PRELIMINARY STUDIES IN SOME ASPECTS OF AMINO ACID BIOSYNTHESIS IN JUVENILES OF *Penaeus monodon* FABRICIUS †

I. INCORPORATION OF ^{14}C FROM (U–^{14}C) ACETATE INTO AMINO ACIDS OF PRECIPITABLE PROTEINS

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ABSTRACT

Ion-exchange chromatography in conjunction with radioisotope labelling techniques were used to investigate the incorporation of ^{14}C from (U-^{14}C) – acetate into amino acids of precipitable proteins in *P. monodon* juveniles. Highest ^{14}C radioactivity was found in the acidic amino acids, aspartic acid and glutamic acid. Little but significant radioactivity was observed in alanine, cysteine, glycine, proline and serine. Amino acids which incorporated little or no ^{14}C were arginine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine. It appears that *P. monodon* juveniles could not synthesize these amino acids from acetate. The essential nature of these amino acids in *P. monodon* is discussed.

INTRODUCTION

The penaeids have increasingly become economically important as food for human consumption. This is the rationale behind protracted efforts to improve the aquaculture of these crustaceans. However, the lack of basic information on the biochemistry, nutrition, physiology, and behaviour of this animal has posed problems in many aspects of developing an effective aquaculture system for this species.

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In the Philippines, the predominantly cultured prawn species is sugpo, *Penaeus monodon* Fabricius, 1798. A major concern of prawn aquaculture is the provision of an efficient artificial diet for optimum growth and survival of juveniles and adults. A logical approach towards the formulation of such a diet for prawns is to determine the dietary needs of the animal in relation to its metabolic activities. Because protein and the composition of such protein are the most important considerations in diet formulation, it is useful to know which amino acids are indispensable in the diet because the prawn cannot synthesize them. Before one can prepare the cheapest combination of available proteins that will meet the demands of the animal for optimal growth, information about the metabolic activity of the animal is desirable.

One highly recommended approach for crustaceans is the use of radioisotope tracer techniques in conjunction with chromatography system in order to determine their amino acid synthesizing capacity (1-5). The technique involves the injection of precursor compound labelled with a tracer such as ^{14}C. The degree of incorporation of the tracer into certain tissue amino acids is taken to indicate the capacity of the animal for *de novo* synthesis of those amino acids. Weakly labelled amino acids are considered essential whereas strongly labelled ones are non-essential. This paper describes the use of U-^{14}C-acetate in labelling the amino acids of precipitable proteins of *P. monodon* juveniles. Ion-exchange column chromatography was utilized to separate ^{14}C-labelled neutral and acidic amino acids since it provides better resolving power and quantitative recoveries than either paper or thin-layer chromatographic techniques.

MATERIALS AND METHODS

Animals. – Juveniles of the prawn, *Penaeus monodon,* used for the radiolabelling experiments were obtained from the Leganes Research Station of the Southeast Asian Fisheries Development Center, Iloilo, Philippines. They were transported to the laboratory in oxygenated plastic bags, at a temperature of 20°C inside a styropor - lined ice chest. They were maintained in the aquarium at room temperature prior to use, given fresh mussel meat at a feeding rate of 8 percent of body weight per day.

Preparation of Sea Water. – Sea water was reconstituted from a salt mixture obtained from Bioresearch, Inc., Manila. The sea water was sterilized by autoclaving 1.5 liter portions in large volume culture flask. After autoclaving, the sea water was allowed to reaerate under aseptic conditions at 27°C for several days. Immediately before use, antibiotics, streptomycin sulfate and sodium benzyl penicillin were added to the sea water at levels of 50 and 40 mg/li respectively.

The Aquarium. – The aquarium, a rectangular glass vessel (40 x 25 x 8 cm), was fitted with a gas-tight, removable top through which were placed

air inlet and outlet tubes. The seawater was aerated by means of a pump; the air leaving the vessel passed through two traps in a series containing carbon dioxide-free sodium hydroxide solution in which all metabolic carbon dioxide leaving the vessel was absorbed. The set-up is shown in Figure 1.

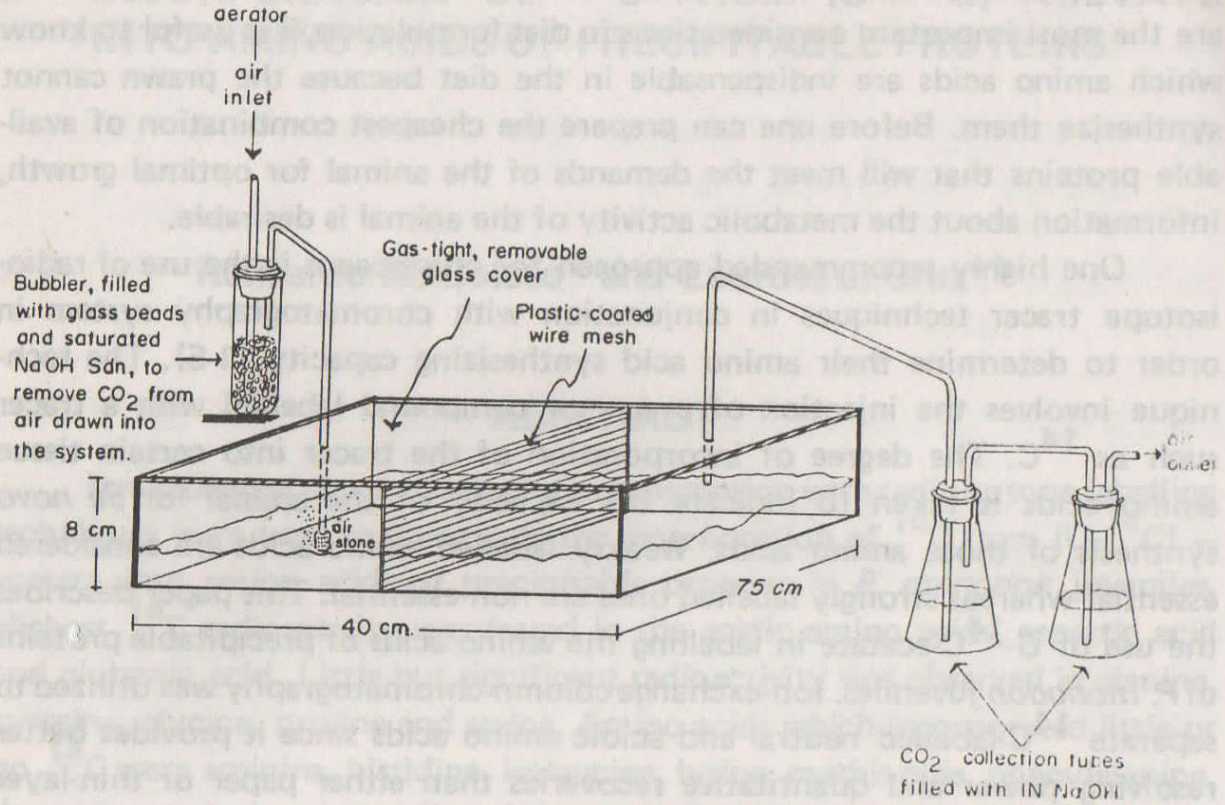

Figure 1. Aquarium for Metabolic CO_2 Collection

Radioisotope Labelling of Prawn Juveniles. – Two prawn juveniles, each weighing about 4 grams, were injected with 5 μli, of a solution of U^{14}C-acetate in physiological saline (5 μCi/μli). The injection was made laterally into the haemocoel, through the articular membrane between the carapace and the first abdominal segment. The prawns were kept separate for the next six days, during which time the expired carbon dioxide was monitored at intervals; the rectangular vessel containing 1.5 liters of sea water with added antibiotics was divided by a piece of plastic coated wire mesh. This prevented the cannibalisation of one prawn by the other during molting. At intervals, the carbon dioxide traps each containing 10 ml of IN sodium hydroxide were removed for analysis; the sea water was changed everyday. Traps containing carbon dioxide-free sodium hydroxide were substituted for those which have been removed. The experiment was carried out at 27^{o}C in a sterile room.

Radioactivity in Tissue Fractions. – The labelled prawn juveniles were minced very finely and to the minced tissue, 0.14M NaCl-0.01M sodium citrate at pH7 .1 was added. The mixture was homogenized for 5 to 10 min

using a Potter-Elvehjem tissue homogenizer. From the tissue homogenate, the TCA Soluble, Lipid, Nucleic Acid, and Protein-Chitin Fractions were prepared. To 0.5ml of each fraction—TCA Soluble, Lipid and Nucleic Acid Fractions – was added 5 ml of scintillation cocktail, Scintisol Complete. Radioactivity due to ^{14}C incorporated in each fraction was determined in a Beckman LS-100 scintillation counter. Total radioactivity in the Protein-Chitin Fraction was determined by solubilizing about 8 mg in 1 ml of NCS tissue solubilizer. Five ml conventional counting medium (toluene/PPO/dimethyl POPOP) was added before counting for radioactivity.

Radioactivity in Expired Carbon Dioxide. – To 0.5 ml sodium hydroxide solution was added 5 ml of scintillation cocktail (toluene/PPO/dimethyl POPOP/Triton X-100). Radioactivity due to ^{14}C expired by the prawns during the interval indicated was measured.

Hydrolysis of Protein for Amino Acid Analysis. – Seventy milligrams of protein-chitin residue was hydrolyzed with 1 ml of 5.7 N HCl in an evacuated sealed tube at 110°C for 24 hours as described by Moore and Stein (6). The hydrolysate was then centrifuged at 10,000 rpm for 10 min. The supernate was evaporated to dryness on a freeze dryer to remove the HCl and the residue was taken up in 1 ml of 0.2N sodium citrate buffer, pH 3.25.

Amino acid Analysis. – One ml of protein hydrolysate was applied on an ion-exchange column of Beckman Type 150-A resin, an 8 percent sulfonated styrene-divinyl benzene copolymer used in the separation of acidic and neutral amino acids. The column was washed by passing one-third bed volume of 0.2N sodium hydroxide containing BRIJ-58 and equilibrated with 0.2N sodium citrate buffer pH 3.25. Elution was carried out using 0.2N sodium citrate buffer, pH 3.25; a change of buffer to pH 4.25 was made at 260 ml elution volume. Fractions were assayed for amino acids using Ninhydrin assay at 570 nm and glycine as standard. Radioactivity due to ^{14}C incorporated in amino acids was also assayed in each fraction.

Purified amino acids were identified using thin layer chromatography on precoated plates of silica gel 60. F254 with concentration zone (precoated layer thickness of 0.25mm). The chromatograms were developed inside a glass vessel containing n-butanol: acetic acid: water/80: 20:20 V/V. After drying the chromatograms, amino acid spots were visualized using ninhydrin spray solution (0.2 percent ninhydrin in n-butanol). The RF values of amino acids were compared to standard amino acids which were similarly chromatographed.

To determine ^{14}C incorporation in individual amino acids of eluates from the ion-exchanger composed of a mixture of amino acids, ascending paper chromatography was used. The chromatograms were developed using t-butanol: formic acid: water/69.5:1:29.5 V/V. After air-drying the chro-

matograms, the paper was cut in 1 cm-squares and radioactivity due to ^{14}C was counted, using 5 ml of scintillation cocktail (toluene/PPO/dimethyl POPOP). Identification of the amino acids was done on paper chromatograms developed similarly and visualized using ninhydrin spray solution. The RF values of the amino acid spots were compared with standard amino acids.

RESULTS

The excretion of $^{14}CO_2$ by juvenile prawns was monitored for six days following the intrahaemacoelic injection of (U-^{14}C)-acetate (25 microcurie) as shown in Figure 2. The curve exhibits a peak 24 hours after the injection. However, the rate of oxidation of acetate to carbon dioxide peaks eight hours following the introduction of carrier acetate. It must be noted that the animals were fasted 12 hours prior to the injection. The amount of excreted $^{14}CO_2$ levelled off on the fourth day and onwards.

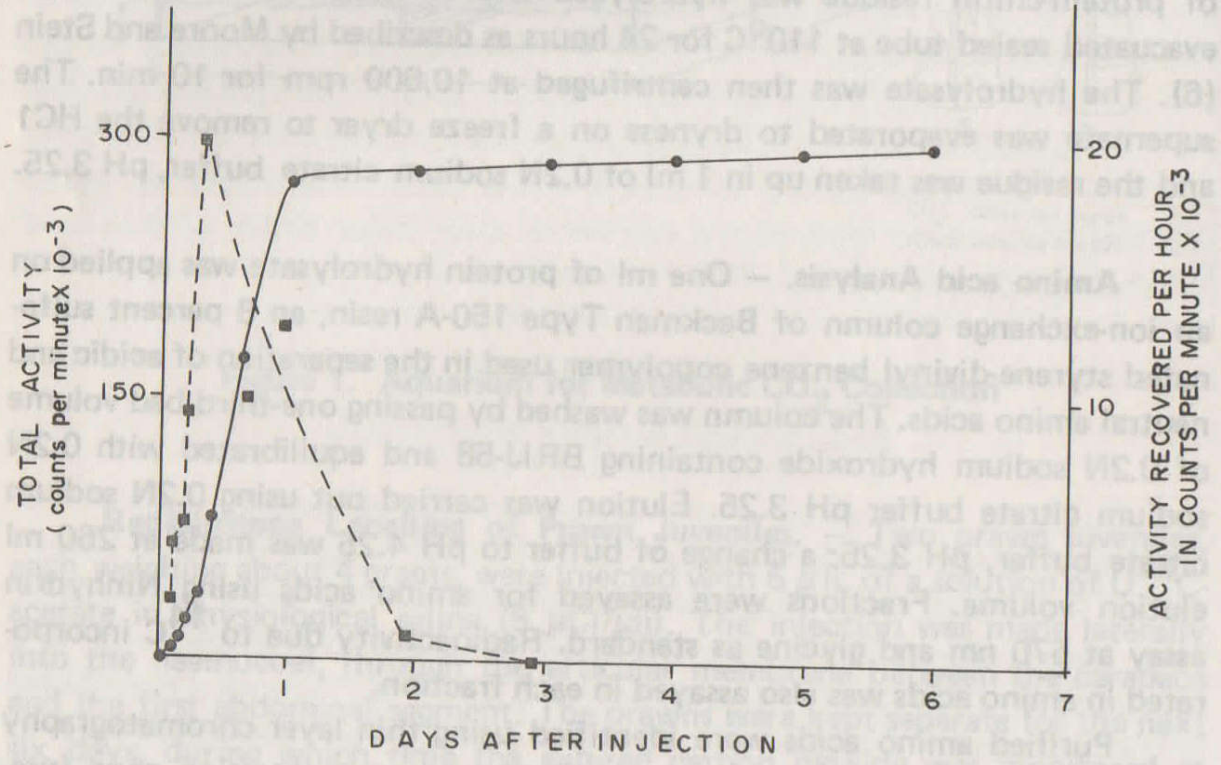

Figure 2. Excretion of $^{14}CO_2$ by juvenile prawns after intrahaemocoelic injection of U-^{14}C-acetate as described in Materials and Methods.

(●—●) total activity due to expired $^{14}CO_2$ as a function of time,
(■—■) rate of $^{14}CO_2$ excretion.

The distribution of radioactivity in tissues of juvenile prawns labelled with (U-^{14}C)-acetate was determined. Table 1 shows the distribution of

radioactivity in the tissue fraction of a prawn sacrificed three days after the injection and another sacrificed six days later. The data show that three days after the injection of carrier, the largest amount of radioactivity was present in the TCA Soluble Fraction wherein low molecular weight substances are soluble. However, six days after the pulse, the protein-chitin residue incorporated the largest amount of radioactivity.

Table 1. Distribution of ^{14}C radioactivity in tissue fractions of *P. monodon* juveniles labelled with U-^{14}C-acetate. Tissue fractionation and assay for radioactivity are described in Materials and Methods.

TISSUE FRACTION	TOTAL RADIOACTIVITY cpm x 10^{-16}	
	3 days	6 days
Trichloroacetic Acid Soluble Fraction	1.38	1.07
Lipid Fraction	0.37	0.40
Nucleic Acid Fraction	0.14	0.08
Protein-Chitin Fraction	0.85	1.44

The result after six days of the pulse is in good agreement with that obtained from juveniles of *Palaemon serratus* (3). In contrast, marine flatfish incorporate the greatest amount of radioactivity in the materials soluble in trichloroacetic acid six days after labelling (7).

From these data it was decided to determine the incorporation of ^{14}C into amino acids of precipitable proteins six days after the pulse. The fact that the excretion of $^{14}CO_2$ has levelled off from the fourth day and onwards and the shift in the fraction containing the highest amount of radioactivity from the TCA Soluble Fraction to the Protein-Chitin Fraction are evidences to support the attainment of a steady-state condition after six days.

Figure 3 shows the elution profile of acidic and neutral amino acids obtained after passing the protein hydrolysates from the Protein-Chitin Fraction of juvenile prawns through Beckman Type 150-A ion exchange resin. The data show significant incorporation into aspartic acid, glutamic acid, serine, proline, glycine, alanine and cysteine. There was little or no significant radioactivity in threonine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. Significant incorporation ranged from 200 cpm to 4,200 cpm. No significant amount of radioactivity was found in the basic

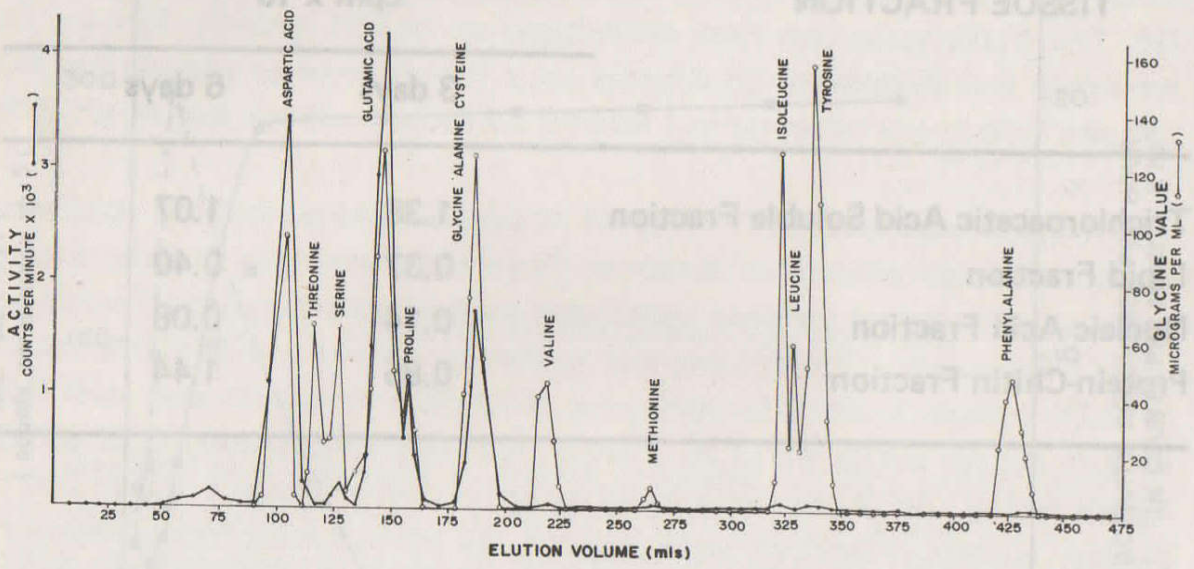

Figure 3. Elution profile of acidic and neutral amino acids of the juveniles after ion-exchange chromatography on Beckman type 150-A resin ○—○. Also shown is the incorporation of ^{14}C into the amino acid ●—●. Refer to Materials and Methods.

amino acids lysine, arginine and histidine. These amino acids remained bound to the ion-exchanger and were eluted out of the column by using

0.2 N NaOH. Portions of the eluate were obtained for liquid scintillation counting (data not shown). Tryptophan was destroyed by acid hydrolysis. These results are summarized in Table 2.

Table 2. Specific activity in individual amino acids of the Protein/Chitin Fraction of *P. monodon* juveniles following the injection of U-^{14}C-acetate.

AMINO ACID	ACTIVITY cpm/micromole C x 10^{-3}
Aspartic Acid	2.66
Glutamic Acid	2.01
Proline	0.70
Glycine	0.41
Cysteine	0.32
Serine	0.24
Alanine	0.24
Threonine	0
Valine	Traces
Methionine	Traces
Isoleucine	Traces
Leucine	Traces
Tyrosine	0
Phenylalanine	0
Lysine	0
Histidine	0
Arginine	0
Tryptophan	Not analyzed*

*Tryptophan is destroyed by acid hydrolysis.

As with other animals already investigated (3, 4, 5, 7), there is a well-defined boundary between amino acids containing radioactivity and those without significant radioactivity. It is believed that those amino acids which can be formed from ordinarily available precursors, in this case, acetate, are

dispensable dietary amino acids of the prawn. The amino acids without significant radioactivity are therefore essential because they cannot be synthesized from acetate.

DISCUSSION

The incorporation of ^{14}C from (U-^{14}C)-acetate into amino acids of precipitable proteins of *P. monodon* juveniles was studied. Those amino acids which incorporated little or no ^{14}C were arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine. It appears that *P. monodon* could not synthesize these amino acids and, thus, has a dietary requirement for them. Moreover, preliminary results indicate that data obtained from ion-exchange chromatography in conjunction with radiotracer assay may be used as a guide in the search for protein sources of optimal amino acid pattern for *P. monodon.*

The accurate use of radiolabelling procedures can add extraordinary perspective into data obtained from conventional techniques. Besides being an elegant approach, its interpretation is usually straight forward.

Primary consideration must be given to the system being labelled. The highest rate of oxidation of acetate to carbon dioxide occurred eight hours following the injection of carrier acetate. The prawn species, *Palaemon serratus,* exhibits the highest rate of oxidation within 1 hour after the injection of carrier acetate (3). It seems that acetate is not as rapidly metabolized by *P. monodon* juveniles as compared to juveniles of *Palaemon serratus.* This may reflect a species difference in the ability of the animal to mobilize nutrients for its energy needs.

Table I shows a shift in the tissue fraction that incorporates the greatest radioactivity from the third day to the sixth day of the pulse. This strongly suggests that acetate is continuously mobilized as a precursor in amino acid synthesis, consequently protein biosynthesis. It can also be inferred that the same precursor is used for chitin synthesis.

^{14}C incorporation from acetate into amino acids of precipitable proteins of *P. monodon* juveniles showed the highest incorporation in acidic amino acids, aspartic acid and glutamic acid. There was smaller but significant radioactivity in serine, proline, glycine, alanine and cysteine. No radiocarbon from acetate was recovered in arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine. From this and other experiments, it may be concluded that there exists a broad similarity between crustaceans and other animals with respect to the biosynthesis of non-essential amino acids via the tricarboxylic acid cycle and its associated pathways.

Glutamate formation is fundamental to the biosynthesis of the non-essential amino acids since it is the main pathway for the generation of alpha-amino groups from ammonia. Glutamate is formed from the reductive ami-

nation of alpha-ketoglutarate derived from the tricarboxylic acid cycle, by the action of glutamate dehydrogenase.

Proline is derived from glutamic acid. Alanine and aspartic acid arise by transamination from glutamate. As shown in Table II, the amount of radiocarbon in aspartic acid is much greater than in alanine. Radiocarbon on alanine comes from pyruvate, that of aspartic acid comes from oxaloacetate. Because there is no direct pathway of synthesis from acetate to pyruvate, the difference reflects a dilution effect of unlabelled pyruvate coming from glycolysis on pyruvate coming from excess labelled oxaloacetate.

Glycine can be formed from carbon dioxide and ammonia by the action of glycine synthetase, a pyridoxal phosphate enzyme. However, it can also come from serine. Although serine can be synthesized from acetate, its formation is relatively low compared to the other amino acids. This is due to the lack of a direct pathway of synthesis from acetate to 3-phosphoglycerate and hydroxypyruvate, the major source for the synthesis of serine. Instead, radiolabels on serine must have come from the transamination of glutamate and labelled 3-phosphohydroxypyruvate.

In higher animals as well as in other crustaceans, cysteine is not an essential amino acid. It is biosynthesized from methionine which is essential and serine which is not through transulfuration, an energy requiring process which involves the formation of cystathionine as a key intermediate.

As in other crustaceans, it is likely that tyrosine can be formed when there is sufficient dietary supply of phenylalanine by hydroxylation of phenylalanine. Zandee (2) has shown that after the administration of 3-^{14}C-phenylalanine to the crayfish, *Astacus astacus,* there was significant incorporation of ^{14}C into tyrosine. As in all crustaceans, *P. monodon* requires arginine, while many vertebrates do not. This is due to the absence of the urea cycle in crustaceans which is the main pathway for arginine biosynthesis from ornithine. Trytophan is probably essential as in *Penaeus aztecus* (4).

Future work using the techniques outlined in this paper must focus on developing a method that would allow the quantitative recovery of the basic amino acids. A most convenient technique is ion-exchange chromatography on a short column of Beckman type 15-A resin for the separation of basic amino acids. If a portion of a protein hydrolysate is introduced into this column, the basic amino acids will elute out while the neutral and acidic amino acids will remain bound to the column. In this way the quantitation of all the amino acids could be achieved. Data on the levels of the essential amino acids in tissue proteins may be used to calculate the proportions of amino acids to be supplied in synthetic diets for growth and survival experiments. Furthermore, it can be used as a guide in looking for natural protein sources of optimal amino acid pattern for the prawn.

In addition, the incorporation of ^{14}C into amino acids can be further studied by using ^{14}C-glucose instead of acetate. If this were done and if all

the members of the glycolytic pathway were present in *P. monodon,* then radiolabels on alanine, serine, glycine and cysteine are expected to increase. Together with the pattern of incorporation from acetate, it should be able to present a more comprehensive picture of non-essential amino acid biosynthesis in this animal.

SUMMARY

The incorporation of ^{14}C from (U-^{14}C)-acetate into amino acids of precipitable proteins in *P. monodon* juveniles was studied using ion-exchange chromatography in conjunction with radioisotope techniques. Highest ^{14}C radioactivity was found in the acidic amino acids, aspartic and glutamic acids. Little but significant incorporation was found in serine, proline, glycine, alanine and cysteine. Amino acids which incorporated little or no ^{14}C were arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine and valine. It appears that *P. monodon* juveniles could not synthesize these amino acids from acetate.

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