AMINO ACID SYNTHESIS IN THE ADULT HONEYBEE

by

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ABSTRACT

Three feeding studies were made to examine the in vivo synthesis of amino acids in the adult honeybee. In the first study amino acid synthesis from uniformly-labelled glucose-C^{14} and sucrose-C^{14} was investigated by the indirect feeding method. The amino acids showing moderate-to-high specific activities, and therefore considered nonessential from a dietary standpoint, were glutamic acid, proline, alanine, aspartic acid, cystine, glycine, and serine. Amino acids which gave no evidence of carbon-14 incorporation from the fed sugars and classified as essential were valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, and arginine. Two exceptions were noted. First, although radioactive threonine was found, previous nutritional studies indicate that it is not synthesized in sufficient quantity to meet the need of the insect and, consequently, remains a dietary requirement. Second, despite the lack of labelling in tyrosine, it has been demonstrated that this amino acid is dispensable in the insect since it can be amply produced from phenylalanine. In the second study glutamic-C^{14} acid was fed to the honeybee to determine the potential of this moiety to serve as a precursor for

nonessential amino acids. The carbon-14 label was incorporated in ornithine, aspartic acid, proline, alanine, threonine, and serine. Finally, in the third study, the amino acids of the ornithine-urea cycle were examined. It was found that arginine very slowly incorporated the labelled carbon of ingested ureido-labelled citrulline-C^{14}; however, there was no radioactive citrulline detected when uniformly-labelled arginine was fed. This suggests that the complete ornithine-urea cycle does not exist in the adult honeybee.

INTRODUCTION

The amino acids essential in the diet of the honeybee were first demonstrated by DeGroot (1). By feeding mixtures of seventeen pure amino acids, each deficient in a specific amino acid, to young honeybees and calculating growth on the basis of dry weight and nitrogen content at fourteen days, DeGroot was able to determine which of these amino acids are indispensible in the food source of the honeybee. Shown to be essential for growth and development are tryptophan, phenylalanine, leucine, isoleucine, threonine, methionine, lysine, valine, histidine, and arginine; nonessential are tyrosine, glycine, glutamic acid, cystine, serine, proline, hydroxyproline, alanine, and aspartic acid. The designation "essential amino acid" usually means that the amino acid is necessary in the diet of the animal to insure full development as well as adult survival and that it is not synthesized by the tissues either from other amino acids or from simple precursors. The growth studies of DeGroot, using the classical deletion method for determining the amino acid requirements, is complicated by the fact that no completely synthetic or chemically defined food has been found that is acceptable to the insect.

An alternate method permitting the determination of essential factors such as amino acids was demonstrated by Steel who showed that carbon-14 appeared in the nonessential but not the essential amino acids when uniformly-labelled sucrose-C^{14} was fed to mice (2). Kasting and McGinnis applied this indirect method of determining the indispensible amino acids to insects by injecting blowfly larvae with uniformly labelled glucose-C^{14} and comparing the results with those of the deletion feeding procedure (3). The radioactive tracer method gave results similar to the classical method and proved to be a useful technique for plant feeding insects which cannot be raised on artificial diets.

To date the amino acid synthetic ability of the honeybee has been studied primarily in the larval stage. The classical deletion method used by DeGroot necessitated the use of the growing honeybee as have the many nutritional studies on pollen substitutes as a protein source for the honeybee. In 1966 Lue and Dixon determined the amino acid requirements of developing honeybee larvae using glucose-C^{14} and sucrose-C^{14}; also they compared the qualitative caste differences with respect to essential amino acids and found the requirements to be the same for the worker and queen larvae (4). Although much information has been presented on the larval and growing stages, little is known of the amino acid metabolism of the fully developed adult.

In addition to being used to determine the essential factors for insects, the radioactive tracer technique has also been employed to study the probable existence of particular pathways. Of specific interest here is the use of carbon-14 to clarify the role of the free amino acid ornithine. Ornithine has been identified in the blood and tissue of the bee, but its metabolic function is unknown. Although Garcia demonstrated the formation of citrulline and ornithine from arginine using in vitro studies and concluded the ornithine-urea cycle existed in the honeybee, nutritional studies by DeGroot show that arginine is an essential amino acid and can be only partly replaced by citrulline and ornithine has no effect as a replacement (5,1). The evidence, thus far, of the participation of ornithine in an ornithine-urea cycle similar to that of vertebrates has been contradictory.

This thesis presents two metabolic studies on amino acid anabolism in the adult honeybee. In the first experiment the biosynthetic transfer of carbon-14 from glucose and sucrose to specific amino acids, and the potential of glutamic acid to serve as an amino acid precursor, are investigated to determine which of the known amino acids are synthesized by the insect and which are necessary in the diet. In the second experiment the existence of an ornithine-urea cycle in the honeybee is examined through a

study of the amino acids resulting from the feeding of uniformly-labelled arginine-C^{14} and ureido-labelled citrulline-C^{14}.

EXPERIMENTAL PROCEDURE

I. Selection and Feeding of Honeybees

The adult worker honeybees used in this experiment were randomly selected from a normal hive maintained at the U.S.D.A. Bee Research Lab, Tucson, Arizona. The bees were isolated from the hive and divided into groups of four bees. Each honeybee was placed in a polyethylene feeding tube one inch in length and one half inch in diameter (Fig. 1). Each tube was closed at one end with a metal staple and fitted at the other end with a one hole plastic cap of sufficient size for the insertion of a pipette tip. In order to insure adequate intake of the feeding solution, the bees were denied feed and water for one hour prior to feeding the labelled compounds. Each radioactive sugar and amino acid to be fed was made palatable for the honeybee by mixing it with a 50:50 sucrose:water solution. The feeding solution was then offered to the bees via a pipette calibrated to 0.001 ml. Each bee was allowed to imbibe 0.020 ml from the pipette. The groups of bees were marked for sacrifice at specific time intervals after feeding. During this period the bees were provided sucrose and water ad libitum.

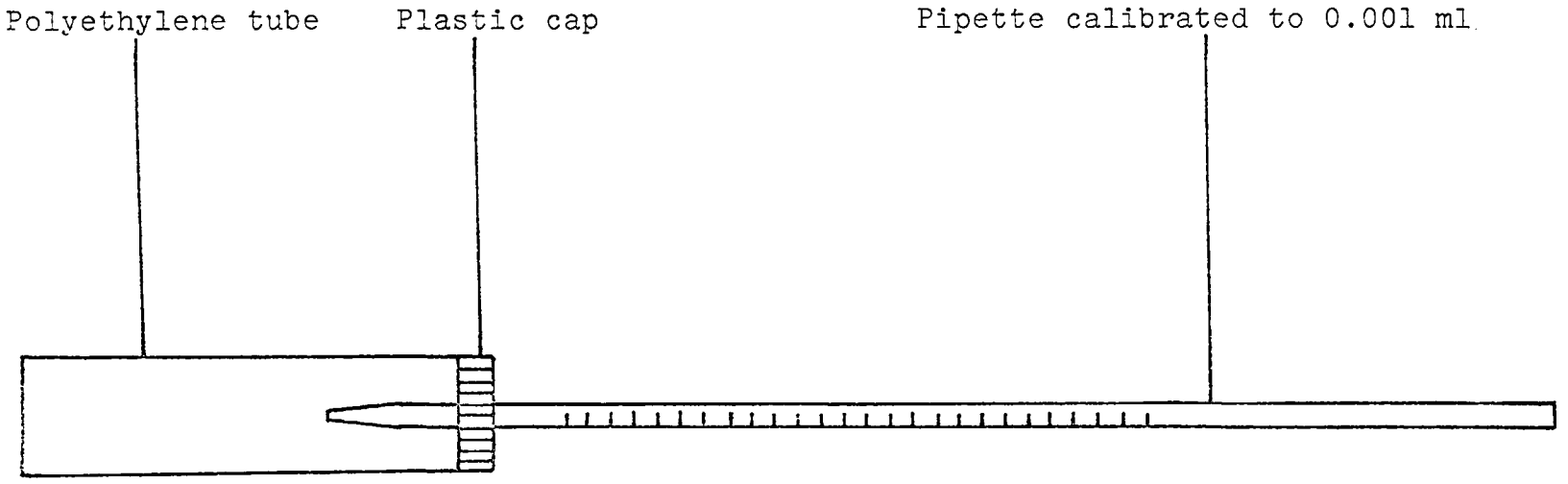

Fig. 1. Feeding apparatus for the administration of labelled compounds to honeybees.

II. Preparation of the Labelled Solutions

a. Uniformly-labelled glucose-C^{14}

D-glucose-UL-C^{14}, specific activity 25-250 millicuries per millimole (mc/mM), was diluted from 1.0 ml to 2.0 ml with sucrose solution to give an activity of 0.5 mc/ml. The labelled solution was fed to three groups of bees; the groups were marked for sacrifice at 2 hr., 4 hr., and 8 hr.

Sucrose-UL-C^{14}, specific activity 10-300 mc/mM, 0.5 mc/ml, was received in 20% ethanol. Because the bees could not tolerate the high alcohol content, it was necessary to dilute 1.0 ml of the labelled sucrose in ethanol to 3.0 ml with sucrose;water to give an activity of 0.17 mc/ml. Four groups of bees were fed and then sacrificed at 1 hr., 2 hr., 3 hr., and 4 hr.

b. Uniformly-labelled glutamic-C^{14} acid

L-Glutamic-UL-C^{14} acid, specific activity 125-200 mc/mM, 0.1 mc/ml, was diluted from 1.0 ml to 2.0 ml with sucrose solution giving an activity of 0.05 mc/ml. Five groups of bees were fed the labelled glutamic acid and were sacrificed at 1 hr., 2 hr., 3 hr., 4 hr., and 5 hr. after feeding.

c. Uniformly-labelled arginine-C^{14}

L-Arginine-UL-C^{14}, specific activity 250 mc/mM, was received packaged in 1.0 ml 0.01 N HCl. 20% NaOH was used

for neutralization. 1.0 ml of the neutralized arginine was mixed with 1.5 ml sucrose solution to give an activity of 0.04 mc/ml. The prepared arginine was fed to five groups of bees. The groups were marked for sacrifice at 3 hr., 6 hr., 9 hr., 24 hr., and 48 hr. from the hour of feeding.

d. Citrulline-ureido-C^{14}

Ureido-labelled L-Citrulline-C^{14}, specific activity 1-5 mc/mM, was received in crystalline form. It was dissolved in 0.6 ml sucrose solution to give a final activity of 0.08 mc/ml. The solution was fed to five groups of honeybees which were marked for sacrifice at 5 min., 1 hr., 2 hr., 4 hr., and 24 hr.

III. Analysis

a. Sample preparation for glucose-UC-C^{14}, sucrose-UL-C^{14}, and glutamic-UL-C^{14} acid feedings

Each group of bees was chloroformed at the designated time after feeding. The intact bees were hydrolyzed in 50 ml 6 N HCl at 106°C for 24 hrs. The filtrate was dried *in vacuo* to remove the HCl. The residue was dissolved in 10.0 ml pH 2.2 sodium citrate buffer.

b. Sample preparation for arginine-UL-C^{14} and citrulline-ureido-C^{14} feedings

The bees were chloroformed at the indicated times and each group was homogenized for 5 min. in 15 ml 80%

ethanol for the extraction of the free amino acids. The ethanol fraction was decanted and dried *in vacuo*. The residue was dissolved in 5.0 ml pH 2.2 sodium citrate buffer.

c. Detection of labelled amino acids

The radioactive amino acids were detected with a Packard Tri-carb Flow Detector which was inserted in the effluent column line of a Beckman 120 B Amino Acid Analyzer. 1.0 ml of an extracted sample or 0.5 ml of a hydrolyzed sample was applied to the amino acid analyzer. The fractionated amino acids passed from the ion exchange column to the scintillation cartridge of the flow detector where the radioactivity was recorded graphically. The effluent from the flow detector was routed back to the Beckman analyzer and the amino acids were determined qualitatively and quantitatively. A comparison of the flow detector graph and the amino acid analyzer graph allowed the identification of the radioactive amino acids. Specific activity, counts per minute per micromole amino acid, was used to express radioactivity. The amino acid micromolar concentrations per 77 mg bee tissue are shown in table 1.

Table 1

Micromoles amino acid/77 mg bee tissue (6)

Amino acid	micromoles
Aspartic acid	6.46
Threonine	2.62
Serine	4.70
Glutamic acid	7.32
Proline	6.78
Glycine	9.78
Alanine	10.8
Cystine	0.385
Valine	2.08
Methionine	1.00
Isoleucine	1.23
Leucine	5.24
Tyrosine	1.69
Phenylalanine	1.77
Lysine	3.54
Histidine	1.54
Arginine	2.08

(6) W. F. McCaughey, Seasonal Changes in Amino Acid Content in Honeybee Tissue, Unpublished.

RESULTS

I. Uniformly-Labelled Glucose-C^{14} and Sucrose-C^{14} Feedings

The scintillation flow monitor permitted the identification of nine amino acids biosynthesized by the honeybee from the labelled sugars. The amino acids showing the highest specific activity were glutamic acid, proline and alanine followed by aspartic acid, cystine, serine, threonine and glycine. Maximum incorporation of the carbon-14 label into alanine, aspartic acid and glycine occurred at 2 hr. from feeding, at 4 hr. into glutamic acid and at 8 hr. in serine and proline (table 2). Another amino acid detected but showing low specific activity was leucine in the 2 hr. sample. Labelled glucosamine, which is a ninhydrine-positive compound, was found in all samples and showed a moderate degree of radioactivity. At 2 hr. from feeding approximately 2% of the labelled carbons had been transferred from the sugar to the amino acids, principally to glutamic acid, alanine and proline. Maximum specific activities of the labelled amino acids are listed in table 3.

Table 2

Time study of radioactive amino acids resulting from the uniformly-labelled glucose-C^{14} feeding to honeybees

Amino Acid	Specific Activity counts per minute/micromole		
	2 hr.	4 hr.	8 hr.
Aspartic acid	7,319	3,288	4,611
Threonine	0	2,374	0
Serine	1,259	2,158	3,228
Glutamic acid	31,606	38,950	20,946
Proline	20,235	22,287	30,021
Glycine	2,260	672	1,106
Alanine	12,469	7,314	4,195
Cystine	9,851	0	0
Leucine	878	0	0

Table 3

Maximum specific activities of amino acids isolated after feeding uniformly-labelled glucose-C^{14} to honeybees

Amino Acid	Specific Activity counts per minute/micromole
Aspartic acid	7,319
Threonine	2,374
Serine	3,228
Glutamic acid	38,950
Proline	30,021
Glycine	2,260
Alanine	12,469
Cystine	9,851
Valine	0
Methionine	0
Isoleucine	0
Leucine	878
Tyrosine	0
Phenylalanine	0
Lysine	0
Histidine	0
Arginine	0

II. Uniformly-Labelled Glutamic-C^{14} Acid Feeding

When glutamic-C^{14} acid was fed to the honeybee the labelled carbons were found incorporated in seven amino acids within three hours (table 4). Ornithine, aspartic acid and proline showed the greatest degree of carbon-14 transfer from the glutamic acid. There was a rapid transfer of label to the aspartic acid within the first hour after feeding, followed by a decrease in radioactivity to almost one fourth within the next hour. Labelling of proline increased gradually in the 1 hr. and 3 hr. samples, reached a maximum at 4 hr., and diminished in the 5 hr. sample. Alanine and threonine exhibited moderate activities. Labelled serine was seen at 2 hr. and a trace amount of methionine was found in the 1 hr. and 2 hr. samples. Labelled ornithine appeared in the 2 hr. and 3 hr. samples at very high specific activity but was not seen in any of the earlier or later samples. Table 5 shows the maximum specific activities of the amino acids produced from glutamic acid.

III. Uniformly-Labelled Arginine-C^{14} and Ureido-Labelled Citrulline-C^{14} Feedings

The production of radioactive arginine, specific activity 22,069, was noted 24 hr. following the feeding of ureido-labelled citrulline. In samples taken at 5 min. and

1 hr. after feeding no labelled arginine was found; trace amounts were seen at 2 hr. and 4 hr.

Samples taken at 3 hr., 6 hr., 9 hr., 24 hr., and 48 hr. after feeding uniformly-labelled arginine showed no evidence of radioactive citrulline.

Table 4

Time study of radioactive amino acids resulting from the uniformly-labelled glutamic-C^{14} acid feeding to honeybees

Amino Acid	Specific Activity counts per minute/micromole				
	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Aspartic acid	4,960	1,346	2,231	2,430	2,299
Threonine	2,316	1,135	799	1,493	1,019
Serine	0	907	0	0	0
Proline	1,181	1,980	2,853	3,739	3,468
Alanine	350	461	289	336	218
Ornithine	0	86,075	304,150	0	0

Table 5

Maximum specific activities of amino acids isolated after feeding uniformly-labelled glutamic-C^{14} acid to honeybees

Amino Acid	Specific Activity counts per minute/micromole
Aspartic acid	4,960
Threonine	2,316
Serine	907
Proline	3,739
Glycine	0
Alanine	461
Cystine	0
Valine	0
Methionine	0
Isoleucine	0
Leucine	0
Tyrosine	0
Phenylalanine	0
Lysine	0
Histidine	0
Arginine	0
Ornithine	304,150

DISCUSSION AND CONCLUSIONS

The amino acids showing the highest specific activities, and therefore most readily synthesized from the ingested carbon-14 sugars, were glutamic acid, proline, alanine, cystine and aspartic acid. Moderate radioactivity was noted in glycine, serine and threonine and very slight activity in leucine. There was no evidence of the presence of the carbon-14 label in valine, methionine, isoleucine, phenylalanine, tyrosine, lysine, histidine or arginine. These results confirm the classification, by the amino acid deletion method, of glutamic acid, proline, alanine, aspartic acid, cystine, glycine, and serine as nonessential since these amino acids can be produced from glucose or sucrose, carbohydrates found in the natural diet of the honeybee.

Of the amino acids showing little or no carbon-14 incorporation, and consequently taken to be essential, one notation should be made. Although tyrosine appears to be essential in the carbon-14 study, the nutritional experiments of DeGroot give evidence to the contrary (1). Phenylalanine has been shown to be a principal precursor of tyrosine in both the rat and the silkworm (7,8). In 1962 the recovery of radioactive tyrosine from the pale western cutworm injected with uniformly-labelled phenylalanine-C^{14}

was demonstrated by Kasting and McGinnis (9). If the honeybee is able to make a similar conversion of phenylalanine to tyrosine, then tyrosine should be considered nonessential despite the lack of labelling in the present study.

The in vivo synthesis of threonine is of interest since this amino acid has been found to be essential in almost all species studied, the honeybee included; and there has been no pathway proposed to account for the production of threonine from glutamic acid or any other precursor. However, recent work on amino acid synthesis in insects fed uniformly labelled glucose-C^{14} has demonstrated the production of threonine from sugar in the pale western cutworm and the phytophagous mite (9,10). Thus, it seems possible that there is a pathway in some insects allowing the production of threonine from glucose, but not in sufficient quantity to meet the need of the insect for growth and maintenance.

Aspartic acid has been generally considered dispensible for the honeybee. However, Gilmour fails to classify aspartic acid as essential or nonessential; and Lue and Dixon, from aspartic-1-C^{14} acid feedings to honeybee larvae, suggest that this amino acid could be essential in certain stages of development (11,4). The high specific activity of the aspartic acid produced from the carbon-14 sugar indicates that this amino acid is easily produced from a carbohydrate

precursor and is therefore classified nonessential for the adult.

It was not possible to classify tryptophan as essential or nonessential since this amino acid was destroyed during hydrolysis in the sample preparation. However, both the amino acid deletion method and the studies of Lue and Dixon have shown tryptophan to be indispensible for the honeybee (1,4).

From the experimental results of the carbon-14-labelled sugar study, and the possible qualifications discussed, the amino acids determined to be essential for the adult honeybee are threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, and tryptophan; nonessential are aspartic acid, serine, glutamic acid, proline, glycine, alanine, cystine, and tyrosine (table 6).

When labelled sucrose and glucose were fed to the honeybee the amino acid showing the greatest incorporation of carbon-14 was glutamic acid. The ease of synthesis of glutamic acid from sugar makes this amino acid an important source of nonessential amino acids. The ability of glutamic acid to serve as a precursor for proline via a several step pathway and for aspartic acid through the citric acid cycle has been demonstrated in several animals including insects. The high activity seen in aspartic acid one hour after

Table 6

Amino acid requirement of the honeybee

Amino Acid	C^{14} Study	Deletion Study (11)
Aspartic acid	-	no report
Threonine	+	+
Serine	-	-
Glutamic acid	-	-
Proline	-	-
Glycine	-	-
Alanine	-	-
Cystine	-	-
Valine	+	+
Methionine	+	+
Isoleucine	+	+
Leucine	+	+
Tyrosine	-	-
Phenylalanine	+	+
Lysine	+	+
Histidine	+	+
Arginine	+	+

(11) Gilmour, Darcy, Biochemistry of Insects, Academic Press, New York, 1961.

+ indicates essential; -, not needed.

feeding the labelled glutamic acid indicates an immediate routing of glutamate to the citric acid cycle. Conditions in the insect which suggest that the early metabolism of glutamic acid would involve its entrance into the citric acid cycle are the high energy demand of the flight muscles, the rapid transamination of glutamic acid which has been shown to occur, and the isolation of glutamic dehydrogenase, the enzyme necessary for the oxidative deamination of glutamate to alpha ketoglutarate. The carbon-14 label which appears in alanine may be derived from the glutamic acid via the citric acid cycle and pyruvate, as in vertebrates; however, Bheemeswar has reported extracts from the silkworm which catalyze the beta-decarboxlyation of aspartate to form alanine, and this possibility should also be considered in the honeybee (12). In all samples taken there was found a moderate degree of radioactivity in the amino acid threonine. This indicates that the transfer of the radioactive carbon from glucose to threonine, noted in one sample of the present study of amino acid synthesis from labelled sugar, occurred through glutamic acid. Finally, the incorporation of the labelled carbon from glutamate was noted in the free amino acid ornithine. The synthesis of ornithine from glutamic acid in vertebrates is well understood as is the ultimate function of ornithine in the ornithine-urea cycle. In insects, animals which excrete little or no urea, ornithine

has been found but its metabolism has not been elucidated. The participation of ornithine in a cycle similar to the ornithine-urea cycle of vertebrates was investigated in the concluding portion of this thesis.

The results of feeding uniformly-labelled arginine-C^{14} and ureido-labelled citrulline-C^{14} suggest that the complete urea cycle does not exist in this insect. The labelled carbon of the ingested citrulline was very slowly incorporated into arginine indicating that the citrulline-argininosuccinate-arginine sequence may be present. No radioactivity was found in citrulline when uniformly-labelled arginine-C^{14} was fed. A block must exist which, either prevents the conversion of arginine to ornithine, or the condensation of ornithine and carbamyl phosphate to form citrulline. Since the enzyme arginase has been demonstrated in several insects, it is suspected that the block in the cycle occurs at the condensation step. Radioactive impurities in the arginine and citrulline preparations prevented the monitoring of urea and ornithine. Further purification of the feeding solution to allow the detection of labelled urea and ornithine produced *in vivo* would give more conclusive evidence of the metabolism of the amino acids involved in this cycle. The information obtained in the present study demonstrates why nutritional experiments show that the essential amino acid arginine can be partially replaced by citrulline but not by ornithine in the diet of the honeybee.

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