β-ALANINE METABOLISM IN THE HOUSEFLY, MUSCA DOMESTICA L.

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This is to certify that the

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ABSTRACT

β-ALANINE METABOLISM IN THE
HOUSEFLY, MUSCA DOMESTICA L.

By

Richard H. Ross, Jr.

Housefly larvae, Musca domestica L., were reared on a β-alanine-free diet. The adults were fed a synthetic diet also lacking β-alanine. At various developmental stages the β-alanine was extracted, purified, derivatized, and quantitatively assayed by gas-liquid chromatography. Prior to pupation, wandering larvae synthesized β-alanine so that a peak concentration of 362.2 ± 27.2 μg/g wet weight was found in the white puparium stage. Another peak of 290.1 μg/g was found in the fourth day of puparial development prior to adult emergence. The adults contained more than 600 μg/g which they maintained for at least the first week after eclosion.

β-Alanine synthesis was studied at the time of pupation in the housefly. The larvae were reared aseptically and upon pupation were injected with uracil-6-^{3}H, aspartate-U-^{14}C, pantothenate-1-^{14}C, propionate-2-^{14}C, or malonate-2-^{14}C. It was found by radioassay that relative to the other compounds tested uracil contributed 56.2% and aspartate 24.2% while pantothenate, propionate, and malonate contributed 9.4%, 7.1%, and 3.1%, respectively, to β-alanine synthesis.

Richard H. Ross, Jr.

Aseptically reared houseflies in the early white puparium stage were injected with β-alanine-1-^{14}C, β-alanine-2-^{14}C, or β-alanine-3-^{14}C to study the utilization of β-alanine during pupal sclerotization. On analysis, less than 5% of the radioactivity was expired as $^{14}CO_2$ in 24 hr and less than 1% was incorporated into the total lipids from each of the radiolabelled compounds. β-Alanine labelled at carbons 1, 2, or 3 gave 1 to 2%, 2 to 4%, and 1 to 2% in the other aqueous fraction, respectively. It was found that the radioactivity in the amino acids decreased from about 60% at 0.5 hr after injection to about 10 to 20% by 4 hr for each of the labelled compounds. The decline in amino acid radioactivity had a corresponding elevation in activity incorporated into the residue; it increased from about 5% to over 40% in the same time period. From 4 hr to 24 hr after injection the amino acid radioactivity remained relatively constant at about 10% while the radioactivity in the residue remained at about 40 to 50%.

β-ALANINE METABOLISM IN THE HOUSEFLY, *MUSCA DOMESTICA* L.

By

Richard H. Ross, Jr.

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PART I: β-ALANINE CONCENTRATIONS IN THE LARVAE, PUPARIA, AND ADULTS

INTRODUCTION

β-Alanine was incorporated into the cuticle of insects during the hardening process (SEKI, 1962; JACOBS, 1966; BODNARYK and LEVENBOOK, 1969; BODNARYK, 1970; DUFFY, 1970; GILBY and MCKELLAR, 1970; and SRIVASTAVA, 1971). LEVENBOOK et al. (1969) found that β-alanine occurred as a dipeptide, sarcophagine, in the fleshfly, Sarcophaga bullata; this dipeptide was not found in the housefly, Musca domestica, or the blowfly, Phormia regina. BODNARYK and LEVENBOOK (1968) reported that β-alanine occurred in carnosine (β-alanyl-L-histidine) in the larvae of P. regina.

PANT and LAL (1970) found that the β-alanine titre in Sarcophaga ruficornis varied during metamorphosis. This was also shown for S. bullata (LEVENBOOK et al., 1969; BODNARYK and LEVENBOOK, 1969) and for P. regina (LEVENBOOK and DINAMARCA, 1966). In order to study the metabolism of β-alanine in the housefly, this study was undertaken to quantitatively determine the β-alanine titre during various developmental stages from larvae to adults.

MATERIALS AND METHODS

The houseflies used were a maximum longevity strain obtained from the Insect Physiology Laboratory, USDA, Beltsville, Maryland. The flies were routinely reared on CSMA medium (ANON., 1959) and the adults fed a 1:1 mixture of sucrose and nonfat dry milk. The eggs were collected and sterilized in 0.1% hypochlorite solution for 20 minutes. The larvae were reared aseptically according to MONROE (1962) and were periodically washed out of the culture flasks to obtain samples. The puparia were collected, weighed and held for various lengths of time, and the adults maintained on a synthetic diet (MONROE and LAMB, 1968). The casein used in both larval and adult diets was obtained from Fisons Pharmaceuticals Limited, Loughborough, England.

The casein was analyzed for β-alanine as well as the dried milk and sugar used in the routine rearing of the flies. Samples were extracted with methylene chloride in a Soxhlet extractor for 24 hrs. The samples were then hydrolyzed in 5 ml 6 N HCl at 110°C for 26 hrs, dried on a rotating evaporator and added to a 1.1 X 7 cm Dowex 50-X12 (H^+ form) cation exchange column. The column was washed with 100 ml water and the amino acids were taken off with 100 ml 10% ammonium hydroxide. The resulting aqueous was evaporated to dryness and the N-TFA-n-butyl esters of the amino acids made according to ROACH and GEHRKE (1969). The samples were then analyzed by gas-liquid chromatography (GLC).

The houseflies were analyzed in triplicate; they were homogenized in water, refluxed for 90 min. in acetone-ethanol (1:1) and vacuum filtered (KAPLANIS et al., 1960). The lipids were then extracted 3 times with ethyl ether, the aqueous added to a 1.1 X 7 cm cation exchange column, and the amino acids analyzed by GLC as above. In a preliminary experiment the β-alanine extracted from a sample of housefly puparia was first partially purified by ascending paper chromatography. The amino acids were streaked on a sheet of Whatman No. 1 filter paper. The mobile phase was the upper layer of an n-butanol-acetic acid-water (250:60:250) mixture. The chromatogram was developed for 12 hrs., dried and redeveloped in the same system. The β-alanine was eluted from the paper with water and the N-TFA-n-butyl derivative made as above. Identity was proven by GLC and mass spectrometry.

The GLC analysis in all experiments was accomplished using a Research Specialties Corp., Series 600 GLC equipped with a dual hydrogen flame detector. A 2 m X 4 mm ID glass column packed with Tabsorb (Regis Chemical Co., Chicago, Illinois) was used to separate the amino acid derivatives. The temperature was programmed from 94°C to 210°C at 3.5°C/min; N_2 was at 94 ml/min. The peak areas were computed by disc integration and compared to a standard curve of β-alanine. Because β-alanine and leucine could not be separated completely (in the samples where the β-alanine peak was smaller than leucine) the areas were compared to those obtained by adding β-alanine to a leucine standard that gave a comparable area to that of the unknown.

RESULTS

Figure 1 shows the mass spectrum of extracted and derivatized β-alanine from housefly puparia which was identical to that obtained for authentic derivatized β-alanine (N-TFA-n-butyl-β-alanine). The peak at m/e 241 represents the molecular ion. The base peak at m/e 55 represents $[CH_2{=}CH{-}CH{-}CH_3]^+$ while its complementary peak is m/e 186. The loss of water from m/e 186 yields m/e 168 ($CF_3CONHCH_2CH_2C{\equiv}O^+$).

Table 1 and Figure 2 show the changes in the titre of β-alanine in various stages of houseflies reared on a β-alanine-free diet. There is a sharp increase in β-alanine concentration beginning in the larval wandering stage (51.4 ± 2.0 µg/g wet wt.) and reaching a peak of 362.2 ± 27.2 µg/g shortly after pupation. There is a second peak of 390.1 µg/g prior to eclosion during the fourth day after puparium formation. The adults also accumulate a very high level of β-alanine which they maintain through at least the first week after eclosion. In a preliminary sample where the adults were fed the dry milk-sugar diet which was found to contain β-alanine, the adults acquired a comparable concentration but at a slightly faster rate. All points on the graph except the 4-day-old puparial peak represent the average of triplicate injections from each of 3 samples containing between 50 and 250 individuals. The peak concentration for the 4-day-old puparia represents the value obtained for 1 sample and indicates that the β-alanine concentration reaches a maximum in pharate adults prior to eclosion.

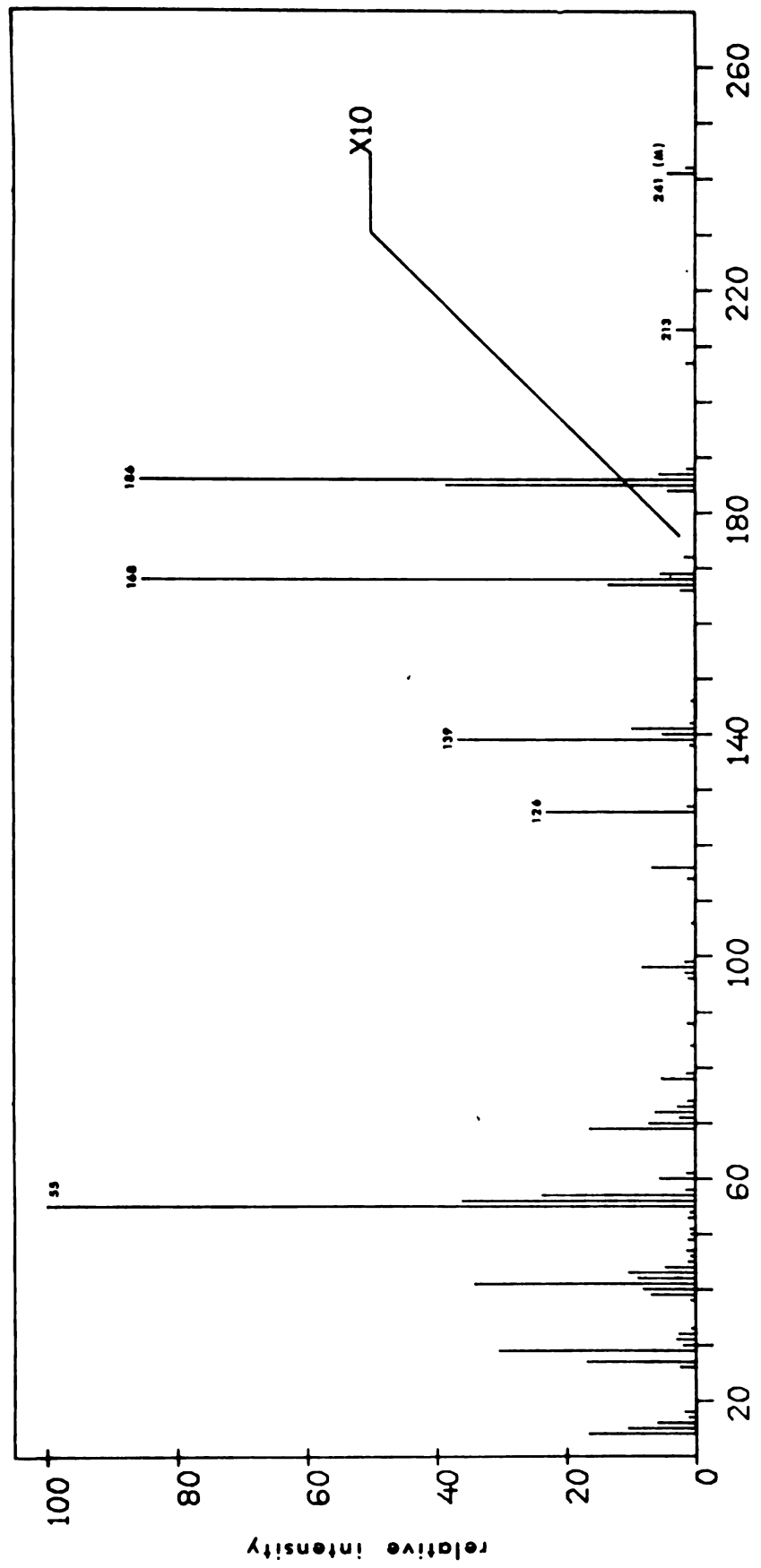

Figure 1.--The mass spectrum of N-TFA-n-butyl-β-alanine.

TABLE 1.--β-Alanine concentrations during the development of larvae, puparia, and adults of Musca domestica reared on an aseptic synthetic diet.

Stage of development (age in hrs.)		β-Alanine concentration	
		µg/individual	µg/g wet weight
Larvae			
72	(3)*	0.7 ± 0.2	38.8 ± 3.2
96	(4)	1.0 ± 0.0	51.4 ± 2.0
Puparia			
1	(0.04)	4.1 ± 0.1	362.2 ± 27.2
6	(0.25)	2.0 ± 0.3	148.0 ± 11.6
12	(0.5)	1.2 ± 0.1	125.1 ± 19.1
24	(1)	1.0 ± 0.4	96.1 ± 4.2
48	(2)	0.5 ± 0.0	64.9 ± 2.4
72	(3)	0.5 ± 0.1	52.0 ± 3.4
84	(3.5)	0.5 ± 0.0	55.4 ± 0.7
96	(4)	0.6 ± 0.1	74.8 ± 1.8
>96	(>4)	3.9**	290.1**
Adults after emergence			
1	(0.04)	1.3 ± 0.1	178.1 ± 13.1
12	(0.5)	0.8 ± 0.2	104.9 ± 9.6
24	(1)	1.2 ± 0.0	139.2 ± 7.2
48	(2)	4.0 ± 0.3	471.4 ± 25.5
72	(3)	5.2 ± 0.6	566.5 ± 40.2
96	(4)	5.5 ± 0.3	602.7 ± 52.0
120	(5)	6.4 ± 0.1	648.2 ± 25.0
144	(6)	6.4 ± 0.2	631.8 ± 24.6
168	(7)	6.6 ± 1.2	633.3 ± 27.3

*For rapid comparison age in days in parentheses.

**Based on one sample.

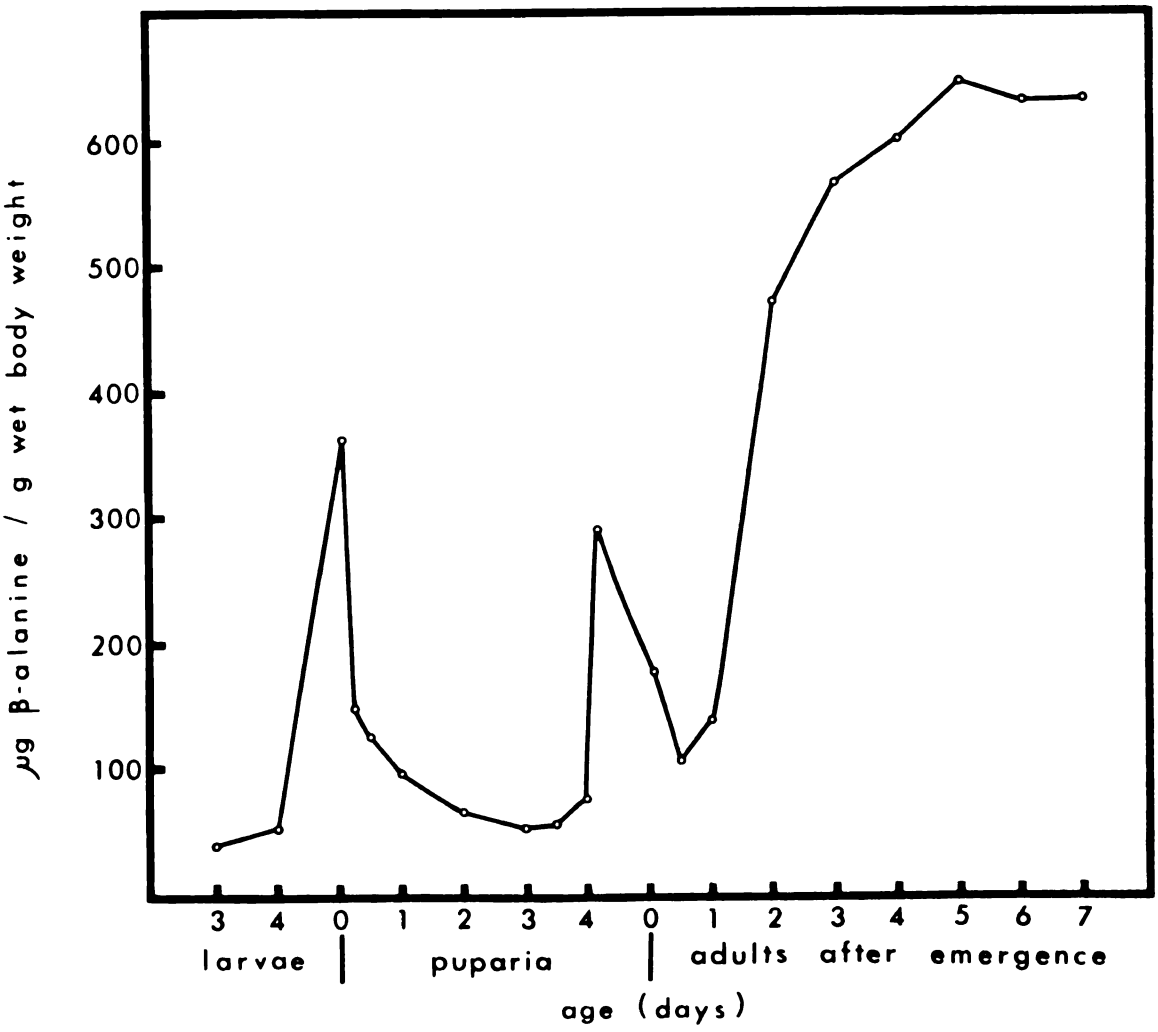

Figure 2.--β-Alanine concentrations in different life stages of houseflies reared on an aseptic synthetic diet.

DISCUSSION

PANT and LAL (1970) found that β-alanine in S. ruficornis reached a peak concentration in the white puparium stage and that it could be related to cuticular protein formation which also occurred at this stage; however, they did not find a peak prior to eclosion. LEVENBOOK et al. (1969) found that β-alanyl-L-tyrosine in S. bullata reached a peak concentration in wandering larvae while BODNARYK and LEVENBOOK (1969) showed that free β-alanine reached a peak sometime after pupation and prior to the initiation of cuticular darkening. LEVENBOOK and DINAMARCA (1966) reported that β-alanine reached a peak concentration in the puparia of P. regina, but they did not find a definite peak before adult emergence. In working with houseflies, LORD and SOLLY (1964) found that β-alanine was a major amino acid in the adults.

This study showed that houseflies maintained a rather low level of β-alanine during larval development and that there was a very rapid synthesis of β-alanine in the late wandering or white puparium stage. Upon the initiation of puparium darkening the β-alanine concentration decreased, which could be due at least partially to the incorporation of β-alanine into the cuticle. The occurrence of β-alanine in the pupal sheath has been well documented for houseflies (SEKI, 1962; FUKUSHI and SEKI, 1965; FUKUSHI, 1967), Drosophila sp. (SEKI, 1962;

JACOBS and BRUBAKER, 1963; JACOBS, 1966; FUKUSHI, 1967; and JACOBS, 1968a), Lucilia cuprina (GILBY and MCKELLAR, 1970), the fleshfly, S. bullata (BODNARYK and LEVENBOOK, 1969) and the wax moth, Galleria mellonella (SRIVASTAVA, 1971).

In one trial, there was also a peak β-alanine concentration in 4-day-old puparia prior to adult emergence. Because the other 3 trials did not show a peak until the newly emerged adult stage, the synthesis of β-alanine occurred rapidly sometime during the fourth day and prior to eclosion; biological variation may have contributed to a variation of several hours even under identical rearing conditions. As the newly emerged adults' cuticle hardened the β-alanine concentration decreased. After the flies had fed for several hours the β-alanine concentration increased to about twice the concentration of either of the peaks present during pupal formation and development. The study showed that this buildup occurred when β-alanine was present or absent in the diet. LORD and SOLLY (1964) also found that β-alanine was a major component of the adult housefly amino acids. The function of β-alanine has not been determined in adult flies although JACOBS (1968a) has shown that injected β-alanine in Drosophila melanogaster adults inhibited CO_2 excretion from glucose and several other sugars, and JACOBS (1968b) indicated that β-alanine may inhibit glucose oxidation by inhibiting phosphorylation. JACOBS (1970) also showed that β-alanine inhibited the catabolism of phenylalanine in D. melanogaster, and VERESHTCHAGIN et al. (1961) showed that β-alanine depressed the electrical activity in the nerve chain of the pine moth caterpillar, Dendrolimus pini. JACOBS and BRUBAKER (1963) and JACOBS (1966) showed the presence of a

β-alanine oxidase in D. melanogaster which indicated that β-alanine may be very active in adults.

Complete understanding of the function of β-alanine in insects, however, awaits further investigations.

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PART II: STUDIES ON β-ALANINE SYNTHESIS IN THE EARLY PUPARIUM

INTRODUCTION

In housefly puparia HIJIKURO (1968) found that β-alanine was synthesized from ^{14}C-aspartic acid and ^{3}H-uracil. A wild type fly strain utilized both precursors, while a black puparium strain was unable to incorporate ^{14}C-aspartic acid into β-alanine. NAKAI (1971) also found that aspartic acid and uracil were precursors of β-alanine in wild type housefly pupae. He concluded that in the wild type strain the puparial β-alanine was probably derived primarily from aspartic acid.

WHITE et al. (1964) stated that β-alanine catabolism could go through malonic semialdehyde, malonic acid, and acetic acid and that an alternate pathway of synthesis and utilization would be through malonic semialdehyde and β-hydroxypropionyl CoA to propionyl CoA. HAYAISHI et al. (1961) showed that in Pseudomonas fluorescens β-alanine transaminated with pyruvic acid to give malonic semialdehyde and L-α-alanine. KUPIECKI and COON (1957) showed that β-alanine also transaminated reversibly with α-ketoglutarate to give malonic semialdehyde, and they also suggested that in plants propionate was a precursor of β-alanine. It has also been demonstrated that propionate may serve as a β-alanine precursor in animal tissues (RENDINA and COON, 1957).

This study reports the relative contributions of uracil-6-^{3}H, D-pantothenate-1-^{14}C, malonate-2-^{14}C, propionate-2-^{14}C, and L-aspartate-U-^{14}C in β-alanine synthesis during the housefly white puparial stage.

MATERIALS AND METHODS

The houseflies used were a maximum longevity strain obtained from the Insect Physiology Laboratory, USDA, Beltsville, Maryland. The flies were routinely reared on CSMA medium (ANON., 1959) and the adults fed a 1:1 mixture of sucrose and nonfat dry milk. For these studies eggs were collected and sterilized in 0.1% hypochlorite solution for 20 min and the larvae were reared aseptically according to MONROE (1962). The wandering larvae were washed out of the diets and allowed to pupate. Upon pupation 1 µl of a given radiolabelled compound was injected into the posterior portion of the newly formed white puparium. The uracil-6-^{3}H and D-pantothenic acid-1-^{14}C (sodium salt) were obtained from New England Nuclear Corp., Boston, Mass. The sodium malonate-2-^{14}C, L-aspartic acid-U-^{14}C, and sodium propionate-2-^{14}C were obtained from Amersham/Searle, Des Plaines, Illinois. The injected puparia were then aged for 1 hr., weighed, and frozen at -32°C until further analysis. From 20 to 30 puparia were used in each test and each compound was analyzed in triplicate. In order to determine the time needed for maximum incorporation, larvae were also injected at intervals of several hours prior to pupation.

The puparia were homogenized in water, refluxed for 90 min in acetone-ethanol (1:1) at 4 times the aqueous volume and vacuum filtered (KAPLANIS *et al.*, 1960). The residue was dried, weighed, and combusted

to $^{14}CO_2$ in a combustion flask previously reported by HOPKINS and LOFGREN (1968). The $^{14}CO_2$ was trapped in 10 ml monoethanolamine-methyl cellosolve (1:2) and radioassayed with a Nuclear Chicago Unilux 1 (model 6850) liquid scintillation spectrometer.

The lipids were extracted 3 times with ethyl ether, dried over sodium sulfate, and radioassayed. The aqueous after lipid extraction was added to a 1.1 x 7 cm Dowex 50-X12 cation exchange column and the wash from 100 ml water (subsequently referred to as other aqueous) was evaporated to 5 ml and radioassayed. The amino acids were eluted with 100 ml 10% ammonium hydroxide (v/v); they were evaporated to 5 ml and radioassayed.

For all samples except those injected with pantothenate-1-^{14}C the amino acids were dried and the N-TFA-n-butyl derivatives made according to ROACH and GEHRKE (1969). The derivatives were then analyzed by a gas-liquid chromatograph equipped with a dual hydrogen flame detector (Research Specialties Corp., Series 600). The separations were accomplished using a 2m x 4mm ID glass column packed with Tabsorb (Regis Chemical Co., Chicago, Illinois). The temperature was programmed from 94 to 210°C at 3.5°C/min. A 10:1 splitter was put on the column outlet and the amino acid derivatives collected with hexane in a dry ice-acetone bath. The relative per cents of β-alanine were then calculated by radioassay.

RESULTS

In all samples where the compounds were injected prior to pupation, if injection was done more than 1 to 2 hrs before the larvae pupated, there was a decrease in total activity recovered as well as a decrease in activity incorporated into β-alanine with increasing time before pupation. The results presented here, therefore, were obtained by injecting the compounds as pupation occurred and holding the puparia for 1 hr prior to analysis. Table 2 summarizes the number of puparia, their weight, and the injection data for each of the radiolabelled compounds analyzed.

TABLE 2.--Treatment data of radiolabelled compounds injected into early puparia of the housefly.

Radiolabelled compound	Radio chemical purity %	No. puparia	Total weight mg	Total dpm injected	Total µg injected
Uracil-6-^{3}H	99	67	990.9	3,507,182	0.015
Aspartate-U-^{14}C	98	90	1,491.6	2,913,030	0.800
Pantothenate-1-^{14}C	99	75	1,349.4	1,152,524	27.825
Malonate-2-^{14}C	98	90	1,021.0	1,162,080	4.185
Propionate-2-^{14}C	>99	79	1,329.6	1,092,254	3.934

Table 3 presents the data on the recovery of the injected radioactivity in the residue, lipids, amino acids, and other aqueous for each of the compounds. These data show that very little radioactivity was recovered in the lipids of all the samples. Uracil-6-^{3}H incorporated most of its label, 32.1% and 25.6%, into the other aqueous and amino acid fractions, respectively, while 6.1% was recovered in the residue. With aspartate-U-^{14}C, 20.4% was found as amino acids, 13.9% as other aqueous, and 6.1% as residue. After injection of pantothenate-1-^{14}C the major portion (70.5%) was recovered in the other aqueous fraction, while only 4.0% and 0.5% were recovered in the amino acids and residue, respectively. Malonate-2-^{14}C had 48.0%, 13.9%, and 3.2% in the other aqueous, amino acids, and residue, respectively. After injection with propionate-2-^{14}C the amino acids had the most radioactivity (24.6%), followed by the other aqueous (14.7%) and the residue (5.0%).

Table 4 presents the amount of radioactivity found as β-alanine after gas-liquid chromatographic separation, subsequent trapping, and radioassay. It was found that relative to the other radiolabelled compounds examined, uracil accounted for 56.2% of the radiolabelled β-alanine and asparate for 24.2%. Pantothenate, propionate, and malonate accounted for 9.4%, 7.1%, and 3.1%, respectively.

TABLE 3.--Recovery of injected radioactivity in the residue,* lipids, amino acids, and other aqueous** from early puparia of the housefly.

Radiolabelled compound	Total recovery %	Residue		Lipids		Amino acids		Other aqueous	
		µg-equivalents	%	µg-equivalents	%	µg-equivalents	%	µg-equivalents	%
Uracil-6-^{3}H	63.8 ± 6.1	0.001 ± 0.000	6.1 ± 0.6	0.000 ± 0.000	0.1 ± 0.0	0.004 ± 0.000	25.6 ± 2.5	0.005 ± 0.001	32.1 ± 5.0
Aspartate-U-^{14}C	40.8 ± 5.3	0.049 ± 0.007	6.1 ± 0.9	0.003 ± 0.000	0.4 ± 0.0	0.163 ± 0.008	20.4 ± 2.5	0.112 ± 0.017	13.9 ± 2.1
Pantothenate-1-^{14}C	75.2 ± 13.1	0.129 ± 0.026	0.5 ± 0.1	0.041 ± 0.032	0.1 ± 0.1	1.124 ± 0.309	4.0 ± 1.1	19.622 ± 3.869	70.5 ± 13.9
Malonate-2-^{14}C	65.8 ± 2.6	0.135 ± 0.025	3.2 ± 0.6	0.030 ± 0.009	0.7 ± 0.2	0.582 ± 0.117	13.9 ± 2.8	2.008 ± 0.201	48.0 ± 4.8
Propionate-2-^{14}C	53.9 ± 10.9	0.198 ± 0.055	5.0 ± 1.4	0.049 ± 0.012	1.2 ± 0.3	0.969 ± 0.323	24.6 ± 8.2	0.579 ± 0.197	14.7 ± 5.0

*Residue after homogenation, refluxing, and filtration.

**Aqueous after amino acid extraction.

TABLE 4.--Incorporation of radioactivity into β-alanine after injection into early puparia of the housefly.

Radiolabelled compound	Radioactivity in β-alanine		
	µg-equivalents	% of injected	Relative %**
Uracil-6-^{3}H	0.003 ± 0.000	23.9 ± 2.3	56.2
Aspartate-U-^{14}C	0.083 ± 0.010	10.3 ± 1.3	24.2
Pantothenate-1-^{14}C*	1.124 ± 0.309	4.0 ± 1.1	9.4
Malonate-2-^{14}C	0.054 ± 0.021	1.3 ± 0.5	3.1
Propionate-2-^{14}C	0.118 ± 0.023	3.0 ± 0.6	7.1

*Based on all of amino acid radioactivity as β-alanine.

**Based on the total radioactivity contributed by all compounds tested.

DISCUSSION

ROSS and MONROE (1972) reported that β-alanine concentration reached its peak in the 1-hr-old white puparium stage. They proposed that β-alanine was synthesized primarily after the start of pupation. These radiolabelled experiments would support the conclusion that β-alanine synthesis occurred primarily upon pupation on at most 1 to 2 hrs prior to its onset.

This study indicated that for this strain of houseflies most of the β-alanine was synthesized from uracil and aspartate with these two compounds accounting for over 80% of the radioactivity recovered in the β-alanine fraction. This per cent probably represented a minimal figure as it was expected that uracil and aspartate were diluted more than the remaining three compounds tested upon their admixing with the biological pool. These results are similar to those found by NAKAI (1971) with his wild housefly strain. He found that uracil incorporated about 50% of the injected radioactivity into β-alanine while aspartate accounted for 10% to 20%. HIJIKURO (1968) also indicated that uracil and aspartate were used in the synthesis of β-alanine in housefly pupae. Because both uracil and aspartate incorporated some radioactivity into the residue, it was possible that a small amount of the synthesized β-alanine had been utilized in cuticle formation in the early pupal stage within 1 hr after the onset of pupation.

These experiments showed that pantothenate contributed nearly 10% to β-alanine synthesis; however, in reality its contribution was probably negligible. Because of the specific activity a relatively large amount was injected thereby increasing the available pantothenic acid in the biological pool by several fold. Simple hydrolysis of pantothenic acid would yield β-alanine and pantoic acid.

Together, malonate and propionate contributed about 10% of the β-alanine found. Because the biological pools of these compounds were theoretically low and higher weights of them were also injected, it was thought that these two compounds also contributed negligibly to β-alanine synthesis.

These experiments indicated conclusively that of the five most probable precursors in β-alanine synthesis by various pathways, that this strain of the housefly, M. domestica, used only uracil and aspartate. The experiments were unable to show, however, whether β-alanine synthesis from aspartate occurred through uracil synthesis and catabolism or via a direct aspartate decarboxylase system.

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PART III: STUDIES ON β-ALANINE UTILIZATION IN THE EARLY PUPARIUM

INTRODUCTION

Injection of labelled β-alanine into rats by PIHL and FRITZSON (1955) showed that β-alanine-1-^{14}C was very rapidly oxidized, β-alanine-2-^{14}C was much more slowly oxidized, and β-alanine-3-^{14}C was intermediate. They suggested that β-alanine was deaminated to formylacetic acid, then decarboxylated to CO_2 and acetaldehyde which was further oxidized to acetic acid. JACOBS and BRUBAKER (1963) and JACOBS (1966) also showed that β-alanine labelled at carbons 1 and 2, after injection into Drosophila melanogaster pupae, was respired as $^{14}CO_2$. They further showed that β-alanine was incorporated into the pupal sheaths. DUFFY (1968) indicated that D. melanogaster adults possessed a β-alanine decarboxylase and a β-alanine oxidase.

BODNARYK (1971a) showed by kinetic studies that β-alanine was probably incorporated as an N-terminal amino acid in the puparia of Sarcophaga bullata. BODNARYK (1971b) showed that over 50% of the bound β-alanine was incorporated in S. bullata puparia within 3 hr after the onset of sclerotization. He also indicated that the amount of incorporation was directly related to the extent of sclerotization.

ROSS and MONROE (1972a, 1972b) showed that β-alanine concentration increased in the white puparium stage of Musca domestica and after the onset of sclerotization decreased over a 24 hr period. They indicated that the β-alanine was primarily synthesized from uracil and aspartic

acid at the time of pupation. The purpose of this study was to determine where the β-alanine was being utilized during the first 24 hr after pupation.

MATERIALS AND METHODS

The houseflies used were a maximum longevity strain obtained from the Insect Physiology Laboratory, USDA, Beltsville, Maryland. Eggs were collected from a stock culture, surface sterilized in 0.1% hypochlorite solution for 20 min and the larvae reared aseptically according to MONROE (1962). The wandering larvae were washed out of the diets and allowed to pupate. Within 15 min after the onset of pupation 1 µl of the radiolabelled β-alanine was injected into the posterior end of the white puparium. The β-alanine-1-^{14}C was obtained from New England Nuclear Corp., Boston, Mass. The β-alanine-2-^{14}C and β-alanine-3-^{14}C were obtained from International Chemical and Nuclear Corp., Irvine, California. After injection, samples were analyzed for $^{14}CO_2$ production in a respiration train modified from that reported by HOPKINS and LOFGREN (1968). The $^{14}CO_2$ was collected at various time intervals in monoethanolamine-methyl cellosolve (1:2) and radioassayed with a Nuclear Chicago Unilux I (model 6850) liquid scintillation spectrometer. Other samples were aged for various time intervals, weighed, and frozen at -32°C until further analysis. Values were obtained from triplicate samples of 20 puparia each.

The puparia were homogenized in water, refluxed for 90 min in acetone-ethanol (1:1) at 4 times the aqueous volume and vacuum filtered (KAPLANIS et al., 1960). The residue was dried, weighed, and combusted

to $^{14}CO_2$ and water in a combustion flask previously reported by HOPKINS and LOFGREN (1968). The $^{14}CO_2$ was trapped in 10 ml monoethanolamine-methyl cellosolve (1:2) and radioassayed.

The lipids were extracted 3 times with ethyl ether, dried over sodium sulfate, and radioassayed. The aqueous after lipid extraction was added to a 1.1 x 7 cm Dowex 50-X12 cation exchange column and the wash from 100 ml water (subsequently referred to as other aqueous) was evaporated to 5 ml and radioassayed. The amino acids were eluted with 100 ml 10% ammonium hydroxide (v/v); they were evaporated to 5 ml and radioassayed.

RESULTS

Table 5 shows the injection data for the β-alanine-^{14}C labelled at each of the carbon atoms. In all tests the same weight was injected into each puparium. Table 6 presents the average live weights and total per cent recovery for each test of 20 puparia. The average weights per puparium ran from 11.8 to 17.9 mg while the average per cent recoveries ranged from 31.3% to 92.8%.

TABLE 5.--Treatment data of radiolabelled β-alanine injected into early puparia of the housefly.

Radiolabelled compound	Radio-chemical purity %	No. puparia per test	Total dpm injected	Total µg injected
β-Alanine-1-^{14}C	99	20	546,680	27.80
β-Alanine-2-^{14}C	99	20	74,180	27.80
β-Alanine-3-^{14}C	98	20	777,880	27.80

TABLE 6.--Average per test of the live weight and per cent recovery for the various times after injection of β-alanine-^{14}C into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C		β-Alanine-2-^{14}C		β-Alanine-3-^{14}C	
	Live weight* mg	Total recovery %	Live weight* mg	Total recovery %	Live weight* mg	Total recovery %
0.5	337.7 ± 12.9	68.0 ± 13.2	332.5 ± 2.2	70.8 ± 9.8	354.5 ± 1.4	92.8 ± 3.4
1	327.3 ± 13.8	31.3 ± 2.6	338.8 ± 7.7	76.5 ± 9.2	336.3 ± 14.1	73.2 ± 7.7
2	314.3 ± 6.0	48.2 ± 31.1	324.1 ± 4.5	74.6 ± 4.5	330.2 ± 23.0	78.4 ± 6.4
3	358.7 ± 8.4	70.1 ± 25.9	311.9 ± 9.0	79.1 ± 5.0	324.5 ± 12.7	58.6 ± 1.8
4	357.6 ± 5.6	53.1 ± 6.4	309.2 ± 0.5	74.3 ± 6.7	323.4 ± 5.1	74.9 ± 15.2
6	341.5 ± 13.0	58.2 ± 13.9	275.0 ± 11.5	82.0 ± 19.8	317.2 ± 6.2	75.3 ± 2.9
8	346.3 ± 11.9	51.1 ± 1.7	251.8 ± 15.5	53.9 ± 12.6	296.8 ± 9.2	81.6 ± 10.2
12	308.5 ± 4.8	64.0 ± 10.5	289.9 ± 2.4	59.2 ± 12.6	290.2 ± 4.9	69.8 ± 4.6
24	289.9 ± 7.8	72.3 ± 2.4	235.7 ± 4.9	54.0 ± 2.2	266.2 ± 1.8	73.5 ± 8.3

*Total weight of 20 puparia per test.

In Table 7, which shows the average accumulative $^{14}CO_2$ production, the data clearly indicate that during the first 24 hr after pupation that little $^{14}CO_2$ was produced from any of the carbon positions of β-alanine. Carbons labelled in the 1 and 3 position produced 4 to 5% while the 2 position produced about 3% of the initial radioactivity into $^{14}CO_2$.

Table 8 shows that virtually no radioactivity was incorporated into the total lipid fraction during the first 24 hr after pupation. In Table 9 the data show that little radioactivity was found in the other aqueous portion of the puparial extracts. β-Alanine-1-^{14}C incorporated about 1% into this fraction while the 2 and 3 positions incorporated 2 to 3% and 1 to 2%, respectively.

Table 10 shows a marked decrease in the radioactivity found in the amino acids following injection of each of the compounds. In all cases the levels decreased over the first 3 hr after pupation and reached fairly constant levels by about the 4th hr. The majority was lost in the first 2 hr after puparial formation. In Table 11 the data indicate a corresponding increase in the radioactivity incorporated into the residue during the first 2 to 3 hr following the onset of pupation. At 0.5 hr there was less than 8% in the residue and by 3 to 4 hr between 40% and 50% of the injected radioactivity had been incorporated into the residue. After 4 hr the radiolabelled residue remained fairly constant for the duration of the experiment.

TABLE 7.--Average per test of the accumulative $^{14}CO_2$ production after injection of β-alanine into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C		β-Alanine-2-^{14}C		β-Alanine-3-^{14}C	
	µg-equivalents $^{14}CO_2$	% of injected dose	µg-equivalents $^{14}CO_2$	% of injected dose	µg-equivalents $^{14}CO_2$	% of injected dose
0.5	0.00 ± 0.00	0.0 ± 0.0	0.07 ± 0.4	0.3 ± 0.2	0.02 ± 0.01	0.1 ± 0.0
1	0.12 ± 0.05	0.4 ± 0.2	0.09 ± 0.09	0.3 ± 0.3	0.07 ± 0.04	0.2 ± 0.1
2	0.54 ± 0.34	1.9 ± 1.2	0.14 ± 0.12	0.5 ± 0.4	0.26 ± 0.06	0.9 ± 0.2
3	0.92 ± 0.66	3.3 ± 2.4	0.21 ± 0.18	0.7 ± 0.6	0.57 ± 0.17	2.0 ± 0.6
4	1.10 ± 0.68	3.9 ± 2.4	0.36 ± 0.29	1.3 ± 1.0	0.95 ± 0.32	3.4 ± 1.1
6	1.15 ± 0.86	4.1 ± 3.0	0.46 ± 0.24	1.6 ± 0.8	1.09 ± 0.32	3.9 ± 1.2
8	1.20 ± 0.90	4.3 ± 3.2	0.61 ± 0.15	2.2 ± 0.5	1.17 ± 0.30	4.2 ± 1.1
12	1.24 ± 0.43	4.4 ± 1.5	0.68 ± 0.12	2.4 ± 0.4	1.23 ± 0.30	4.4 ± 1.1
24	1.26 ± 0.48	4.5 ± 1.7	0.78 ± 0.07	2.8 ± 0.3	1.27 ± 0.25	4.6 ± 0.9

TABLE 8.--Average per test of the incorporation of the radiolabel into the total lipids after injection of β-alanine-^{14}C into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C		β-Alanine-2-^{14}C		β-Alanine-3-^{14}C	
	µg-equivalents lipid	% of injected dose	µg-equivalents lipid	% of injected dose	µg-equivalents lipid	% of injected dose
0.5	0.02 ± 0.01	0.1 ± 0.0	0.07 ± 0.05	0.2 ± 0.1	0.04 ± 0.01	0.1 ± 0.0
1	0.00 ± 0.00	0.0 ± 0.0	0.12 ± 0.03	0.4 ± 0.1	0.06 ± 0.01	0.2 ± 0.0
2	0.04 ± 0.03	0.1 ± 0.1	0.12 ± 0.04	0.4 ± 0.1	0.10 ± 0.02	0.4 ± 0.1
3	0.00 ± 0.00	0.0 ± 0.0	0.14 ± 0.02	0.5 ± 0.1	0.09 ± 0.02	0.3 ± 0.1
4	0.01 ± 0.01	0.0 ± 0.0	0.12 ± 0.04	0.4 ± 0.1	0.11 ± 0.04	0.4 ± 0.1
6	0.01 ± 0.01	0.0 ± 0.0	0.13 ± 0.06	0.5 ± 0.2	0.11 ± 0.01	0.4 ± 0.0
8	0.02 ± 0.02	0.1 ± 0.1	0.13 ± 0.03	0.5 ± 0.1	0.11 ± 0.03	0.4 ± 0.1
12	0.01 ± 0.00	0.0 ± 0.0	0.14 ± 0.02	0.5 ± 0.1	0.13 ± 0.01	0.5 ± 0.0
24	0.01 ± 0.00	0.0 ± 0.0	0.09 ± 0.04	0.3 ± 0.1	0.11 ± 0.03	0.4 ± 0.1

TABLE 9.--Average per test of the incorporation of the radiolabel into the other aqueous* after injection of β-alanine-^{14}C into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C		β-Alanine-2-^{14}C		β-Alanine-3-^{14}C	
	µg-equivalents other aqueous	% of injected dose	µg-equivalents other aqueous	% of injected dose	µg-equivalents other aqueous	% of injected dose
0.5	0.37 ± 0.08	1.3 ± 0.3	0.77 ± 0.09	2.8 ± 0.3	0.71 ± 0.10	2.6 ± 0.4
1	0.25 ± 0.02	0.9 ± 0.1	0.96 ± 0.02	3.5 ± 0.1	0.64 ± 0.14	2.3 ± 0.5
2	0.36 ± 0.25	1.3 ± 0.9	1.12 ± 0.13	4.0 ± 0.5	0.68 ± 0.12	2.4 ± 0.4
3	0.40 ± 0.17	1.4 ± 0.6	0.91 ± 0.18	3.3 ± 0.6	0.44 ± 0.08	1.6 ± 0.3
4	0.27 ± 0.07	1.0 ± 0.3	0.88 ± 0.06	3.2 ± 0.2	0.50 ± 0.04	1.8 ± 0.2
6	0.27 ± 0.06	1.0 ± 0.2	0.77 ± 0.25	2.8 ± 0.9	0.40 ± 0.05	1.4 ± 0.2
8	0.22 ± 0.03	0.8 ± 0.1	0.53 ± 0.14	1.9 ± 0.5	0.39 ± 0.02	1.4 ± 0.1
12	0.29 ± 0.05	1.0 ± 0.2	0.52 ± 0.18	1.9 ± 0.6	0.30 ± 0.02	1.1 ± 0.1
24	0.32 ± 0.02	1.1 ± 0.1	0.38 ± 0.05	1.4 ± 0.2	0.33 ± 0.06	1.2 ± 0.2

*Aqueous after amino acid extraction.

TABLE 10.--Average per test of the incorporation of the radiolabel into the amino acids after injection of β-alanine-^{14}C into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C		β-Alanine-2-^{14}C		β-Alanine-3-^{14}C	
	µg-equivalents amino acids	% of injected dose	µg-equivalents amino acids	% of injected dose	µg-equivalents amino acids	% of injected dose
0.5	17.14 ± 3.15	61.7 ± 11.3	17.54 ± 2.63	63.1 ± 9.4	22.83 ± 1.12	82.1 ± 4.0
1	5.90 ± 0.78	21.2 ± 2.8	16.78 ± 2.75	60.3 ± 9.9	12.84 ± 1.58	46.2 ± 5.7
2	6.49 ± 3.89	23.3 ± 14.0	8.62 ± 1.10	31.1 ± 4.0	6.20 ± 0.57	22.3 ± 2.0
3	4.86 ± 2.45	17.5 ± 8.8	6.86 ± 1.66	24.7 ± 6.0	3.28 ± 0.58	11.8 ± 2.1
4	2.32 ± 0.84	8.4 ± 3.0	4.79 ± 0.90	17.2 ± 3.2	3.44 ± 0.78	12.4 ± 2.8
6	1.68 ± 0.28	6.0 ± 1.0	5.23 ± 1.97	18.8 ± 7.1	2.80 ± 1.03	10.1 ± 3.7
8	1.80 ± 0.08	6.5 ± 0.3	4.91 ± 2.21	17.7 ± 8.0	3.14 ± 0.10	11.3 ± 0.4
12	2.22 ± 0.34	8.0 ± 1.2	2.42 ± 1.04	8.7 ± 3.7	1.81 ± 0.57	6.5 ± 2.1
24	3.02 ± 0.96	10.9 ± 3.5	2.91 ± 0.31	10.5 ± 1.1	3.70 ± 1.61	13.3 ± 5.8

TABLE 11.--Average per test of the weight and incorporation of the radiolabel into the residue* after injection of β-alanine-^{14}C into early puparia of the housefly.

Time after injection hr	β-Alanine-1-^{14}C			β-Alanine-2-^{14}C			β-Alanine-3-^{14}C		
	weight** mg	μg-equivalents residue	% of injected dose	weight** mg	μg-equivalents residue	% of injected dose	weight** mg	μg-equivalents residue	% of injected dose
0.5	72.7 ± 4.7	1.37 ± 0.92	4.9 ± 3.3	64.4 ± 1.0	1.23 ± 0.13	4.4 ± 0.5	68.5 ± 1.4	2.19 ± 0.27	7.9 ± 1.0
1	67.9 ± 2.2	2.43 ± 1.32	8.7 ± 4.7	68.2 ± 3.0	3.32 ± 0.60	12.0 ± 2.2	66.3 ± 1.4	6.74 ± 0.86	24.2 ± 3.1
2	69.2 ± 1.3	5.98 ± 2.49	21.5 ± 9.0	67.8 ± 2.3	10.75 ± 1.19	38.7 ± 4.3	66.1 ± 2.7	14.55 ± 1.37	52.3 ± 4.9
3	70.3 ± 1.3	13.51 ± 4.83	48.6 ± 17.4	62.5 ± 2.3	13.89 ± 0.55	50.0 ± 2.0	63.6 ± 1.7	12.06 ± 0.63	43.4 ± 2.3
4	72.1 ± 3.0	11.25 ± 1.15	40.5 ± 4.2	65.8 ± 2.2	14.66 ± 1.02	52.7 ± 3.7	67.2 ± 1.3	16.21 ± 3.41	58.3 ± 12.3
6	69.9 ± 6.0	13.21 ± 3.61	47.5 ± 13.0	63.1 ± 2.2	16.38 ± 3.35	58.9 ± 12.1	66.1 ± 2.5	16.85 ± 0.40	60.6 ± 1.4
8	69.4 ± 3.4	11.08 ± 0.52	39.8 ± 1.9	63.0 ± 1.8	9.06 ± 1.19	32.6 ± 4.3	64.4 ± 2.3	18.09 ± 2.85	65.1 ± 10.3
12	70.3 ± 4.3	14.13 ± 2.98	50.8 ± 10.7	66.0 ± 1.6	12.91 ± 2.35	46.4 ± 8.4	64.4 ± 3.1	16.10 ± 0.73	57.9 ± 2.6
24	67.6 ± 2.9	15.49 ± 0.89	55.7 ± 3.2	65.4 ± 5.0	10.87 ± 0.33	39.1 ± 1.2	60.8 ± 0.9	15.02 ± 1.88	54.0 ± 6.7

*Residue after homogenization, refluxing, and filtration.

**Total residue weight of 20 puparia per test.

DISCUSSION

These experiments indicated that during puparial formation and subsequent sclerotization β-alanine was almost exclusively incorporated into the residue. The data showed that very little radioactivity was recovered as $^{14}CO_2$, total lipids, and other aqueous. Because the original radiolabelled compounds had 1 to 2% impurities, the data show that less than 6% was recovered in the total of these three fractions in the first 24 hr after pupation. This indicated that the pathways suggested by PIHL and FRITZSON (1955) in rats and DUFFY (1968) in D. melanogaster were not being used during puparial formation and sclerotization.

These experiments showed that β-alanine was incorporated directly into the residue and that this process occurred rapidly during the initial period of sclerotization. These data showed that fully 50% of the β-alanine was incorporated within 3 hr after pupation which agrees with that found by BODNARYK (1971b) for S. bullata.

ROSS and MONROE (1972a) indicated that β-alanine reached a maximum concentration in 1 hr old white puparia and that the level had dropped off very rapidly by 6 hr but that it took 12 - 24 hr for it to level off. These experiments indicated that the maximum rate of incorporation occurred from 0.5 - 3 hr after injection of early puparia within 15 min after the onset of pupation. The data for M. domestica

tend to support the findings of BODNARYK (1971a, 1971b) that S. bullata rapidly incorporated β-alanine into the puparial case during sclerotization and that β-alanine was found as an N-terminal amino acid in cuticular proteins.

The metabolic fates of β-alanine in the housefly at times other than during puparial formation and sclerotization await further investigations.

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APPENDIX

LITERATURE REVIEW

The metabolism of β-alanine has been studied in a variety of microorganisms and animals. CAMPBELL (1957) showed that β-alanine was a product of uracil degradation in Clostridium uracilicum, and Pseudomonas fluorescens was shown to have a β-alanine transaminase (HAYAISHI et al., 1961). Deamination was also shown to be a route for catabolism in rats (GRAFF and HOBERMAN, 1950). PIHL and FRITZSON (1955) showed that β-alanine was rapidly decarboxylated in rats and postulated that the degradation occurred by deamination to formyl-acetic acid, subsequent decarboxylation to acetaldehyde, which was oxidized to acetic acid.

Insects also contain β-alanine. SEKI (1962) showed that the pupal sheaths of certain flies contain β-alanine and this was supported by the studies of KUME and SEKI (1962), FUKUSHI and SEKI (1965), and HIJIKURO (1968b). In certain strains of Drosophila melanogaster, Bombyx mori, and Musca domestica β-alanine might have been related to puparium color (SEKI et al., 1966; HIJIKURO, 1967; FUKUSHI, 1967). JACOBS (1966) showed that wild type D. melanogaster pupae incorporated more β-alanine than did the ebony strain. GILBY and MCKELLAR (1970) found β-alanine in the blowfly puparial sheath and SRIVASTAVA (1971) also found it in the cuticular proteins of Galleria mellonella pupae. JACOBS and BRUBAKER (1963) demonstrated that ebony D. melanogaster

deposited less ^{14}C from labelled β-alanine in pupal sheaths and decarboxylated and oxidized it much more rapidly than did non-ebony flies.

The concentrations of β-alanine vary during metamorphosis in Sarcophaga ruficornis (PANT and LAL, 1970). They found that the β-alanine concentration decreased upon pupation, and this was also found to be true in Phormia regina (LEVENBOOK and DINAMARCA, 1966). BODNARYK and LEVENBOOK (1968) found that β-alanyl-L-histidine (carnosine) was a constituent of P. regina, and LEVENBOOK et al. isolated β-alanyl-L-tyrosine (sarcophagine) from the larvae of Sarcophaga bullata. BODNARYK and LEVENBOOK (1969) demonstrated that β-alanine from the dipeptide was incorporated into the cuticle after pupation, and BODNARYK (1970) hypothesized that the sarcophagine was cleaved prior to the incorporation of β-alanine into the cuticle. BODNARYK (1971a) demonstrated that the utilization was under hormonal control via a dipeptidase and DOPA-decarboxylase which were ecdysterone-induced enzymes. BODNARYK (1971c) showed that fully 50% of the total amount of bound β-alanine in the empty pupal case had been incorporated within 3 hr after the onset of pupation. HIJIKURO (1968a) had suggested that the β-alanine was an N-terminal amino acid in D. melanogaster pupae, and this was further shown by kinetic studies in S. bullata (BODNARYK, 1971b).

Few studies have been done on β-alanine biosynthesis in insects. JACOBS (1968a) has indicated that uracil was a precursor to β-alanine in D. melanogaster, and uracil and aspartic acid were found to be precursors in M. domestica puparia (NAKAI, 1971).

β-Alanine is metabolically active in adults also. CURTIS et al. (1959) showed that β-alanine depressed spinal neurones in the cat. The inhibition of motor activity was also shown in worms and gypsy moth caterpillars (VERESHTCHAGIN et al., 1963) and also in the pine moth caterpillar (VERESHTCHAGIN et al., 1961). LORD and SOLLY (1964) showed that β-alanine was an abundant amino acid in M. domestica adults. DUFFY (1968) demonstrated the presence of a β-alanine oxidase and a β-alanine decarboxylase in D. melanogaster adults. β-Alanine inhibited glucose but not glucose-6-phosphate metabolism (JACOBS, 1968a, 1968b) and JACOBS (1970) showed that β-alanine also inhibited the metabolism of other compounds also. The overall importance of β-alanine in the metabolic scheme of insect adults remains to be studied and understood.

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