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BIOSYNTHESIS OF 2-AMINO-4-METHYLHEX-4-ENOIC ACID IN *AESCULUS CALIFORNICA*: THE PRECURSOR ROLE OF ISOLEUCINE

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Abstract—The biosynthesis of 2-amino-4-methylhex-4-enoic acid in *Aesculus californica* has been studied using ^{14}C-labelled precursor feeding techniques. Isoleucine was the most effective precursor tested, but its carboxyl-carbon atom was not incorporated. Two alternative biosynthetic mechanisms consistent with these observations are discussed.

INTRODUCTION

SEED of *Aesculus californica* (California buckeye) contains four unusual amino acids and one related γ-glutamyl peptide.[1] Quantitatively, the most significant of these compounds is 2-amino-4-methylhex-4-enoic acid (I, AMHA), present in fresh mature seed at a level of about 5g/kg, which represents approximately half of the total soluble-nitrogen fraction present in seeds. 2-Amino-4-methylhexanoic acid (II, a homoisoleucine) forms a very minor component of the free amino acid pool. This report presents evidence concerning the biogenetic origin of the branched C_7 skeleton common to these two amino acids.

A mechanism effecting carbon chain elongation is responsible for the formation of leucine from valine,[2] of homomethionine from methionine,[3] and of γ-phenylbutyrine from phenylalanine[4] in various plant systems. On this basis, isoleucine can be envisaged as a possible precursor of homoisoleucine (II) and of AMHA following dehydrogenation. Another possible biosynthetic route could involve the transfer of a C_1-group from methionine, or from serine, and its attachment to one of the terminal C-5 methyl groups of leucine. A less likely possibility is that AMHA arises by the transfer of a methyl group to 2-aminohexa-4,5-dienoic acid, an allenic amino acid isolated from the fungus, *Amanita solitaria*.[5] In reporting the isolation of this allene, the authors made the suggestion that it might act as a precursor of hypoglycin A [β-(methylenecyclopropyl)alanine], which possesses a branched C_7 structure essentially similar to that of AMHA. These and other alternative possibilities

[1] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[2] L. FOWDEN, in *Plant Biochemistry* (edited by J. BONNER and J. A. VARNER), p. 361, Academic Press, New York (1965).
[3] M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem.* **42**, 1033 (1964).
[4] E. W. UNDERHILL, *Can. J. Biochem.* **43**, 179 (1965).
[5] W. S. CHILTON, G. TSOU, L. KIRK and R. G. BENEDICT, *Tetrahedron Letters* 6283 (1968).

have been examined by supplying ^{14}C-labelled precursors to *A. californica* and measuring the extent to which each was incorporated into AMHA and homoisoleucine.

RESULTS AND DISCUSSION

The first attempts to investigate the biosynthesis of AMHA were performed using young, rapidly expanding leaves attached to seedling trees of *A. californica* grown in a glasshouse in London. However, the concentration of AMHA in the leaves of the plant was very low in comparison with that associated with the maturing seed and negligible radioactivity was present in AMHA after leaves had assimilated $^{14}CO_2$ for either a 1- or 2-week period. Neither [U-^{14}C]glucose nor [U-^{14}C]isoleucine effected more than a trace labelling of AMHA when infiltered into leaf petioles over a period of a week. Although ^{14}C-label was translocated throughout the five leaflets of the compound leaves, this failure to observe any significant labelling of AMHA from compounds that rapidly label a wide range of intermediary carbon metabolites in growing plants indicated that growing leaves were not an important site for the synthesis of AMHA.

Further biosynthetic investigations employed developing seeds. Solutions containing ^{14}C-labelled precursors were infiltered via a cotton wick into the short stalks subtending individual buckeye fruits. Normally, the uptake of labelled compounds was completed in a few hours. Almost all the absorbed radioactivity was moved rapidly by translocation processes into the fruits, where metabolism was permitted to proceed for 7 days before harvest and extraction. To ensure that labelled precursor feeding coincided with an active period of amino acid biosynthesis, the procedure was repeated four times at approximately 14-day intervals during July and August as fruits developed on trees growing at Davis, California. On each occasion, glucose formed one of the ^{14}C-compounds supplied. By ensuring the distribution of label widely among compounds of the carbon intermediary pool, the feeding of relatively large quantities of ^{14}C-glucose should mark those periods of active synthesis of AMHA, irrespective of the biosynthetic pathway involved.

The percentage incorporation of ^{14}C-label into AMHA from each compound examined is recorded in Table 1. The experiments in which labelled glucose was provided indicated that synthesis of AMHA became a more dominant feature of metabolism as fruits developed. The same trend is apparent when the incorporation of ^{14}C from other labelled substances is examined.

The possibility that homoisoleucine and AMHA arise by the transfer of a C_1 group from either methionine or serine to a leucine skeleton was discounted by these radioisotopic tracer experiments. No ^{14}C-label was detected in either of the C_7 amino acids after metabolism of [U-^{14}C]leucine, [methyl-^{14}C]methionine or [3-^{14}C]serine. In contrast, ^{14}C-label was incorporated into AMHA more efficiently from [U-^{14}C]isoleucine than from any other compound tested. In the experiment with stage 3 fruits, activity incorporated into homoisoleucine from [U-^{14}C]isoleucine was approximately 1 per cent of that in AMHA: the concentration of AMHA was slightly more than a hundred times that of homoisoleucine, so the two amino acids had acquired almost equal specific activities.

The complete absence of label from AMHA following [1-^{14}C]isoleucine feeding indicated that the carboxyl-C atom was lost, presumably by decarboxylation, during biosynthesis. The remaining C_5-skeleton, together with a two-carbon fragment from acetate, could furnish the required branched C_7 carbon chain common to homoisoleucine and AMHA. In practice, ^{14}C from acetate, supplied either as the methyl- or carboxyl-labelled forms, was incorporated into AMHA. As commonly found in acetogenesis, more activity was incorporated from

TABLE 1. THE INCORPORATION OF ^{14}C-LABELLED COMPOUNDS INTO AMHA AT VARIOUS STAGES OF FRUIT DEVELOPMENT

Fruit growth stage	1	2	3	4
Fruit f. wt. (g.)	3–5	15–23	46–55	65–85
^{14}C-Labelled precursor*	% ^{14}C Incorporation into AMHA			
D-[U-^{14}C]Glucose (200μc)	t	0·029	0·045	0·051
[1-^{14}C]Acetate (125μc)	t	0·005	—	0·008
[2-^{14}C]Acetate (125μc)	t	0·012	—	0·037
L-[U-^{14}C]Isoleucine (50μc)	0·0013	0·13	0·51	0·42
L-[1-^{14}C]Isoleucine (50μc)	—	0	—	0
L-[U-^{14}C]Leucine (50μc)	0	0	0	0
L-[methyl-^{14}C] Methionine (50μc)	0	0	0	0
L-[3-^{14}C]Serine (50μc)	—	—	—	0
[1-^{14}C]Glycine (50μc)	—	—	—	0·003
[2-^{14}C]Glycine (50μc)	—	—	—	0·012
DL-[2-^{14}C]-2-Aminohexa-4,5-dienoic acid (40μc)	—	—	—	0·009

Data are expressed as % of the radioactivity supplied entering AMHA during a 1-week metabolic period: in the case of 2-aminohexa-4,5-dienoic acid, percentage incorporations are calculated on the basis of the L-form only.

t = Detectable activity but less than 0·001 % incorporation.

— Experiments in which particular ^{14}C-precursor was not supplied.

* The specific activity of the labelled compounds was 20μc/μmole, except [1-^{14}C] isoleucine (10μc/μmole) and [2-^{14}C]-2-aminohexa-4,5-dienoic acid (6·1μc/μmole).

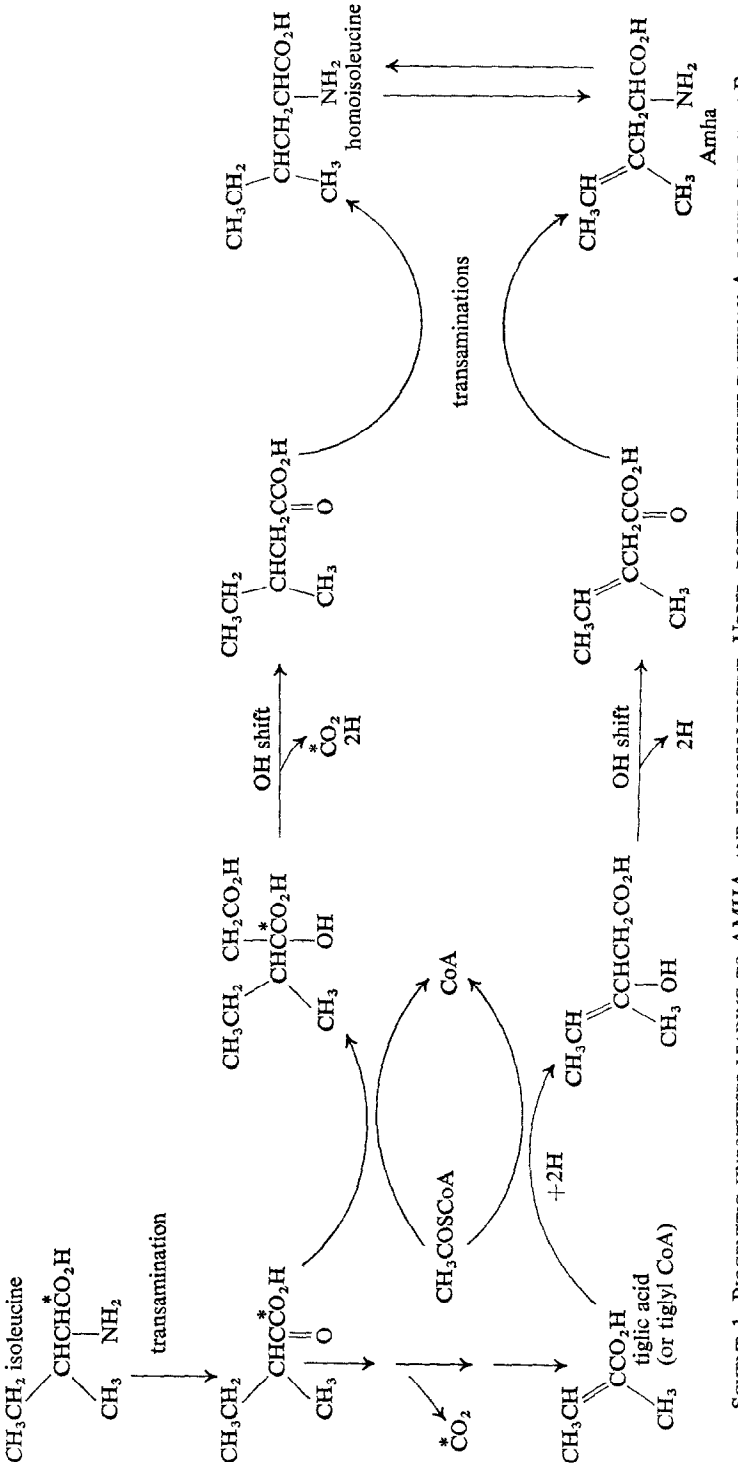
[2-^{14}C]-acetate than from [1-^{14}C]-acetate, presumably because the carboxyl-carbon of the acetate molecule was lost from the system as respiratory $^{14}CO_2$ considerably more rapidly than the methyl-C atom. A specific labelling of the C-1 and C-2 atoms of AMHA from [1-^{14}C]- and [2-^{14}C]-acetate, respectively, would not be anticipated because acetate also serves as a precursor of the isoleucine skeleton.[2] Incorporation of radioactivity from specifically labelled glycines into AMHA was less than that from correspondingly labelled acetates. It is then unlikely that glycine is utilized as an intact unit to provide the polar end of the AMHA molecule.

The allenic 2-aminohexa-4,5-dienoic acid was infiltered only into stage 4 fruits. Analysis after a 7-day metabolic period showed the compound to be largely unchanged, although a slight labelling of AMHA (see Table 1) and a somewhat stronger labelling (0·05 ^{14}C-incorporation) of norleucine had occurred. The low degree of incorporation into AMHA was not consistent with the view that the allenic amino acid might act as a direct precursor.

The maximum incorporation of isotope from isoleucine into AMHA was about 0·5 per cent during the normal assimilation period of 1 week. This degree of incorporation compares very favourably with those reported for many biogenetic investigations concerned with alkaloids, but it is low when compared with reported conversions of certain amino acids into cyanogenetic glucosides.[6] However, it is pertinent to remember that seeds approaching their mature size are synthesizing protein intensively and that this process represents a major sink for the infiltered ^{14}C-isoleucine.

When young (stage 1) fruits were examined, the incorporation of radioactivity into AMHA was very low, irrespective of the ^{14}C-compound supplied (Table 1). However,

[6] E. E. CONN and G. W. BUTLER, in *Perspectives in Phytochemistry* (edited by J. B. HARBORNE and T. SWAIN), p. 47, Academic Press, London (1969).



SCHEME 1. BIOGENETIC HYPOTHESES LEADING TO AMHA AND HOMOISOLEUCINE. UPPER ROUTE REPRESENTS PATHWAY A, LOWER PATHWAY B.

several other amino acids became labelled in these young fruits after metabolism of leucine, isoleucine or methionine (Table 2). At this developmental stage, intermediary metabolic processes of a catabolic nature must be relatively more important, in comparison with protein synthesis, than they are in the maturing seeds. The data in Table 2 shows that the pattern of labelling of amino acids after ^{14}C-leucine feeding was very similar to that arising after supplying [2-^{14}C] acetate. This result conforms to the idea that leucine can undergo a stepwise degradation that leads principally to acetate. By comparison, carbon from isoleucine and the methyl group of methionine is introduced into other amino acids in a more restricted manner.

TABLE 2. THE DISTRIBUTION OF ^{14}C FROM VARIOUS LABELLED COMPOUNDS SUPPLIED TO STAGE 1 FRUITS AMONG SOME COMPONENTS OF THE SOLUBLE-NITROGEN FRACTION

Labelled compound supplied	% ^{14}C Incorporation into Asp	GluA	$GluNH_2$	Ala	Ser	Gly	Val	Leu/Isol	γ-AB
[1-^{14}C]Acetate (125μc)	t	t	0·009	0·002	t	t	t	0·003	t
[2-^{14}C]Acetate (125μc)	0·003	0·003	0·022	0·007	0·002	0·003	0·001	0·006	0·003
L-[U-^{14}C]Leucine (50μc)	0·002	0·002	0·017	0·004	t	t	t	0·004	0·003
L-[U-^{14}C]Isoleucine (50μc)	0	t	0·002	t	0	0	0	†	0
L-[Methyl-^{14}C] methionine (50μc)	0·002	0	0	0	0·002	0	0	0	0

Data are expressed as % of the radioactivity supplied entering each amino acid during a 1-week metabolic period.

* Specific activity of all compounds was 20μc/μmole.

† Residual radioactivity of ^{14}C-isoleucine supplied.

t = Detectable activity but less than 0·001 % incorporation

The labelling experiments performed to date have eliminated a number of projected pathways and indicated that isoleucine is probably an important precursor from which biosynthesis of the branched C_7 amino acids commences. The detail of the intermediary steps has not been revealed except that a mechanism eliminating the carboxyl-carbon of isoleucine must be implicated. Other experiments have shown that a transamination reaction may occur as the last step in the synthesis of AMHA.[7] Using these guidelines, two alternative pathways may be formulated for the synthesis of AMHA and homoisoleucine (see Scheme 1): the pathways implicate either reactions known to be involved in isoleucine metabolism in plants or reaction sequences having established parallels in the intermediary metabolism of protein amino acids.

Pathway A is modelled on the analogous conversion of valine into leucine (see earlier) and would result in the initial formation of homoisoleucine. Pathway B follows the known degradative steps of isoleucine metabolism as far as the production of tiglyl CoA, and then AMHA arises from steps effecting reductive condensation with acetyl CoA, hydroxyl-group shift, dehydrogenation to yield an α-keto acid, and finally transamination to produce AMHA. The observation that the specific activities of AMHA and homoisoleucine were almost equal after [U-^{14}C]isoleucine assimilation, even though the concentration of AMHA in seeds was about 100 times that of homoisoleucine, renders pathway A unlikely and favours AMHA as the primary C_7 amino acid product. However, nothing is known concerning the sub-cellular distribution of the large quantity of AMHA present in maturing seeds or the

[7] J. E. BOYLE and L. FOWDEN, unpublished experiments.

possibility that discrete sub-cellular pools of AMHA may not be in metabolic equilibrium with homoisoleucine. Further support for the primary nature of AMHA then is desirable, and will be sought in future tracer feeding experiments utilizing ^{14}C-tiglate. In addition, ^{14}C-AMHA and ^{14}C-homoisoleucine will be infiltered into developing seeds to assess the degree of interconversion and the preferred direction of the reaction. Eventually, enzymic studies will be required to examine further the nature of the metabolic intermediates.

EXPERIMENTAL

Radioactive Compounds

Labelled glucose, sodium acetate, and most of the ^{14}C-amino acids were supplied by the Radiochemical Centre, Amersham or by Calbiochem, Los Angeles. In general, specific activities were adjusted to be 20μc/μmole by addition of carrier compounds: [1-^{14}C]isoleucine was used as supplied (10 μc/μmole).

[2-^{14}C]-2-Aminohexa-4,5-dienoic acid was prepared by a micro-scale synthesis modified from the procedure of Black and Landor.[8] The formamidocyanoacetate used in the original synthesis was replaced by ethyl [2-^{14}C]acetamidocyanoacetate (8 mg; 6·1 μc/μmole) supplied by NEN Chemicals GMBH, West Germany. Condensation with 1-bromobuta-2,3-diene (6·4 mg), dissolved in 0·2 ml dry ethanol containing 1·1 mg Na, was effected by standing 24 hr at laboratory temp. followed by heating in a sealed tube at 100° for 2 hr. Ethanol was removed by evaporation and the residue was hydrolϕyzed with HCl at 100° for 2 hr. The labelled product was readily separated from a small amount of radioactive glycine (resulting from the hydrolysis of ^{14}C-acetamidocyanoacetate remaining after the condensation) on a paper chromatogram developed in *n*-BuOH–HOAc–H_2O (90:10:29, v/v). The yield of [2-^{14}C]-2-aminohexa-4,5-dienoic acid was about 60% theoretical.

Infiltration and Extraction Procedures

Labelled compounds were supplied to individual developing fruits in the amounts shown in Table 1. Each compound was dissolved initially in 0·25 ml water (containing 20 μg/ml streptomycin sulphate to prevent bacterial contamination) in a closed vial and transferred to the plant via a cotton wick sheathed over much of its length by a glass capilliary. The end of the wick was inserted through an incision in the stalk carrying a suitable fruit at a distance of about 1 cm from the fruit. Uptake of the initial solution was usually complete in 3–5 hr at the normal shade temperature of 30–35°: subsequently the container and wick forming the infiltration system were flushed through with a further ml of water containing streptomycin to ensure the complete transference of radioactivity to the plant tissue. One week later the individual fruits were harvested, coarsely chopped and then macerated in 75% (v/v) ethanol. Extraction proceeded with constant shaking for 24 hr, when the residue was separated by centrifuging. The residue was re-extracted twice with more 75% ethanol, after which the three extracts were combined and evaporated to dryness at laboratory temperature. AMHA, homoisoleucine and other compounds of the soluble-nitrogen fraction were separated by the chromatographic procedures described below.

Chromatographic Separations

A combined amino acid fraction was separated from each extract by employing cation-exchange resin (Zeokarb 225) columns as described by Dunnill and Fowden.[9] Subsequently paper chromatography was employed to separate the individual amino acids present in an aliquot of the combined fraction from 1 g of plant material. Homoisoleucine was separated most effectively from other amino acids in *t*-AmOH–HOAc–H_2O (15:1:15, by vol.; upper phase). AMHA cannot be separated completely on paper chromatograms from leucine-isoleucine by any solvent system so far examined and so for quantitative determinations it was converted into homoisoleucine by catalytic hydrogenation using Adam's platinum oxide catalyst. The amount of AMHA in extracts, and the ^{14}C-radioactivity associated with it, were determined by difference measurements on the homoisoleucine present in aliquots of the combined amino acid fraction before and after catalytic hydrogenation. Additional chromatograms were developed in butanol-acetic acid-water and in 75% (w/w) phenol to effect the separation of those components of the amino acid fraction which were not well resolved during the separation of homoisoleucine.

Quantitative amino acid assay was performed by the method of Atfield and Morris;[10] suitable internal standards were run simultaneously on the chromatograms. ^{14}C determinations were made directly on developed chromatograms using a Packard Radiochromatogram Scanner; a graded series of ^{14}C-labelled amino acid standards, chromatographed simultaneously, served for calibration.

[8] D. K. BLACK and S. R. LANDOR, *J. Chem. Soc. C*, 288 (1968).
[9] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 933 (1965).
[10] G. N. ATFIELD and C.J.O.R. MORRIS, *Biochem. J.* **81**, 606 (1961).

Anionic and unionized compounds present in the plant extracts were not retained by the Zeokarb columns. These two types of compound were separated using anion-exchange resin (Dowex-1) columns, and the total radioactivity present in each fraction was determined.

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