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BIOSYNTHESIS OF L-β-(METHYLENECYCLOPROPYL)-ALANINE (HYPOGLYCIN) IN *BLIGHIA SAPIDA*

ECCLESTON A. KEAN and CYNTHIA E. LEWIS

Biochemistry Department, University of the West Indies, Mona, Kingston 7, Jamaica

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Key Word Index—*Blighia sapida*; Sapindaceae; ackee; β-(methylenecyclopropyl)-alanine; hypoglycin; biosynthesis.

Abstract—Threonine and C_1 units from methionine contribute to form an intermediate in the biosynthesis of L-β-(methylenecyclopropyl)-alanine, the toxic amino acid hypoglycin in *Blighia sapida*. Thereafter, the pathway followed duplicates that for leucine biosynthesis, utilizing identical or analogous enzymes.

INTRODUCTION

A search of the literature revealed only one biosynthetic study on L-β-(methylenecyclopropyl)-alanine (hypoglycin), although this amino acid was recognized over two decades ago as a toxic component of ackee fruit (*Blighia sapida*) [1, 2]. In that work, ^{14}C from methyl-labelled methionine and [U-^{14}C]acetate were found in hypoglycin of sycamore seeds (*Acer pseudoplatanus*) [3]. The suggestion was made that C_2 units condensed into a linear C_6 skeleton, followed by cyclopropyl ring formation through addition of a C_1 unit at a position of 4,5-unsaturation. The same worker proposed elsewhere [4, 5] a scheme leading from isoleucine, which lost carboxyl-C before adding a C_2 unit from acetate. 2-Oxo-4-methylhex-4-enoic acid was considered as a possible intermediate, but results with labelled isoleucine failed to support these hypotheses [3].

The present investigation produces evidence favouring a different hypothesis which links the biogenesis of hypoglycin to that of L-α-(methylenecyclopropyl)-glycine (MCPG), the lower homologue. The latter, first recognized in *Litchi chinensis* (Sapindaceae), has only now been identified in ackee fruit. The results of ^{14}C incorporation experiments, and also enzyme activities demonstrated in extracts of ackee seeds and leaves provide the basis for a reasonably complete biogenetic scheme.

RESULTS AND DISCUSSION

MCPG in ackee fruit

Rigorous proof that MCPG occurs in ackee will be difficult to achieve, a major problem being that the concentration appears to be very low. Preliminary searches using 2-D chromatography with various solvent combinations were unsuccessful; the compound gives a characteristic brown colour with ninhydrin [6]. Eventually, an ethanolic extract of seeds from immature fruit was processed to isolate a neutral amino acid fraction, and a portion containing *ca* 1.4 μmol mixed acids run on an amino acid analyser. An unknown trace component was eluted at 117 min, after alanine and before valine. When the analysis was duplicated with added authentic MCPG [6] (a kind gift from Dr. L. Fowden) only the location of the unknown showed an increased peak height. This strongly suggests that the unknown was MCPG. If this identification is correct, MCPG comprised *ca* 0.07% of the neutral amino acids. A reasonable expectation is that the homology between MCPG and hypoglycin would be reflected in their biogenesis.

Incorporation of ^{14}C-labelled compounds

Cut ackee shoots bearing immature fruit were fed potential hypoglycin precursors which were radioactive. Aliquots of extracts from the pooled seeds and arilli derived from each separate shoot—one shoot for each experiment—were processed to yield pure *N*-benzoyl hypoglycin. The incorporation of radioactivity observed in a representative set of experiments is expressed in two ways in Table 1. One method shows the ratio of product to precursor specific activity (specific incorporation). The other (relative % incorporation) is calculated from (dpm in hypoglycin/g fr. wt of ackee fruit part) × 100/total dpm fed. This method was adopted to compensate for variation in tissue mass. Data on the original hypoglycin content of each relevant fruit part were therefore needed, because variable losses occurred during product purification. A convenient specific analytical procedure [7] was available for this purpose.

Table 1 shows that the two criteria of incorporation are in general agreement. Hypoglycin in seeds and arilli incorporated ^{14}C from all the precursors listed. The validity of the data is reinforced by results of parallel experiments (not shown) in which it was found, as expected, that only acetate and pyruvate donated ^{14}C to glutamate.

[U-^{14}C]Threonine was similar to [*methyl*-^{14}C]-methionine as an effective labelling agent for hypoglycin. An apparent anomaly is the greater uptake of label from [U-^{14}C]acetate in arilli than in seeds. This may be related to an active fatty acid metabolism in the lipid-rich arilli, dependent in part on effective mobilization of C_2 units supplied by acetate. Least incorporation was obtained with [2-^{14}C]pyruvate.

Results for methionine, acetate and to some extent, pyruvate, are in accord with those previously either

Table 1. Radioactivity in hypoglycin of ackee fruit after feeding ^{14}C-labelled compounds

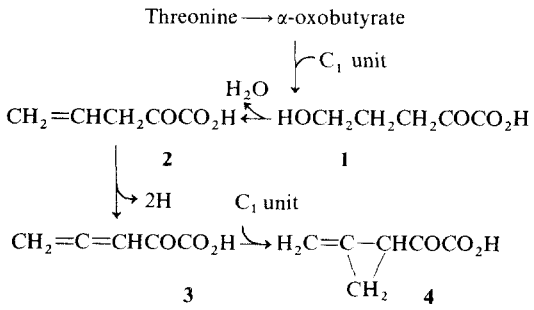
Precursor fed	Source of hypoglycin	^{14}C incorporation Specific* ($\times 10^3$)	Relative† (%)
L-[Me-^{14}C]Methionine	Seeds	3.11	0.194
	Arilli	0.440	0.026
L-[U-^{14}C]Threonine	Seeds	2.51	0.225
	Arilli	0.444	0.030
[U-^{14}C]Acetate	Seeds	0.297	0.015
	Arilli	0.447	0.017
[2-^{14}C]Pyruvate	Seeds	0.270	0.008
	Arilli	0.169	0.012

*(dpm/mmol hypoglycin)/(dpm/mmol precursor fed).
†(dpm in hypoglycin/g fr. wt fruit part)/(total dpm fed).

reported [3] or privately communicated (R. J. Suhadolnik, quoted in ref. [3]). The results with threonine are, however, of special interest because they support a new hypothesis which is partially summarized in Scheme 1.

We propose that α-oxobutyrate, a known product of threonine metabolism in plants [8], accepts a C_1 unit which originates from methionine and which is probably carried by THF at the hydroxymethyl level of oxidation. Subsequent steps of dehydration, dehydrogenation and addition of a second C_1 unit across an ethylenic bond, respectively yield **2**, **3** and **4**. Formation of **4** would resemble the methionine-dependent genesis of cyclopropane fatty acids in certain micro-organisms [9]. Methylenes preferentially add at the more substituted double bond of allenes *in vitro* [10] and this may also be the case *in vivo*. While allenic compounds are rare in biological systems, the higher homolgue of **3** is clearly related structurally to 2-aminohex-4,5-dienoic acid which was identified in the fungus *Amanita solitaria* [11].

By our hypothesis, formation of MCPG from **4** is accomplished by transamination. Of greater quantitative significance in ackee, however, is the ultimate conversion of **4** to hypoglycin which the results suggest. Incorporation of radioactivity into the amino acid from [U-^{14}C]acetate and [2-^{14}C]pyruvate is best explained as follows. These precursors are converted to acetyl-CoA, which then adds to **4** by the same mechanism that forms α-isopropylmalate in the leucine biosynthetic pathway [12, 13]. The product (3-hydroxy-3-carboxy-4,5-cyclopropylhex-5-enoate, **5**) then exactly follows the remaining steps of that pathway [12, 14], the sequence being isomerization, and dehydrogenation with decarboxylation. Finally, transamination of β-(methylenecyclopropyl)-pyruvate yields hypoglycin. Relevant enzymes of the leucine pathway appear not to be absolutely specific, as discussed below.



Scheme 1. Postulated early steps in hypoglycin biosynthesis.

It is evident that the carboxyl-C originating from threonine is lost in this sequence, in contrast to the carboxyl-C atom from acetate or from C-2 of pyruvate. However, pyruvate appears to be a relatively poor source of acetyl-CoA for this pathway.

Information privately communicated by Dr. R. J. Suhadolnik strongly supports the proposed scheme. Radioactive hypoglycin isolated from ackee fruit fed [Me-^{14}C]methionine was treated with a reagent [15] which selectively removes terminal methylene groups. Only *ca* 50% of the hypoglycin ^{14}C content was thereby lost. This is entirely consistent with Scheme 1, where only the first C_1 unit adds to form the terminal methylene.

Ackee α-isopropylmalate synthetase (EC 4.1.3) and amino acid: α-oxo acid aminotransferase

Activities of these two enzymes, which clearly could play a role in hypoglycin synthesis, were investigated. The first was extracted from seeds, and the latter from seeds and leaves. To obtain substrate for the demonstration of the condensation with acetyl-CoA which leads from **4** to **5**, we aimed to isolate MCPG from litchi seeds and to prepare **4** from it. However, attempts to isolate pure MCPG were unsuccessful because of persistent contamination with valine, a problem noted in [6]. We therefore used the next higher homologue of **4**, i.e. β-(methylenecyclopropyl)-pyruvate (MCPP), prepared from pure hypoglycin, in place of **4**. Extracts of immature seeds showed measurable synthetase activity in a system designed for assay of the bacterial enzyme. Comparing MCPP with α-oxoisovalerate, each used at the same concentration in separate assay mixtures, enzyme activity with the former was only *ca* one-eighth of that with the latter (Table 2). The mixed-substrate method [16] was applied to a partially purified enzyme preparation and indicated that the same enzyme acts on both substrates to catalyse condensation with acetyl-CoA. Because of its closer isosteric relationship to the 'natural' substrate α-oxoisovalerate, **4** would be expected to give higher

Table 2. Activity of α-isopropylmalate synthetase derived from ackee seeds

Substrate	Activity (units/mg protein)
OIV*	0.839
MCPP†	0.102
OIV + MCPP	0.763

*α-Oxoisovalerate.
†Methylenecyclopropylpyruvate.

reaction rates than MCPP. Therefore, this step appears highly probable in hypoglycin biosynthesis.

Both alanine and glutamate appear to be effective amino group donors for hypoglycin synthesis. Acetone powders prepared from ackee leaves and immature seeds yielded aminotransferase activity following extraction with buffer, salting out and redissolving of the precipitate. Each amino acid produced leucine from α-oxoisocaproate, and hypoglycin from MCPP upon incubation with the enzyme, as verified by TLC. Aspartate, even after incubations prolonged up to 18 hr, gave negative results. Quantitative assay of these crude enzyme preparations gave the activities shown in Table 3, based on rates of pyruvate formation using alanine as donor. As in the case of the synthetase reaction, it appears that a single enzyme was involved. At present there are no parallel results with glutamate as amino group donor to justify a similar claim, but in rat liver [17], a single enzyme catalyses the reaction between glutamate and either α-oxoisocaproate or MCPP.

Our conclusions verify, in a sense, the suggestion [18] that hypoglycin may be formed from MCPG. In that paper, the co-occurrence of hypoglycin and MCPG in ackee was also forecast, based on the case of *Billia hippocastanum*, a related species [18]. However, our conclusions contradict the other suggestion then advanced, that branched-chain proteinogenic amino acids were the likely precursors of MCPG and hypoglycin.

One, or at most, two early steps of our scheme appear novel. In these may lie an explanation for the limited distribution of these amino acids. The biosynthesis of γ-glutamyl hypoglycin, a dipeptide which also occurs in the ackee [1, 5], has been described elsewhere [19].

Table 3. Activity of alanine aminotransferase derived from ackee tissues

Substrate	Activity (units/mg protein) × 10^3 Seeds	Leaves
OIC*	2.54	2.66
MCPP†	3.33	1.27
OIC + MCPP	2.87	1.94

*α-Oxoisocaproate.
†Methylenecyclopropylpyruvate.

EXPERIMENTAL

Chemicals were from commercial sources, except for hypoglycin and MCPP. The former was prepared via *N*-γ-glutamyl-hypoglycin [5]. MCPP was prepared from hypoglycin using L-amino acid oxidase [20].

Protein in extracts was determined by the method of ref. [21]. Amino acid analysis was by the standard hydrolysate procedure using an autoanalyser.

^{14}C *Incorporation into hypoglycin.* Leafy shoots bearing 4–6 immature fruit were obtained from ackee trees on the University grounds. Stems, cut under H_2O in the lab, were kept with the cut ends in solns (1 ml) of the labelled precursors (50 μmol, *ca* 6 × 10^6 dpm) contained in a vial. The prepns were exposed to normal environmental light, temp. (22–25°) and upward draught in a fume chamber. The original 1 ml soln virtually disappeared within 2 hr, but vials were recharged with H_2O throughout 60 hr. Arilli and seeds were then separated, pooled and homogenized and the neutral amino acid fraction isolated from the extracts [22]. The following procedure aimed to recover sufficient leucine-free hypoglycin derivative for weighing and radioassay. An aliquot was removed for hypoglycin assay [7], and to the remainder, 50 or 100 mg hypoglycin was added. Notwithstanding this addition of cold hypoglycin, the amount of labelled hypoglycin in the purified derivative was known because it formed a calculable fraction of the wt of final product. Recrystallization (× 3) yielded impure hypoglycin, which was then benzoylated [23] and the derivative recrystallized (× 3) to constant sp. act. and mp (114–115°). The weighed product was dissolved in scintillation fluor (toluene–Triton X-100–PPO) and radioassayed. The external standard ratio method was used to determine dpm. Tests involving addition of 1 part [U-^{14}C]leucine to 10 parts non-radioactive hypoglycin showed that only *ca* 4 ppm derivatized leucine remained in *N*-benzoyl-hypoglycin after this procedure. Both dpm/mmol extracted hypoglycin and dpm in total extracted hypoglycin were calculated to provide the data of Table 1.

A fraction of the eluates from anion-exchange columns contained glutamate free of other amino acids [19, 22], and aliquots were radioassayed directly without purification.

Enzyme preparation and assays. α-Isopropylmalate synthetase activity was determined [24] using *ca* 0.1–0.4 mg enzyme protein/ml assay medium. α-Oxo acid was omitted from blanks, and the reaction time prolonged to 10 min. A unit of enzyme activity = 1 μmol CoA released/hr. The enzyme was partially purified starting from Me_2CO powder prepared from immature seeds. Powder was extracted with 20 mM KPi (pH 8) and from the extract, a fraction [25–45% satn with $(NH_4)_2SO_4$] was percolated through a column of Sephadex G-200 equilibrated and washed with extraction buffer. Enzyme activity was not retarded, suggesting that the enzyme was itself of relatively high MW (> 150 000) or part of such a complex. Active enzyme was absorbed on alumina Cγ gel (gel:protein ratio, 1:2, pH 6.5), and eluted with extraction buffer containing 10% (w/v) $(NH_4)_2SO_4$. Protein in the eluate was ppted at 60% satn with $(NH_4)_2SO_4$ and this purified enzyme was taken up in extraction buffer. This procedure gave only 2-fold increase in sp. act. based on values for Me_2CO powder extracts.

To determine aminotransferase activity, a method for alanine-α-oxoglutarate transaminase in serum [25] was modified using either α-oxoisocaproate or MCPP in place of α-oxoglutarate. *Ca* 0.5 mg crude enzyme protein/ml assay medium was required. A unit of enzyme activity = 1 μmol pyruvate produced/min. To prepare enzyme, Me_2CO powders of leaves and of immature seeds were extracted with 0.12 M KPi (pH 7.5), the eluate brought to 80% satn with $(NH_4)_2SO_4$, and the resulting ppt. dissolved in extraction buffer.

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