Arginine Metabolism in Insects

ROLE OF ARGINASE IN PROLINE FORMATION DURING SILKMOTH DEVELOPMENT

By S. RAGHUPATHI RAMI REDDY AND JAMES W. CAMPBELL

Department of Biology, Rice University, Houston, Tex. 77001, U.S.A.

(Received 11 June 1969)

1. Ornithine δ -transaminase (L-ornithine-2-oxo acid aminotransferase, EC 2.6.1.13) and Δ^1 -pyrroline-5-carboxylate reductase [L-proline-NAD(P) 5-oxido-reductase, EC 1.5.1.2] were demonstrated in fat-body and flight-muscle tissues of the silkmoth *Hyalophora gloveri*. Arginase (L-arginine ureohydrolase, EC 3.5.3.1) is also present in these tissues. 2. Arginase, ornithine transaminase and pyrroline-carboxylate reductase are generally considered to make up the catabolic pathway for the conversion of arginine into proline. The conversion of L-[U- 14 C]arginine into [14 C]proline by intact fat-body tissue was used to show that the enzymes in insect fat body also function in this capacity. 3. Of the three enzymes of the catabolic pathway, only arginase increased during adult development and the increase coincided with the emergence of the winged adult moth. Since proline appears to be a major substrate utilized in insect flight metabolism, the increase in arginase activity at this stage suggests a major role for arginase in proline formation.

Arginine is a nutritional requirement for several insects (House, 1965). In some species this requirement can be replaced by citrulline, but not ornithine (Hinton, 1956; Davis, 1962). These results indicate that insects may have lost the ability to synthesize arginine during their evolutionary transition to uricotelism in much the same way as have birds (Tamir & Ratner, 1963*a,b*). Several insects do possess arginase (Garcia, Tixier & Roche, 1956; Kilby & Neville, 1957; Szarkowska & Porembska, 1959), and in this respect they are also similar to birds and other uricotelic vertebrates. Since the ornithine formed by the action of their arginase cannot be recycled through the arginine pathway for arginine or urea synthesis as it is in ureotelic species, the arginase present in insect tissue must function in a strictly catabolic role.

In a previous study of arginase in the silkmoths *Hyalophora gloveri* and *Antheraea polyphemus* (Reddy & Campbell, 1969) we noted as much as 30 times more fat-body arginase in adult moths than in pupae. As shown below, the most marked increase in arginase activity that occurs during development of pupae to adults coincides with the emergence of the winged adults. Proline appears to have a special role in insect flight metabolism in penetrating the mitochondria to form precursors of oxaloacetate and thus allowing the complete oxidation of pyruvate via the tricarboxylic acid cycle (Sacktor & Childress, 1967). Because of the

interrelationship between arginine and proline, it seemed likely that the increase in arginase activity accompanying the emergence of winged adults might be concerned with the conversion of arginine, formed via protein catabolism, into proline for flight metabolism. This study was therefore directed towards a demonstration of the presence of other enzymes of the pathway for converting arginine into proline, their cellular localization and any changes in activity that they might undergo during adult development.

EXPERIMENTAL

Animals. Commercial samples of *H. gloveri* and *A. polyphemus* pupae were chilled for at least 3 months at 4°. They were brought to room temperature (23–25°) to initiate development.

Chemicals. L-Arginine hydrochloride, L-ornithine hydrochloride, sodium pyruvate, sodium α -oxobutyrate, oxaloacetic acid, α -oxoglutaric acid, pyridoxal 5'-phosphate, NADH, NADPH and horse heart cytochrome *c* were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Δ^1 -Pyrroline-5-carboxylic acid was prepared as described by Strecker (1960) and Feisach & Strecker (1962) from γ -acetamido- γ -diethoxycarbonylbutyraldehyde phenylhydrazone. The latter compound was synthesized from 2-acetamido-2-ethylmalonate (K & K Laboratories, Plainview, N.Y., U.S.A.) by the procedure of Moe & Warner (1948). A modification of the described procedure was

used for recovering the synthetic Δ^1 -pyrroline-5-carboxylate. The HCl hydrolysate of γ -acetamido- γ -diethoxycarbonylbutyraldehyde was chromatographed on a column of the sulphonic resin Dowex 50 (H^+ form) as described by Strecker (1960). Δ^1 -Pyrroline-5-carboxylate was detected in the fractions eluted from the column by its reaction with *o*-aminobenzaldehyde (Strecker, 1957). The fractions containing this compound (in 0.5M-HCl) were stored at 4°. The product was stable under these conditions as judged by its reactivity with *o*-aminobenzaldehyde and its ability to act as a substrate for the reductase. Before use the excess of HCl was removed *in vacuo* at 2° over saturated NaOH. The residue was dissolved in water and the pH adjusted to 6.8 with NaOH. The lowest recovery from this step was 60%.

o-Aminobenzaldehyde was prepared from *o*-nitrobenzaldehyde as described by Albrecht, Sher & Vogel (1962).

L-[U- ^{14}C]Arginine hydrochloride and L-[U- ^{14}C]ornithine hydrochloride were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Enzyme assays. (a) Arginase. The assay previously described (Reddy & Campbell, 1969) was used for this activity.

(b) Ornithine δ -transaminase. Tissues were ground by hand in 9 vol. of 0.1M-KH₂PO₄, adjusted to pH 8.0 with KOH, and containing 10mM-mercaptoethanol, in a Ten-Broeck tissue grinder kept cold in crushed ice. The reaction mixture was essentially as described by Peraino & Pitot (1963) and contained, in 3ml.: L-ornithine sodium salt, pH 8.0, 300 μ moles; sodium α -oxoglutarate, pH 7.4, 60 μ moles; K₂HPO₄-KH₂PO₄ buffer, pH 8.0, 750 μ moles; pyridoxal 5'-phosphate, 1 μ mole; *o*-aminobenzaldehyde, 20.7 μ moles [in 0.1 ml. of 40% (v/v) ethanol]; 0.4 ml. of the tissue homogenate. Incubation was for 60 min. at 25°. Control tubes contained 0.4 ml. of heat-inactivated homogenate (3–5 min. at 100°). The reaction was stopped by adding 2 ml. of 10% (w/v) trichloroacetic acid. The mixture was then kept for 1 hr. at room temperature. It was filtered through Whatman no. 1 filter paper and the extinction of the filtrate was measured at 440 nm. The amount of pyrroline-5-carboxylate formed was calculated from the millimolar extinction coefficient reported by Strecker (1965).

(c) Δ^1 -Pyrroline-5-carboxylate reductase. The activity of this enzyme was determined by the NADH-dependent disappearance of Δ^1 -pyrroline-5-carboxylate. Tissues were ground as described above for ornithine transaminase in 9 vol. of 50mM-KH₂PO₄ adjusted to pH 6.8 with KOH, and containing 1mM-EDTA and 2mM-mercaptoethanol (Peisach & Strecker, 1962). The reaction mixture contained, in 3 ml.: sodium Δ^1 -pyrroline-5-carboxylate, pH 6.8, 15 μ moles; NADH, 4 μ moles; K₂HPO₄-KH₂PO₄ buffer, pH 6.8, 100 μ moles; 0.2 ml. of tissue homogenate. Incubation was for 10 or 15 min. at 25°. NADH was omitted from control tubes. The reaction was stopped with 2 ml. of 10% trichloroacetic acid. The mixture was clarified by centrifuging and portions of the supernatant fluid were analysed for Δ^1 -pyrroline-5-carboxylate with *o*-aminobenzaldehyde (Strecker, 1957).

(d) Cytochrome oxidase. This activity was measured as described by Smith (1955).

Protein measurements. Protein was estimated in tissue homogenates as described by Oyama & Eagle (1956), with

standard crystalline bovine serum albumin (Sigma Chemical Co.) that had been previously dried over anhydrous CaCl₂.

Free amino acid analysis. The free amino acids in pooled fat-body tissue were extracted with 5% (w/v) trichloroacetic acid and were isolated from this extract with the sulphonic resin Dowex 50 (H^+ form) as described by Campbell & Speeg (1968a). To free the tissue of haemolymph amino acids, it was rinsed twice in lepidopteran saline (described below) and blotted before extraction. The individual amino acids were determined with an automatic amino acid analyser (Technicon).

Metabolism of ^{14}C -labelled compounds. The fat-body tissue from pupal and adult moths was pooled and rinsed in the high-K⁺ low-Na⁺ lepidopteran saline formulated by Reddy & Wyatt (1967) to which was added 5mM-glucose. The tissue was blotted and 200mg. portions (196–213 mg.) were incubated with shaking for 20 min. in air at 25° in 2 ml. of the lepidopteran saline-glucose solution containing either 5 μ moles of L-[U- ^{14}C]arginine (1 μ C/ μ mole) or 5 μ moles of L-[U- ^{14}C]ornithine (1 μ C/ μ mole). The reaction was stopped by adding 2 ml. of 10% trichloroacetic acid and the free amino acids were extracted as described above. The free amino acids were separated on Whatman 3MM paper by two-dimensional chromatography with butan-2-ol-formic acid (88%, w/v)–water (15:3:2, by vol.) and phenol-water (3:1, w/w) in an ammoniacal atmosphere. [^{14}C]Proline and [^{14}C]glutamate were detected on the chromatograms by radioautography with non-screen X-ray film (Anscoc). These two amino acids were eluted from the paper with water. Portions of the eluates were counted in a liquid-scintillation spectrometer in Bray's (1960) solution. Other portions were used for the determination of proline (Chinard, 1952) and glutamate (Lee & Takahashi, 1966).

RESULTS

Arginase activity during development. The increase in fat-body arginase activity during development of *H. gloveri* and *A. polyphemus* is shown in Fig. 1. There is a 30-fold increase in *H. gloveri* fat-body arginase between the pupal and the winged adult stage, and the major increase occurs after the emergence of the winged adult. There is also an increase in *A. polyphemus* fat-body arginase although the magnitude is less. The results given in Fig. 1 are based on both fat-body tissue weight and protein content. Arginase also occurs in other tissues: in adult male *H. gloveri* flight muscle the activity is 19–50 μ moles of arginine hydrolysed/hr./g. or 0.19–0.29 μ mole/hr./mg. of protein. To show that the increase in fat-body arginase in the adult reflects an actual increased potential for arginine hydrolysis, the total soft tissues from individual pupae and adult *H. gloveri* were removed and assayed for activity. The arginase activity for adults was 900–1483 μ moles/hr./individual and for pupae it was 81–208 μ moles/hr./individual. There was complete recovery of the added arginase activity when pupal and adult fat-body enzyme preparations were mixed. It was previously shown that no apparent differences exist between

Fig. 1. Fat-body arginase activity during development of *H. gloveri* (a) and *A. polyphemus* (b). Activity is expressed as $\mu\text{moles hydrolysed/hr./g.}$ of tissue (●) and as $\mu\text{moles/hr./mg.}$ of protein (○).

Table 1. Ornithine δ -transaminase activity in *H. gloveri* fat-body

The assay mixture contained 0.4 ml. of 10% (w/v) tissue homogenates containing 2.91 mg. of protein for the adult and 5.61 mg. of protein for the pupa. Incubation was for 60 min. at 25° with the standard reaction mixture described in the text. —, Not determined.

Reaction mixture modifications	Δ^1 -Pyrroline-5-carboxylate formed (μmoles)	
	Pupa	Adult
None	4.19	3.37
Minus L-ornithine	0	0
Minus α -oxoglutarate	0.06	0.06
Minus pyridoxal 5'-phosphate	2.04	2.47
Minus enzyme	—	0.03
Heat-killed enzyme	0	—

the pupal and adult enzymes (Reddy & Campbell, 1969).

Ornithine δ -transaminase activity. The requirements for ornithine δ -transaminase activity in *H. gloveri* fat-body preparations are shown in Table 1. Omission of pyridoxal 5'-phosphate decreased the activity by 30–50%, which indicates that the pyridoxal derivative serves as a cofactor for the insect enzyme as it does in other species (Vogel & Kopac, 1960; Katunuma, Matsuda & Tomino,

Fig. 2. Effect of pH on pupal *H. gloveri* fat-body ornithine δ -transaminase activity in phosphate buffer (○) and tris-chloride buffer (●).

Fig. 3. Effect of L-ornithine concentration [S] on pupal *H. gloveri* fat-body ornithine δ -transaminase activity (v). The α -oxoglutarate concentration was 20 mM. The L-ornithine concentration was varied as indicated.

1964; Hill & Chambers, 1967). Under the conditions used for the assay of ornithine δ -transaminase, the formation of glutamic γ -semialdehyde was linear with both time of incubation (up to 60 min.) and amount of enzyme extract (up to 0.8 ml.).

The pH optimum of fat-body ornithine δ -transaminase with α -oxoglutarate as the amino acceptor is about pH 8 in phosphate buffer and about pH 7.6 in tris-chloride buffer (Fig. 2).

In addition to α -oxoglutarate, other α -oxo acids were tried as amino acceptors with both fat-body and flight-muscle ornithine δ -transaminase. These were freshly prepared solutions of α -oxobutyrate, oxaloacetate and pyruvate. The activity with all but pyruvate was questionable. There was some transamination with pyruvate, but this activity, which showed a pH optimum at 6.8–7.0 in both potassium phosphate and tris-chloride buffer, amounted to no more than 4 μ moles/hr./g. of fat-body and less than 1 μ mole/hr./g. of flight muscle in adult *H. gloveri*. With adult male and female *H. gloveri* the ornithine δ -transaminase activity of flight muscles with α -oxoglutarate as the acceptor was 20–61 μ moles/hr./g. of tissue or 0.13–0.51 μ mole/hr./mg. of protein.

The apparent K_m for L-ornithine of fat-body (pupal) ornithine δ -transaminase was estimated to be 13mM and L-ornithine concentrations higher than 0.1M were inhibitory (Fig. 3). With a saturating L-ornithine concentration (0.1M), the apparent K_m for α -oxoglutarate was about 3mM (pupal fat-body enzyme).

The activities of ornithine δ -transaminase measured in fat-body tissue of male and female *H. gloveri* during development are shown in Table 2. In contrast with arginase there is little or no change in the activity of the fat-body enzyme during development. If the activity changes at all, it may possibly decrease slightly.

Δ^1 -Pyrroline-5-carboxylate reductase activity. The requirements for reductase activity are shown in Table 3. The cofactor requirement is shown in Table 4. NADH gives about twice the activity

obtained with NADPH. In this respect the insect enzyme is more like the mammalian enzyme (Smith & Greenberg, 1957; Peisach & Strecker, 1962) than the protozoan enzyme (Hill & Chambers, 1967) or the fungal enzyme (Yura & Vogel, 1959). Under the conditions of the standard assay, the formation of proline was linear with both time of incubation (up to 25 min.) and amount of enzyme extract (up to 0.45 ml.).

To show that proline was the product of the reaction, it was separated from the reaction mixture by one-dimensional chromatography on Whatman 3MM paper with butan-2-ol-formic acid (88%, w/v)–water (15:3:2, by vol.). Proline was identified by its R_F and characteristic yellow colour after the chromatograms were sprayed with ethanolic 0.2% ninhydrin. The amount of proline formed, as judged by the intensity of the ninhydrin reaction, increased with increased incubation time. No proline was detected in control tubes (Table 3).

The pH optimum for pupal fat-body pyrroline carboxylate reductase was rather broad with some increase in activity in the region pH 6.5–6.8; this is the range reported for the mammalian enzyme (Smith & Greenberg, 1957; Peisach & Strecker,

Table 2. Ornithine δ -transaminase activity in *H. gloveri* fat-body during development

Ornithine δ -transaminase was measured as described in the text. The activities as μ moles/hr./mg. of protein are given in parentheses. The results for seven female and four male pupae in diapause and five adult males are given as means $\pm$ s.d.; otherwise the values are for individuals.

Developmental stage	Δ^1 -Pyrroline-5-carboxylate formed (μ moles/hr./g. of tissue)	
	Female	Male
Diapause (pupae chilled 4–6 months)	59.9 $\pm$ 21.2 (0.41 $\pm$ 0.18)	80.0 $\pm$ 27.6 (0.83 $\pm$ 0.20)
Day 2	70.6 (0.54) 68.0 (0.61)	92.8 (0.95) 76.5 (0.93)
Day 7	28.4 (0.22) 38.6 (0.25)	58.0 (0.64) 51.0 (0.57)
Day 14	38.6 (0.29) 32.9 (0.27)	35.0 (0.26) 51.0 (0.47)
Adult moth	45.8 (0.53) 25.4 (0.31)	47.7 $\pm$ 27.2 (0.56 $\pm$ 0.35)

Table 3. Δ^1 -Pyrroline-5-carboxylate reductase activity in adult male *H. gloveri* fat-body

The assay mixture contained 0.2 ml. of a 10% (w/v) tissue homogenate containing 4.4 mg. of protein. Incubation was at 25° for 10 min. with the standard assay conditions described in the text.

Reaction mixture modifications	Proline formed (μ moles)
None	2.10
Minus NADH	0.30
Minus Δ^1 -pyrroline-5-carboxylate	0
Minus enzyme	0
Heat-killed enzyme	0

Table 4. Cofactor requirement of pupal female *H. gloveri* fat-body Δ^1 -pyrroline-5-carboxylate reductase

The assay mixture contained 0.2 ml. of a 10% (w/v) tissue homogenate. Incubation was for 10 min. in the standard reaction mixture described in the text, in which the nicotinamide nucleotide content was varied as indicated.

Nicotinamide nucleotide	Proline formed (μ moles)
NADH (4 μ moles)	2.94
None	0
NADPH (4 μ moles)	1.46
NADH + NADPH (4 μ moles each)	2.76

1962) and the fungal enzyme (Yura & Vogel, 1959). The protozoan enzyme shows an optimum at about pH 9 (Hill & Chambers, 1967).

The apparent K_m for Δ^1 -pyrroline-5-carboxylate (NADH concn. 1.33 mM) of the fat-body enzyme is 1.6 mM. The apparent K_m for NADH (Δ^1 -pyrroline-5-carboxylate concn. 5 mM) is 0.5 mM.

As shown in Table 5, there were no marked differences in the reductase activity between pupal and adult fat-body tissue.

Δ^1 -Pyrroline-5-carboxylate reductase activity is

Table 5. Δ^1 -Pyrroline-5-carboxylate reductase activity in pupal and adult *H. gloveri* fat-body

Enzyme activity was determined as described in the text. The activities as μ moles/hr./mg. of protein are given in parentheses. The values are for individuals.

	Proline formed (μ moles/hr./g. of tissue)	
	Male	Female
Pupa (chilled 6-8 months)	495.0 (4.42)	426.0 (2.94)
	832.5 (6.94)	439.0 (3.21)
		882.0 (6.90)
		502.0 (4.52)
Adult	540.0 (7.89)	1050.0 (11.90)
	660.0 (9.30)	967.5 (10.60)
	665.0 (12.60)	
	570.0 (7.35)	

also present in adult flight muscle. Two male moths gave activities of 555 and 510 μ moles/hr./g. of tissue (3.47 and 3.10 μ moles/hr./mg. of protein) and two females gave activities of 600 and 713 μ moles/hr./g. of tissue (3.66 and 4.83 μ moles/hr./mg. of protein). The product of the reaction was not identified in flight muscle and these values could be high since they were determined by substrate disappearance. Flight muscle contains pyrroline-5-carboxylate dehydrogenase activity (Brosemer & Veerabhadrapa, 1965) and part of the apparent reductase activity could be due to the utilization of the NAD⁺ formed by the reductase reaction for the conversion of pyrroline-5-carboxylate into glutamate.

Subcellular distribution of enzymes. Fat-body arginase activity occurs mainly in the soluble cellular fraction (Reddy & Campbell, 1969). As shown in Table 6, this is also true for both fat-body and flight-muscle ornithine δ -transaminase and pyrrolinecarboxylate reductase activities. The transaminase is found exclusively in the soluble fraction whereas some reductase activity is found in other fractions, especially in flight muscle. The occurrence of these enzymes for the conversion of arginine into proline in the extramitochondrial fraction is consistent with the proposed role of proline penetrating the mitochondria to form precursors of oxaloacetate to bring about the complete oxidation of pyruvate (Sacktor & Childress, 1967).

Table 6. Subcellular distribution of ornithine δ -transaminase and Δ^1 -pyrroline-5-carboxylate reductase in adult *H. gloveri* tissues

Tissues were ground by hand in 9 vol. of 0.25 M-sucrose in 50 mM-KH₂PO₄, adjusted to pH 6.8 with KOH, and containing 2 mM-mercaptoethanol and 1 mM-EDTA, in a TenBroeck tissue grinder kept in a crushed-ice bath. Fractionation by centrifuging was performed at 0° in a Sorvall RC2-B centrifuge with a fixed-angle rotor (SS-34). Centrifugal forces given are approximate averages. The residues obtained by centrifuging were resuspended in the original volume of the sucrose-phosphate solution for determination of enzyme activity. The standard assay conditions described in the text were used. Cytochrome oxidase activity was determined as $\Delta E_{550}/2$ min./25 μ l.

Fraction	Ornithine δ -transaminase		Pyrrolinecarboxylate reductase		Cytochrome oxidase
	(μ moles/hr./0.4 ml.)	(% of total)	(μ moles/hr./0.2 ml.)	(% of total)	(% of total)
Fat-body:					
Homogenate	2.66	100	11.4	100	100
500g residue	0	0	0	0	35.3
15000g residue	0	0	0.7	6.1	70.6
40000g residue	0	0	0.4	3.5	11.8
40000g supernatant fluid	2.74	103	13.6	119	17.6
Flight muscle:					
Homogenate	1.16	100	7.2	100	100
500g residue	0	0	0.8	11.1	25
15000g residue	0	0	0.8	11.1	62.5
40000g residue	0	0	1.0	13.9	12.5
40000g supernatant fluid	1.23	106	6.6	91.7	4.2

Ornithine transaminase is a particulate enzyme in the rat (Peraino & Pitot, 1963), *Tetrahymena* (Hill & Chambers, 1967), the sunflower (J. E. Smith, 1962) and the chicken (Vecchio & Kalman, 1968). Only in *Neurospora* (Vogel & Kopac, 1960) has it

previously been inferred to be a soluble enzyme. Its location in the extramitochondrial fraction in insects is thus unique for animal tissues.

Fat-body free amino acids. As shown in Table 7, the concentration of most free amino acids in fat-body tissue of post-emergent adults is lower than in diapausing pupae. With certain amino acids, such as lysine, this concentration difference is very pronounced. Glutamine and tryptophan are exceptions in showing increased concentration in adults. The general decrease in the free amino acid content of the fat-body of the winged adult may indicate the extreme terminal stages of protein catabolism in which the utilization of amino acids exceeds their rate of replenishment in the pool from protein breakdown. Saturniid silkmoths do not feed as adults and live only long enough to reproduce. The adults are presumed to normally die of starvation and desiccation (Williams, 1963; Krishnakumaran & Schneiderman, 1964). Although information does not appear to be available on this point, it seems reasonable to assume the winged adults are in a state of extreme protein catabolism.

The decrease in free arginine in adult fat-body is not reflected in a concomitant increase in free ornithine. Nor is there an increase in free proline and glutamate, the main conversion products of ornithine. These findings are consistent with a utilization during development of the ornithine formed from arginine via the action of arginase.

Conversion of arginine and ornithine into proline and glutamate. The overall pathway for the conversion of arginine into proline in intact fat-body

Table 7. Concentrations of free amino acids in pupal and adult *H. gloveri* fat-body

The tissue was pooled from several individuals for analysis.

Amino acid	Concentration (μ moles/g.)		Ratio: Pupa Adult
	Pupa	Adult	
Alanine	1.53	1.36	1.13
Arginine	5.17	0.99	5.22
Aspartic acid	0.26	0.11	2.36
Glutamic acid	2.97	1.17	2.54
Glutamine	2.88	6.26	0.46
Glycine	0.98	0.53	1.85
Histidine	5.04	1.00	5.04
Isoleucine	0.73	0.12	6.08
Leucine	0.74	0.16	4.63
Lysine	12.23	0.22	55.59
Methionine	0.11	Trace	
Ornithine	0.15	0.15	1.00
Phenylalanine	0.66	0.14	4.71
Proline	6.40	1.32	4.85
Serine	6.77	1.50	4.51
Threonine	0.86	0.31	2.77
Tryptophan	1.15	1.84	0.63
Tyrosine	1.27	0.45	2.82
Valine	1.89	0.25	7.56

Table 8. Metabolism of L-[U- 14 C]arginine and L-[U- 14 C]ornithine to proline and glutamate by pupal and adult *H. gloveri* fat-body tissues

From 196 to 213 mg. of fat-body tissue was incubated for 20 min. at 25° with either 5 μ moles of L-[U- 14 C]-ornithine [2.08×10^6 d.p.m./ μ mole; corrected for average endogenous ornithine (Table 7)] or 5 μ moles of L-[U- 14 C]-arginine (1.68×10^6 d.p.m./ μ mole, ornithine carbon only; corrected for average endogenous arginine content). Proline and glutamate were determined as described in the text.

Substrate	Pupa		Adult		Product concn. ratio ($\frac{\mu\text{moles/g. of pupa}}{\mu\text{moles/g. of adult}}$)	Specific radioactivity ratio ($\frac{\text{d.p.m./}\mu\text{mole of adult}}{\text{d.p.m./}\mu\text{mole of pupa}}$)
	(nmoles incorporated)	(d.p.m./ μmole)	(nmoles incorporated)	(d.p.m./ μmole)		
Proline						
L-[U- ¹⁴ C]-Ornithine	2.20	17340	6.10	185588	3.86	10.70
L-[U- ¹⁴ C]-Arginine	7.83	33813	7.81	188955	4.76	5.59
Glutamate						
L-[U- ¹⁴ C]-Ornithine	0.50	7500	1.35	23275	1.20	3.10
L-[U- ¹⁴ C]-Arginine	1.99	17806	1.60	22965	1.18	1.29

tissue is documented in Table 8: both L-[U- ^{14}C]-arginine and L-[U- ^{14}C]-ornithine are converted into [^{14}C]proline as well as [^{14}C]glutamate. Other ^{14}C -labelled compounds were formed from these two substrates, but they were not identified. Because the incorporation of ^{14}C from both [^{14}C]-arginine and [^{14}C]-ornithine into proline is about four times that into glutamate in both pupae and adults, one might conclude that the catabolic pathway in the silkworm is directed more towards proline formation than towards glutamate formation. This is similar to the situation in *Tetrahymena* (Hill & Chambers, 1967). In mammalian liver (Katunuma, Okada, Matsuzawa & Otsuka, 1965) and perhaps micro-organisms (Ramaley & Bernlohr, 1966) the pathway is directed more towards glutamate synthesis.

From the results obtained in this experiment with intact tissue (Table 8) it appears that the increase in arginase activity of the adult moth is not reflected by an increased rate of conversion of arginine into either proline or glutamate. Both pupal and adult fat-body tissue converted approximately the same amounts of [^{14}C]arginine into these products. As shown by the product concentration/specific radioactivity ratios, the greater specific radioactivity of the products in the adult is a result of less dilution by the endogenous proline and glutamate of the tissue. On the other hand, there was an increased conversion of [^{14}C]-ornithine into proline and glutamate by the adult tissue. This increase is about three times that of the pupa. In this case the greater specific radioactivity of the products cannot be accounted for by less dilution due to endogenous proline and glutamate.

There also appears to be compartmentation of ornithine metabolism in fat-body tissue. In pupae, the rate of conversion of ornithine derived from arginine into proline and glutamate is almost four times the rate of conversion of exogenously supplied ornithine. Even in the adult, when the rate of conversion of exogenously supplied ornithine is increased, it is still less than the conversion of ornithine derived from arginine. Since all three enzymes of the catabolic sequence are located mainly in the extramitochondrial fraction, this differential utilization of endogenously formed and exogenously supplied ornithine could be due either to a permeability difference between the two amino acids that changes with development or to a compartmentation phenomenon.

DISCUSSION

Although arginase is widely distributed in uricotelic animals, most of which, with the exception of land snails (Campbell & Speeg, 1968a), appear to be incapable of arginine biosynthesis, its physio-

logical function in these organisms has received little attention. In mammalian liver, arginase functions in urea biosynthesis and also as a catabolic enzyme for the utilization of the ornithine carbon atoms of arginine. Ornithine δ -transaminase is a mitochondrial enzyme in mammals, and ornithine is converted by mitochondria into aspartate and glutamate (Katunuma *et al.* 1965). Arginase functions mainly as a catabolic enzyme in mammalian liver cells in tissue culture that have lost the ability to synthesize arginine (Eliasson & Strecker, 1966) and in micro-organisms that are capable of arginine biosynthesis but do not synthesize urea as a metabolic end product (Laishley & Bernlohr, 1968). Both arginase (Brown, 1966) and ornithine δ -transaminase are present in avian liver and show some response to conditions that stimulate gluconeogenesis (Vecchio & Kalman, 1968). This suggests that arginase functions mainly as a catabolic enzyme in this uricotelic species.

Insects in general are uricotelic. We have been unable to demonstrate either ornithine transcarbamylase or argininosuccinate lyase in fat-body tissues of *H. gloveri* (S. R. R. Reddy & J. W. Campbell, unpublished work) using assay methods that have been used to demonstrate these activities in other invertebrates (Campbell & Speeg, 1968b). This, plus the nutritional requirement for arginine shown by several insect species, suggests that, in general, insects cannot synthesize arginine. The present results indicate that the arginase present in *H. gloveri* fat-body functions as a catabolic enzyme for the conversion of arginine into proline. Flight muscle also possesses arginase as well as ornithine δ -transaminase and pyrroline-5-carboxylate reductase, so this may also hold true for this tissue.

The catabolic role of arginase in proline formation during states of protein catabolism that presumably exist in winged adult saturniid moths may be especially important because of the role proline appears to play in insect flight-muscle metabolism. Saturniid moths do not feed as adults, but must survive long enough to mate, a process involving flight. Insect tissues show very high proline concentrations (Price, 1961; Corrigan & Kearns, 1963) and in flight muscle proline is rapidly utilized during flight (Bursell, 1963). Δ^1 -Pyrroline-5-carboxylate dehydrogenase is present in flight-muscle mitochondria (Brosemer & Veerabhadrapa, 1965), and the findings of Sacktor & Childress (1967) indicate that proline penetrates flight-muscle mitochondria and forms precursors of oxaloacetate, allowing for the complete oxidation of pyruvate. Glutamate is not effective in restoring oxygen consumption in depleted mitochondria. The orientation of the catabolic pathway towards proline formation rather than towards glutamate formation and the

localization of the enzymes for the conversion of arginine into proline in the extramitochondrial fraction reported here are consistent with this function of proline in insect metabolism. Proline formed from arginine outside the mitochondrion could enter the mitochondrion and be converted into oxaloacetate via glutamate (Bursell, 1966).

The control of the catabolic pathway in insects appears to be complex. The increase in fat-body arginase activity after emergence of the winged adult is a response typical of the enzyme in mammals during states of high protein catabolism. However, ornithine δ -transaminase does not increase along with arginase in the silkworm as it does in the mammal. Arginase and ornithine transaminase respond separately in mammalian cells in tissue culture (Eliasson & Strecker, 1966), so this independent response shown by the insect enzymes may be characteristic of cells incapable of arginine biosynthesis. In the chicken arginase activity has been reported to show some increase after 30 hr. starvation whereas ornithine transaminase activity remains constant (Vecchio & Kalman, 1968).

The results on the metabolism of [^{14}C]arginine and [^{14}C]ornithine by intact fat-body tissue indicate that the rate of conversion of exogenous arginine into proline *in vivo* is not increased in spite of the increase in measurable arginase activity. This suggests that the formation of ornithine from arginine may not be rate-limiting as it is in *Tetrahymena* (Hill & Chambers, 1967). The rate of conversion of exogenous ornithine into proline does, however, increase in intact adult fat-body in the absence of any apparent increase in measurable ornithine δ -transaminase or pyrroline-5-carboxylate reductase activity. These results are more consistent with ornithine δ -transaminase being rate-limiting *in vivo*. If this were true, the change in the free amino acid content of fat-body tissue might be invoked to explain the more rapid conversion of ornithine into proline in the adult. The decrease in such amino acids as valine, which inhibit ornithine δ -transaminase (Katunuma *et al.* 1964), would effectively increase the reaction catalysed by ornithine transaminase *in vivo* and this increase might not be manifest by enzyme assays *in vitro*, where the inhibitory effect of high concentrations of free amino acids is diluted out. The decrease in the concentration of free lysine could also be expected to affect arginase *in vivo*: this amino acid is an effective competitive inhibitor of insect fat-body arginase (Reddy & Campbell, 1969). If arginase is not rate-limiting, the effect of the concentrations of inhibitory amino acids on it *in vivo* might not influence the overall conversion of arginine into proline. However, since exogenous and endogenous ornithine appear to be metabolized

differently, the question of control of the catabolic sequence must remain open.

This work was supported by grants from the U.S. National Science Foundation (GB-4524 and GB-8172) and the U.S. Public Health Service (AI05006). S.R.R. is the recipient of a Fulbright travel grant from the U.S. Educational Foundation (India). J.W.C. is a U.S. Public Health Service Career Development Awardee (5-K3-GM-6780).

REFERENCES

- Albrecht, A. M., Sher, W. I. & Vogel, H. J. (1962). *Analyt. Chem.* **34**, 398.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Brosemer, R. W. & Veerabhadrapa, P. S. (1965). *Biochim. biophys. Acta*, **110**, 102.
- Brown, G. W., jun. (1966). *Arch. Biochem. Biophys.* **114**, 184.
- Bursell, E. (1963). *J. Insect Physiol.* **9**, 439.
- Bursell, E. (1966). *Comp. Biochem. Physiol.* **19**, 809.
- Campbell, J. W. & Speeg, K. V., jun. (1968a). *Comp. Biochem. Physiol.* **25**, 3.
- Campbell, J. W. & Speeg, K. V., jun. (1968b). *Z. vergl. Physiol.* **61**, 164.
- Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
- Corrigan, J. J. & Kearns, C. W. (1963). *J. Insect Physiol.* **9**, 1.
- Davis, G. R. F. (1962). *J. Insect Physiol.* **8**, 377.*
- Eliasson, E. E. & Strecker, H. J. (1966). *J. biol. Chem.* **241**, 5757.
- Garcia, I., Tixier, M. & Roche, J. (1956). *C.R. Soc. Biol., Paris*, **150**, 632.
- Hill, D. L. & Chambers, P. (1967). *Biochim. biophys. Acta*, **148**, 435.
- Hinton, T. (1956). *Arch. Biochem. Biophys.* **62**, 78.
- House, H. L. (1965). In *Physiology of Insecta*, vol. 2, p. 769. Ed. by Rockstein, M. New York: Academic Press Inc.
- Katunuma, N., Matsuda, Y. & Tomino, I. (1964). *J. Biochem., Tokyo*, **56**, 499.
- Katunuma, N., Okada, M., Matsuzawa, T. & Otsuka, Y. (1965). *J. Biochem., Tokyo*, **57**, 445.
- Kilby, B. A. & Neville, E. (1957). *J. exp. Biol.* **34**, 276.
- Krishnakumaran, A. & Schneiderman, H. A. (1964). *J. exp. Zool.* **157**, 293.
- Laishley, E. J. & Bernlohr, R. W. (1968). *J. Bact.* **96**, 322.
- Lee, Y. P. & Takahashi, T. (1966). *Analyt. Biochem.* **41**, 71.
- Moe, O. A. & Warner, D. T. (1948). *J. Amer. chem. Soc.* **70**, 2763.
- Oyama, V. I. & Eagle, H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 305.
- Peisach, J. & Strecker, H. J. (1962). *J. biol. Chem.* **237**, 2255.
- Peraino, C. & Pitot, H. C. (1963). *Biochim. biophys. Acta*, **73**, 222.
- Price, G. M. (1961). *Biochem. J.* **80**, 420.
- Ramaley, R. F. & Bernlohr, R. W. (1966). *Arch. Biochem. Biophys.* **117**, 34.
- Reddy, S. R. R. & Campbell, J. W. (1969). *Comp. Biochem. Physiol.* **28**, 515.
- Reddy, S. R. R. & Wyatt, G. R. (1967). *J. Insect Physiol.* **13**, 981.

- Sacktor, B. & Childress, C. C. (1967). *Arch. Biochem. Biophys.* **120**, 583.
- Smith, J. E. (1962). *Biochim. biophys. Acta*, **57**, 183.
- Smith, L. (1955). In *Methods in Enzymology*, vol. 2, p. 732. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Smith, M. E. & Greenberg, D. E. (1957). *J. biol. Chem.* **226**, 317.
- Strecker, H. J. (1957). *J. biol. Chem.* **225**, 825.
- Strecker, H. J. (1960). *J. biol. Chem.* **235**, 2045.
- Strecker, H. J. (1965). *J. biol. Chem.* **240**, 1225.
- Szarkowska, L. & Porembaska, Z. (1959). *Acta biochim. pol.* **6**, 273.
- Tamir, H. & Ratner, S. (1963a). *Arch. Biochem. Biophys.* **102**, 249.
- Tamir, H. & Ratner, S. (1963b). *Arch. Biochem. Biophys.* **102**, 259.
- Vecchio, D. A. & Kalman, S. M. (1968). *Arch. Biochem. Biophys.* **127**, 376.
- Vogel, R. H. & Kopac, M. J. (1960). *Biochim. biophys. Acta*, **37**, 539.
- Williams, C. W. (1963). *Biol. Bull., Wood's Hole*, **124**, 355.
- Yura, T. & Vogel, H. J. (1959). *J. biol. Chem.* **234**, 335.