catalysed by enzymes.

it is clear that this enzymic study is in general agreement with the conclusion reached on the basis of amino acid distribution studies, namely that the section Macrothyrsus resembles more closely the section Pavia, rather than the section Calothyrsus.

When the distribution of amino acids is viewed in the wider context of relationships between the families Sapindaceae and Hippocastanaceae, it is quite clear that on these chemical grounds any split of the genera into two families is quite artificial. A complex network of inter-related biosynthetic processes spans the genera, although no one species elaborates all possible products resulting from these pathways. *Billia hippocastanum* produces compounds, hypoglycins A and B and α-(methylenecyclopropyl)glycine, that form important components of the free amino acid pool of seed of the sapindaceous genera, *Blighia* and *Litchi*, respectively. There would seem also to be more similarity between the amino acid biogenetic potential of (i) *Aesculus* species of the section Calothyrsus and the genera *Blighia* and *Euphoria*

(Sapindaceae); and of (ii) *Aesculus* spp. assigned to the section Pavia and *Litchi chinensis* (Sapindaceae), than exists between the spp. forming the various sections of the *Aesculus* genus. It would be revealing to have the views of authoritative taxonomists on those observations, more especially because opinions obviously were divided until relatively recent times.

EXPERIMENTAL

Amino Acid Extraction and Chromatography

Ground seed material was extracted with 75% (v/v) ethanol (10 ml/g seed). The clarified extracts were treated on small Zeokarb-225 cation-exchange resin columns to obtain an amino acid fraction.[7] After concentration, volumes containing the amino acids from 0·5 g of original seed were applied to sheets of Whatman 3MM chromatographic-grade filter paper. The chromatograms were developed in the first direction 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) as second solvent. Spots were revealed using 0·1% ninhydrin in ethanol. Occasionally, one-dimensional chromatograms were developed in *tert*-amyl alcohol–acetic acid–water (20:1:20, v/v; upper phase): this solvent effects a better resolution of those amino acids having relatively high R_fs in butan-1-ol–acetic acid–water.

Identification of Amino Acids

Generally, amino acids were identified by their position on chromatograms and by other characteristic features. Several substances gave spots of an unusual colour after ninhydrin treatment, e.g. unsaturated compounds such as IIa and IIb gave yellow-brown chromophores, VI gave a brown-purple colour, whilst VIII reacted to yield a brilliant yellow colour. In contrast to proline which reacts sensitively with isatin to give a blue chromophore, *cis*-3,4-methanoproline (VIII) gave no colour with isatin but tended to bleach the natural orange-yellow colour of the reagent. The identity of certain other substances could be confirmed by checking the nature of their reduction or hydrolysis products. For instance, α-(methylenecyclopropyl)glycine yields a mixture of leucine, norleucine and *allo*isoleucine after catalytic hydrogenation, whilst hypoglycin A gives a similar series of homoleucines after hydrogenation. Hydrolysis with 2 N-HCl at 100° for 2 hr converts γ-glutamyl peptides such as Ib, IIc and IXb into mixtures of glutamic acid and the appropriate second amino acid product.

Studies with Phenylalanyl-tRNA Synthetases

A full description of the methods used to purify and assay the phenylalanyl-tRNA synthetases from the different *Aesculus* species has been given by Anderson and Fowden.[18]

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