THE LIPOGENIC ACTIVITY OF FIVE AMINO ACIDS IN ASEPTICALLY REARED HOUSEFLIES, MUSCA DOMESTICA L.

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

ROBERT THOMAS KON

1968

ABSTRACT

THE LIPOGENIC ACTIVITY OF FIVE AMINO ACIDS IN ASEPTICALLY REARED HOUSEFLIES, MUSCA DOMESTICA L.

By

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Five different free, radiolabelled amino acids comprising five treatments were blended into a synthetic casein diet and fed to aseptically reared larvae of Musca domestica L. After four replications, analysis of pupae under 24 hours old showed that per mg of total lipid, leucine contributed 25.1% of the weight, glutamate 22.1%, alanine 4.3%, phenylalanine 4.2%, and methionine 0.3%. The values for fatty acids alone were 27.5%, 22.0%, 4.2%, 4.0% and 0.4%, respectively. Except for leucine and methionine these values had good linearity with the corresponding amino acid per cent values naturally occurring in casein. The fatty acids found by gas-liquid chromatographic analysis were, myristic (1.7%) palmitic (28.7%), palmitoleic (39.7%), stearic (trace), and oleic (29.9%). A treatment average of from 84% to 96% of the radiolabel in the total lipids was found in the fatty acids, but it is believed that 95% more accurately represents actual biochemical occurrences. Among the unsaponifiable lipids,

from 69% to 76.6% of the activity behaved as hydrocarbons, from 16.5% to 29.9% behaved as high molecular weight alcohols, from 0% to 4.5% behaved as free sterols (column data, without additional analysis), and from 1.1% to 5.4% behaved as more polar compounds. The level of metabolites contributed to the bio-fluids by each radiolabelled amino acid was found to be quite linear with the corresponding per cent value of each in casein and a hypothesis about this observation was discussed.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Entomology

1968

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INTRODUCTION

A complete knowledge of insect biochemistry could possibly lead to the development of a specific insecticide for any insect or small group of insects based upon slight metabolic variations. As a step toward further clarification of the biochemistry of the housefly, Musca domestica L., a study was made of the conversion of five radiolabelled amino acids into the fatty acids of aseptically reared housefly pupae.

As the lipogenic activities of all the amino acids become known, the more important question of why they are different can be attacked. Because housefly larvae need only cholesterol supplemented protein as a carbon source (Brookes and Fraenkel, 1957; Monroe, 1962), they provide an excellent study subject by avoiding the variables of dietary carbohydrates and fats (assuming also that they possess no significant free CO_2 fixation mechanism).

The amino acids chosen represent several structural classes. In this study the radiolabel was supplied as Alanine-U-C^{14}, Glutamate-U-C^{14}, Leucine-U-C^{14}, Methionine-G-H^3, and Phenylalanine-U-C^{14}.

LITERATURE REVIEW

Studies have revealed that lipids serve as more than a reserve energy source for insects. Hobson (1935) discovered a dietary requirement for cholesterol by the blowfly, Lucilia sericata, which has since been accepted as a generality for all insects (Albritton, 1953).

Clayton (1964) has reviewed the literature on cholesterol requirements by insects, and Monroe et al. (1967) have shown the benefit of cholesterol in houseflies for growth and reproduction. Ecdysone, the molting hormone of insects, was found to be a metabolite of cholesterol (Karlson and Hoffmeister, 1963).

Another dietary requirement for a lipid was reported by Fraenkel and Blewett (1946). It was shown that the moth, Ephestia kuehniella, needed, but could not synthesize linoleic acid for successful emergence. Dadd (1961) found that Locusta migratoria did not mature on a linoleic deficient diet, while from 10% to 56% of Schistocerca gregaria did mature on the same diet, but with damaged wings. Fast (1964) reviewed the literature on insect lipids and found no consistent requirement for a lipid other than sterols.

Structurally, lipoproteins were components of the nerve axons of insects studies by Richards (1944).

Watanabe and Williams (1951) demonstrated that mitochondria from the flight muscle of Phormia regina contained 29% lipid (dry weight). The insect cuticle was found to reduce water loss by using layers of hydrocarbons and waxes as waterproofing mechanisms (Ramsay, 1935; Gilby and Cox, 1963).

Lipids have also been implicated in resistance to insecticides. Munson et al. (1954) presented numerous observations to show that temperature induced differences in saturation of lipids were related to insecticide resistance. The data were offered in support of the view that unsaturation increased the holding capacity of lipids for DDT and related compounds, rendering them non-toxic. Neri et al. (1958) found a tendency for higher lipid content in DDT resistant strains of Anopheles atroparvus compared to susceptible strains.

Recently, Moore et al. (1967) found differences in fatty acid per cent composition between boll weevils, Anthonomus grandis, which died and those which survived exposure to insecticides. A higher percentage of palmitic and oleic acids were found in those which survived and a higher percentage of stearic, linoleic, and linolenic acids in those which died. These results contradicted those of Munson et al. (1954).[1]

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[1] In the two papers, different species were studied. Neither worker analyzed lipids by fractions to determine exactly where the differences in per cent composition occurred when such differences appeared related to insecticide resistance.

The nutritional importance of amino acids, both essential (Mendel, 1914) and unessential (Snyderman, 1962) has been recognized. Now, elementary work is being done to characterize the mechanisms of insecticides by observing changes in free amino acid content of an insect.

Lord and Solly (1964) studied Diazion^{R} in concentrations which killed 50% and 90% of resistant and of susceptible houseflies. They found a decrease in α- and β-alanine as well as proline in the dead flies. This effect was absent in surviving flies. A decrease in glycine proline, and glutamate was noticed in susceptible, malathion-poisoned roaches, Blatella germanica, by Mansingh (1965), while only a slight reduction occurred in poisoned, resistant roaches. Corrigan and Kearns (1963), monitored 14 amino acids and found only proline to decrease progressively with time in DDT poisoned roaches, Periplaneta americana, at 15°C.

Despite the possible relationships of insecticide resistance with both amino acids and lipids, there is a lack of good information concerning the lipogenic activities of amino acids in insects. Clements (1959) presented data on the incorporation of glycine and leucine into fats by whole tissue preparations of fat body from the desert locust, Schistocerca gregaria. Glycine was 1.6 times as lipogenic as leucine. However, in vitro studies do not consider total body needs for amino acids, or the variation in composition of a total diet.

In vivo studies of insect fatty acid synthesis from precursors included that by Louloudes et al. (1961) from acetate-1-C^{14} injected into *Periplaneta americana*. Robbins et al. (1960) performed a conversion study of injected acetate-1-C^{14} into lipids by adult houseflies. The synthesis of adult housefly lipids from injected mevalonate-2-C^{14} was reported by Kaplanis et al. (1961).

Several studies have been performed to determine the amino acid requirements of insects (Vanderzant, 1957; Rock and King, 1967a, b; Earle et al., 1966). However, no reports were found describing from an *in vivo* study, with insect materials, the relative tendencies of various amino acids to donate carbon to the body lipids.

MATERIALS AND METHODS

The parent flies for the pupae in this study were of a laboratory strain derived from the U.S.D.A., Insect Physiology Laboratory colony at Beltsville, Maryland. A synthetic housefly larval diet described by Monroe (1962) was modified to the following composition by omitting sodium oleate:

Ingredient	Parts
Casein (vitamin free)[1]	72
Alphacel[2]	3
Wesson's salts[2]	4
RNA[2]	1
Agar[1]	20

An aqueous misture of B-vitamins was prepared to be added as the diet was used. Four to five drops of concentrated ammonium hydroxide were added to keep the vitamins in suspension. The composition per 10 ml of vitamin mixture was:

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[1]Supplied by Calbiochem, Los Angeles, California.

[2]From Nutritional Biochemicals Corp., Cleveland, Ohio.

Vitamin[1]	mg.
Thiamine hydrochloride	50
Riboflavin	25
Nicotinic acid	100
Pantothenic acid	50
Pyridoxine hydrochloride	25
Choline chloride	1000
Inositol	500
Folic acid	5
Biotin	1

The vitamin-free dry mixture was ball milled 4 hours before adding 0.1% by weight of cholesterol in dichloromethane which was then evaporated in a warm oven with frequent stirring. Radiolabelled amino acids[2] were mixed with unlabelled carrier amino acid[3] and distilled water to form 1mM solutions. Data for the amino acids were as follows:

Amino Acid	dpm/μg[4]	Quantity[5]	Radiochemical Purity
Alanine-U-C^{14}	162	12 μC	99%
Glutamate-U-C^{14}	22	12 μC	96%
Leucine-U-C^{14}	53	12 μC	98%
Methionine-G-H^{3}	1423	120 μC	96%
Phenylalanine-U-C^{14}	97	12 μC	96%

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[1]All vitamins were from Nutritional Biochemicals Corp., Cleveland, Ohio.

[2]From Nuclear Chicago, Des Plaines, Illinois.

[3]Nutritional Biochemicals Corp., Cleveland, Ohio.

[4]Determined for each flask and based on the amino acid percentages for casein given by Hawk et al. (13th ed. 1954).

[5]Added per flask.

Radiochemical purity was determined by paper chromatography employing a system of n-butanol-acetic acid-HOH (125-30-125 v/v).

Ten ml of modified Bray's fluor solution were used per vial for counting on a Nuclear Chicago Unilux I (model 6850) liquid scintillation counter. The formula for 2 liters of modified Bray's solution was:

Ethylene glycol monomethyl ether	500 ml
Toluene	1500 ml
POPOP (1, 4-bis-[2-(5 phenyloxazoly)]-benzene)	100 mg
PPO (2,5-Diphenyloxazole)	8 gm

Diet Preparation

Twenty synthetic larval diets were prepared in pyrex flasks (wide mouth, 250 ml) containing 7.5 g of dry diet, 26 ml of HOH, 24 ml of 1mM amino acid solution and 0.75 ml of B-vitamin mixture. The flasks were sealed with a diSPo^{R} plug (Scientific Products, Evanston, Illinois) and autoclaved at 15 pounds pressure (121°C) for 15 minutes. After sterilization, vigorous aggitation in ice water dispersed the diet as the agar gelled.

Rearing Procedure and Aseptic Culture

For oviposition, a Petri dish holding a muslin bag wet with ammonium carbonate, was placed in cages containing adult flies reared by the CSMA method (Anonomous,

1959). Six hours later, the collected eggs were loosely wrapped in moist paper toweling until used.

The eggs were washed twice in water by shaking in a 50 ml Erlenmeyer flask and pouring off the eggs which floated. A 20 minute wash in 0.1% sodium hypochlorite (Chlorox^{R}) was then given the eggs before aseptic transfer to the flasks with a pipette calibrated to deliver about 300 eggs.

The innoculated flasks were incubated at 30°C for 2 days followed by 28°C until pupation began. A 600 ml beaker under each flask insured that no migrating larvae would be lost from any sample. Pupating larvae were collected by breaking up the remaining diet with a jet of water and washing it through a No. 20 standard screen. Larvae and pupae were then placed on Whatman filter paper in Petri dishes to set overnight at 38°C. The resulting pupae were counted, weighed,[1] and frozen at -30°C until analyzed. Fluid thioglycollate medium was utilized to determine asepsis, and only proven sterile cultures were used in these studies.

Extraction and Chromatographic Fractionation of Lipids

The method of Kaplanis et al. (1961) for extracting total lipids from adult houseflies was adopted for pupal

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[1]All gravimetric determinations were made on a Mettler Gram-atic balance.

extraction. The pupae were homogenized in a glass Tenbroeck homogenizer with water. The homogenate and rinses were collected in a 300 ml round bottom flask and refluxed for 1.5 hours with acetone-ethanol (1:1 v/v) at 4 times the volume of water used.

After reflux, the material was quantitatively[1] vacuum filtered through a Büchner funnel. The acetone-ethanol were then removed in vacuo and the total lipids extracted from the remaining aqueous (after acidification with HCl) with 3 equal washes of anhydrous ethyl ether. The ether was then dried over anhydrous sodium sulfate, collected in a tared flask, and removed in vacuo. Total lipids were weighed, radioassayed in 10 ml of a standard toluene-fluor mixture,[2] and frozen in benzene under a nitrogen atmosphere.

Radioactivity in the aqueous fraction was determined in modified Bray's solution. A 2.5 ml sample from each of the 5 pooled aqueous fractions was chromatographed on a cation exchange column (1.1 x 7.5 cm) of Dowex 50-X12 ground to pass a No. 100 standard sieve. The column was successively washed with 25 ml of water followed by 30 ml

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[1]After every transfer of material throughout this study, the glassware yielding the material was rinsed at least 3 times into that which received it as an effort to be quantitative.

[2]Made as reported for modified Bray's solution, but replacing the ethylene glycol monomethyl ether with toluene.

of 10% ammonium hydroxide and each fraction was assayed radiometrically.

Saponification of the total lipids was accomplished by a slight modification of Kaplanis et al. (1961). The total lipids were transferred into a 45 ml glass stoppered centrifuge tube with ethyl ether. A stream of nitrogen removed the ether and 5-6 ml of 10% (w/v) potassium hydroxide in 90% ethanol were added. The tube was flushed with nitrogen, stoppered and refluxed for 1.5 hours. A quantitative transfer was made from the centrifuge tube to a separatory funnel with water and ethyl ether and the unsaponifiable lipids extracted with anhydrous ethyl ether. The extracts were placed into another separatory funnel, acidified to neutralize any potassium hydroxide, and then washed to neutrality with water. Anhydrous sodium sulfate was used to dry the ether as it was collected into a tared flask. After removing the solvent *in vacuo*, the weight of the unsaponifiable lipids were obtained and samples taken for radioassay. This fraction was then frozen in benzene in a nitrogen atmosphere for later analysis.

The aqueous from the unsaponifiable fraction above was acidified to free the fatty acids from their potassium salts and then extracted with ethyl ether as described for the unsaponifiable fraction. The fatty acids were assayed radiometrically and gravimetrically before storage under nitrogen at -30°C.

The fatty acids were pooled by treatment and the solvent removed *in vacuo*. Transfer was made with ether to a 45 ml glass stoppered centrifuge tube and the ether removed by a stream of nitrogen. A boron trichloride-methanol solution[1] was added and the tube was nitrogen flushed and refluxed 2 minutes (Metcalf and Schmitz, 1961). Transfer of the methyl esters to a separatory funnel was made with small aliquots of water (total 20 ml) and quantitatively extracted with anhydrous ethyl ether. The ether was then dried over anhydrous sodium sulfate and assayed gravimetrically in a nitrogen atmosphere. The fatty acid methyl esters were then stored under nitrogen at -30°C.

A dual column analysis was made of the unsaponifiable lipids (Monroe et al., 1968). Woelm alumina (7.5g), neutral Grade I, deactivated with 1.5% water was packed in *n*-hexane in a series of two 1.1 cm x 7.5 cm columns. About 1.5 g of anhydrous sodium sulfate was also added to the top of the column. The samples were evaporated *in vacuo* and placed onto the first columns in small aliquots of benzene. The columns were then washed with 100 ml of benzene (eluting hydrocarbons and high molecular weight alcohols), anhydrous ethyl ether (eluting free sterols),

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[1]A prepared estrification kit from Applied Science Laboratories, Inc., State College, Pennsylvania.

and 50 ml of methanol (eluting more polar molecules) (Kaplanis et al., 1961).

The benzene fractions were evaporated *in vacuo* and then placed onto the second columns in n-hexane. These columns were washed with 100 ml each of n-hexane (eluting hydrocarbons), benzene (eluting high molecular weight alcohols), and 50 ml of methanol. The methanol fractions from both columns were pooled through a sintered glass funnel into one flask and thereafter considered as a single fraction. Weight and radiometric determinations were made on all fractions eluted.

Gas-Liquid Chromatography of Fatty Acids

The gas-liquid chromatograph used was a Research Specialities Model 600 Series equipped with a hydrogen-flame detector. The column used was 4.3 ft x 4 mm ID stainless steel with 12% HI-EFF 1B (Diethyleneglycol succinate)[1] filter coated onto Gas-Chrome Q.[1] Detailed operation procedures are presented under Results, and all peaks were identified by comparing the retention times of the unknown acids with those of authentic standards.[1] The standards were:

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[1]Applied Science Laboratories, Inc., State College, Pennsylvania.

K 108		N.I.H. Mix E	
Fatty acid	Per cent	Fatty acid	Per cent
C - 16	20	C - 8	6.28
C - 18	20	C - 10	9.26
C - 18-2H	20	C - 12	12.08
C - 18-4H	20	C - 14	23.29
C - 18-6H	20	C - 16	49.09

Relative per cent compositions were determined with a disc integrator which was checked against a standard. Samples were adjusted to 4µg/ul and 2µl injections were used. A 5µl injection served as a template for trapping individual peaks for radio-assay. These peaks were trapped in n-hexane contained in glass tubes which were immersed in acetone and dry ice. The collecting tubes were rinsed into counting vials and the solvent evaporated by nitrogen before the addition of the fluor mixture and subsequent counting.

RESULTS

All rearing flasks proved to be sterile when the mature larvae were collected, and the gross data for the resulting pupae are presented in Table 1. The average weight per pupa was 16.7 mg compared to 20.5 mg reported by Monroe (1962), for the original version of this diet used with different rearing conditions. The high average weight per pupa from the alanine treatment was consistent through 4 replications.[1]

TABLE 1.--Gross data for housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	No. of Pupae**	Total wt. (mg)**	Ave. wt./ Pupa
Alanine-U-C^{14}	472	8,336	17.7
Glutamate-U-C^{14}	811	13,372	16.5
Leucine-U-C^{14}	815	13,530	16.6
Methionine-G-H^{3}	607	10,020	16.5
Phenylalanine-U-C^{14}	688	11,506	16.7

*Pupae less than 24 hr. old.

**Total from 4 replications per amino acid.

[1]Subsequent tests were made with varying concentrations of free alanine and glutamate to look again for weight differences, but the greatest concentration (2 mM alanine) evidently was not great enough to produce a

The average lipid content (Table 2) was 6.5% of live weight. The average residue fraction was 21.3%, and the aqueous fraction was 72.2% of the live weight. Aqueous fraction values were determined by subtracting the sum of residue and total lipid weight from total live weight.

TABLE 2.--Fresh body weight data of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	Residue** mg	Residue** %	Lipid** mg	Lipid** %	Aqueous*** mg	Aqueous*** %
Alanine-U-C^{14}	1,804	21.6	580	7.0	5,952	71.4
Glutamate-U-C^{14}	2,883	21.6	828	6.2	9.661	72.2
Leucine-U-C^{14}	2,824	20.9	901	6.6	9,805	72.5
Methionine-G-H^{3}	2,159	21.6	642	6.4	7,219	72.1
Phenylalanine-U-C^{14}	2,399	20.9	720	6.3	8,387	72.8

*Pupae less than 24 hr. old.

**Total from 4 replications per amino acid.

***Determined by subtracting (residue + lipid) from total weight.

The activity found in the total lipid fraction is presented as microgram equivalents (µg eq) of amino acid utilized by treatment in Table 3. Although glutamate was

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difference from the other treatments. However, it was shown that free amino acid is as effective as sodium oleate in dispersing the growth medium, thus the effect is physical rather than chemical.

TABLE 3.--Radioactivity in total lipid fraction of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	Specific Activity (dpm/µg)**	Total µg-eq†	Average µg eq/mg lipid
Alanine-U-C^{14}	162	25,051	43.7 ± 1.8
Glutamate-U-C^{14}	22	183,754	221.1 ± 21.4
Leucine-U-C^{14}	53	225,973	250.8 ± 29.2
Methionine-G H^{3}††	1,423	1,930	3.0 ± 0.8
Phenylalanine-U-C^{14}	97	30,839	42.4 ± 5.0

*Pupae less than 24 hr. old.

**Within each diet flask.

†From 4 replications per amino acid.

††120 µC per flask, others with 12 µC each.

2.4 times as abundant as leucine in each rearing flask, the leucine treatment produced more µg eq/mg total lipid than did glutamate (250.8 µg eq and 221.1 µg eq, respectively). Alanine and phenylalanine were approximately 1/6 as lipogenic as leucine. The methionine (3.0 µg eq) was converted to the total lipids at 1/84 the rate of leucine.

Figure 1 shows the relationship between per cent composition and radioactivity incorporated into total lipids for the amino acids studied. Although alanine, phenylalanine and glutamate fall on the line, methionine

falls below and leucine above the linear curve. Table 4 shows that the fatty acids contained over 80% of total lipids' radioactivity. A comparison of activity determined after pooling fatty acid replications is shown in parentheses. The pooled values represent a more accurately weighted estimate of the radioactivity per mg of fatty acid than the average obtained before pooling.

Table 5 presents data on the distribution of radioactivity among the unsaponifiable lipids after column chromatography. From 69% to 76.6% of the carbon-^{14} was found in the n-hexane fraction (hydrocarbons), 16.5% to 29.9% in the benzene fraction (high molecular weight alcohols), 0.0% to 4.5% in the ethyl ether fraction (free sterols), and 1.1% to 5.4% in the methanol fraction (more polar compounds). No detectable activity was recovered from the columns for the methionine treatment.

Results of gas-liquid chromatographic analysis of the fatty acid methyl esters are presented in Table 6. Only myristic (C-14), palmitic (C-16), palmitoleic (C-16:1), stearic (C-18), and oleic (C-18:1) acids were found.

By disc integration, the most abundant fatty acid was palmitoleic (39.7%), followed by oleic (29.9%), palmitic (28.7%), myristic (1.7%) and stearic acids (trace amount). The distribution of radioactivity

TABLE 4.--Radioactivity after saponification of total lipids of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled amino acid	Saponfiable				Unsaponifiable	
	Total µg eq**	µg eq / mg fatty acid		% of activity recovered	Total µg eq**	% of activity recovered
Alanine-U-C^{14}	16,565	40.3	(41.9)	84.0	3222	16.0
Glutamate-U-C^{14}	137,414	213.7	(219.8)	95.0	7249	5.0
Leucine-U-C^{14}	196,800	280.2	(274.5)	95.0	10,404	5.0
Methionine-G-H^{3}	1,651	4.8	(4.1)	94.2	102	5.8
Phenylalanine-U-C^{14}	20,874	38.7	(40.0)	86.3	3318	13.7

*Pupae less than 24 hr. old.

**Total from 4 replications per amino acid.

†120 µC per diet flask; others with 12 µC each.

††Values in parentheses determined after pooling of replications.

TABLE 5.--Relative distribution of radioactivity in unsaponifiable lipids from housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	Relative Per Cent Activity**			
	n-hexane	Benzene	Ethyl ether	methanol
Alanine-U-C^{14}	76.6	17.9	4.4	1.1
Glutamate-U-C^{14}	69.0	29.9	0.0	1.1
Leucine-U-C^{14}	76.2	16.5	4.5	2.7
Methionine-G-H^3	--	--	--	--
Phenylalanine-U-C^{14}	70.9	21.2	2.5	5.4

*Pupae less than 24 hr. old.

**Ave. values for 4 replications. Separation on dual columns of 7.5 g of Woelm aluminum oxide, Grade 1, neutral activity, deactivated with 1.5% water, packed in n-hexane as 1.1 x 7.5 cm columns. Elutions in order were 100 ml benzene (placed on second column and eluted with 100 ml n-hexane, then 100 ml benzene and 50 ml methanol), 100 ml ethyl ether, and 50 ml methanol.

generally agrees with the relative per cent composition. However, a difference of approximately 2% is noted between composition and radioactivity (radioactivity is greater) for both alanine and phenylalanine in the C-14 peak and 3% in the C-16 peak. For the same two treatments the activity is approximately 9% less than per cent composition in the C-16:1 peak. The radioactivity collected from methionine

TABLE 6.--Gas-liquid chromatographic analysis of fatty acids of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acids.*

Labelled Amino Acid	Relative Per Cent Fatty Acids**, †					
	C-14	C-16	C-16:1	C-18††	C-18:1	Solvent Front
Alanine-U-C^{14}	1.2 (3.2)	30.3 (33.4)	39.8 (30.7)	- (2.6)	28.7 (29.3)	(0.81)
Glutamate-U-C^{14}	1.2 (2.8)	28.4 (28.0)	40.5 (38.0)	- (3.1)	29.9 (26.0)	(1.61)
Leucine-U-C^{14}	2.5 (2.1)	28.5 (29.1)	38.4 (37.1)	- (2.6)	30.6 (27.9)	(1.10)
Methionine-U-C^{14}°	2.4 (2.9)	28.5 (32.1)	39.5 (44.6)	- (5.1)	30.3 (14.0)	(1.29)
Phenylalanine-U-C^{14}	1.4 (3.4)	28.1 (31.7)	40.4 (31.1)	- (3.4)	30.0 (28.2)	(2.12)

*Pupae less than 24 hr. old.

**Composition determined from average of three 2 µl replications (4 µg/ml) by disc integration; radioactivity trapped in n-hexane as a series of 5 µl replications.

†Analytical conditions: Column, 12% HI-EFF1B on 100/120 mesh Gas-Chrome Q in 4.3 ft. stainless steel column at 175°C, nitrogen flow 43.5 ml/min., vaporizer 220°C, H.F. detector 280°C, output 220°C, attenuation 100.

††Obtained in trace amounts with 5 µl replication.

°120 µC per flask; others with 12 µC each.

does not agree well with the per cent composition except in the C-14 peak.

The data in Table 7 was taken from the body aqueous fraction (hereafter referred to as bio-fluids). It shows that the incorporation of radioactivity from each amino acid into body bio-fluids is strongly correlated to the natural per cent of that amino acid in casein. Figure 2 makes this point very clear.

TABLE 7.--Radioactivity in the aqueous fraction (bio-fluids) of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	Total Bio-fluid mg**	Total µg eq**	Average µg eq/mg Bio-fluids
Alanine-U-C^{14}	6,225	3,895.7	0.63
Glutamate-U-C^{14}	9,661	47,559.0	4.92
Leucine-U-C^{14}	9,805	19,852.8	2.02
Methionine G-H^{3}†	7,219	3,932.8	0.55
Phenylalanine-U-C^{14}	8,061	11,044.0	1.32

*Pupae less than 24 hr. old.

**From 4 replications per amino acid.

†120 µC per flask. Others with 12 µC each.

An analysis of the bio-fluid fraction by cation exchange chromatography gave the data presented in Table 8. Alanine and glutamate retained only 29.2% and 26.1%, respectively, as amino acids, while leucine and phenylalanine retained 59.5% and 61.9% respectively, as amino acid. No detectable radioactivity was recovered from the methionine analysis.

TABLE 8.--Division of radioactivity between two fractions of bio-fluids of housefly pupae aseptically reared on a synthetic diet containing free, radiolabelled amino acid.*

Labelled Amino Acid	Per Cent in Each Fraction**	
	Amino Acids	Other
Alanine-U-C^{14}	29.2	70.8
Glutamate-U-C^{14}	26.1†	73.9†
Leucine-U-C^{14}	59.5	40.5
Methionine-G-H^{3}	--	--
Phenylalanine-U-C^{14}	61.1	38.9

*Pupae less than 24 hr. old.

**Separated on a 1.1 x 7.5 cm column of Dowex 50-X12 cation exchange resin ground from 40/60 mesh to 100/120 and eluted with 25 ml distilled water (neutral and negative molecules) followed by 30 ml. 10% ammonium hydroxide (amino acids).

†Identical results from two replications. Other values based on only one.

DISCUSSION

Pupae

Pearincott (1960) found lipids of M. domestica pupae (approximately the same age as in this study) to be 6.3% of live weight. This value is essentially the same as recorded in these studies.

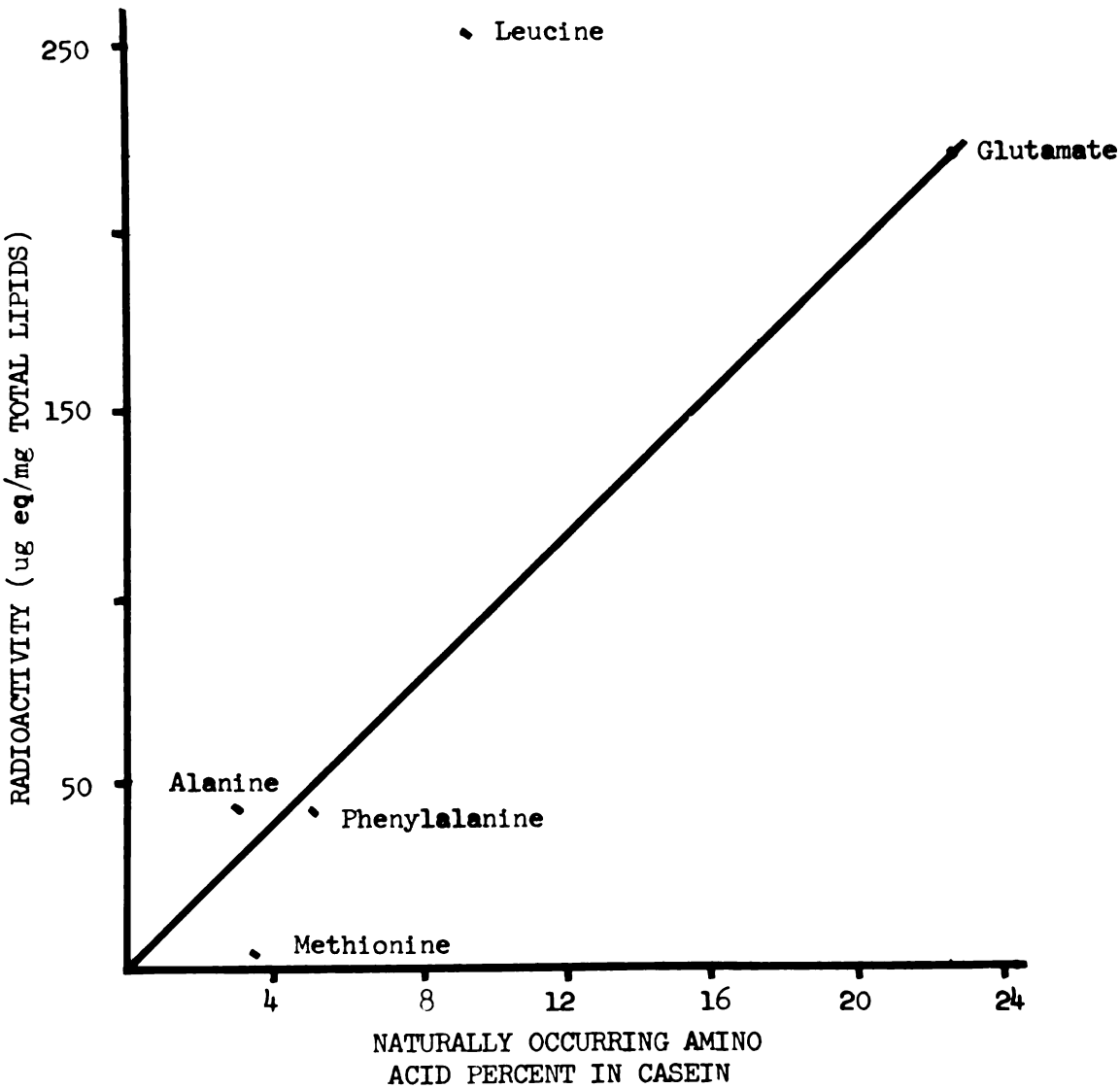
Levinson and Silverman (1954) determined an average moisture content of 73.2% for immature housefly pupae and 69.2% for mature pupae. Data were unavailable for comparison with the average residue fraction obtained in these studies (21.3%), but it appears from gross considerations that the pupae analyzed were normal though of small size.

Lipids

Two broad classes of lipogenic amino acids were apparent from these investigations. In the first class, contributions to fatty acid synthesis were made in close accord with the natural per cent of that amino acid in the casein of the diet. The second class consists of those amino acids which did not contribute to fatty acids at all in relation with their concentration in the casein (Figure 1).

By carrying the dietary concentration of any amino acid out to the theoretical limit, 100%, its contribution

Fig. 1. Relationship between casein composition and radioactivity in the total lipids of housefly pupae (under 24 hours old) aseptically reared on a casein diet containing free, radiolabelled amino acids.



to 1 mg of total lipid would be 1,000 µg, and the slope of the resulting linear graph would be 10 units of rise/run. If contributions from every amino acid in casein were plotted, the sum would have to be 1000 µg/mg of total lipid. A curve of best fit would require the same slope as indicated in Figure 1 (rise/run = 10 units). It is logical to expect that in these studies glutamate would contribute 22.4% of the carbon found in 1 mg of total lipids. It does, in fact, account for 221.1 µg eq or 22.1% and falls exactly on the line (Figure 1).

Such evidence strongly implies that 1 µg eq accurately represents 1 µg of material from amino acids. Therefore, moving the decimal point to the left one place shows the per cent of carbon contributed by each amino acid studied for total lipids (Table 3) and to fatty acids (Table 4).

The departure of leucine and methionine from linearity in Figure 1 may be a reflection of the amount of each present relative to body requirements, directness of degradative pathways to fat precursors, and final breakdown products. From the known pathways of degradation (Meister, 1965), methionine finds many more possible intermediary uses than does leucine. The latter in degradation goes in six steps to acetoacetate which may form two acetate units, and acetyl-CoA. The only fat precursor known formed from glutamate, alanine or methionine is a single acetate unit. Phenylalanine forms acetoacetate,

but it is also used in the tanning of insect cuticle (Brunet, 1963) as well as protein synthesis. Because of its additional use in the cuticle, we could expect phenylalanine to fall slightly below the line (Figure 1), which it did. If the greatest non-protein use of alanine were to form pyruvate, its carbons would be of little metabolic use elsewhere. Acetate for fatty acid synthesis would be very available.

In addition to the factor of quantity vs. use and degradation, another possible reason for the non-linearity of leucine and methionine can be explored. Responding to normal diets used by housefly larvae in nature, a mechanism could have evolved to handle essential amino acids normally in abundance or scarcity relative to requirements.

Selective transportability across the fat body membranes may exist, increasing the uptake of leucine (assuming it is generally superabundant) and decreasing the uptake of methionine (assuming it is generally scarce) relative to alanine, glutamate and phenylalanine. The last three amino acids would be of the same order of transportability.

No weights were recorded for the unsaponifiable lipids in these studies. Weights were taken but indicated that incomplete saponification occurred in at least one replication of both the alanine and phenylalanine

treatments. Data by Levinson and Silverman, 1954, indicated that the unsaponifiable lipids from similar aged housefly pupae comprised 5% or less of total lipid weight. The distribution of radioactivity for all treatments of this study approximated that value in at least one replication (8% or less for all replications of the glutamate, leucine and methionine treatments). It is likely that nearly 95% of the carbon used for lipid synthesis becomes fatty acid.

A previous report of fatty acid composition of housefly pupae (Barlow, 1963) showed the presence of linoleic and linolenic acids. Since that paper did not claim asepsis of growth on a fat-free diet, the reported results may not indicate the biochemical capability of the larvae. Lack of a reported dietary requirement for linoleic or linolenic acids by housefly larvae coupled with the absence of these acids by GLC analysis in this study indicated that they were not involved in development or metamorphosis.

The difference between composition and activity found for both alanine and phenylalanine in the C-14 peak (radioactivity 2% greater) and in the C-16 peak (radioactivity 3% greater) might represent contamination from adjacent peaks (except for C-14 which stood alone). However, such differences could indicate that at some point prior to pupation there was a change toward increased utilization of alanine and phenylalanine for fatty acid

synthesis with the increased activity appearing in the first two peaks. This increase could have resulted from a decreased demand on these amino acids for growth coupled with an inability to have their acetate units enter the Kreb's cycle against the pressure of acetate from other amino acids. It is not believed that the distribution of tritium seen among the fatty acids was a consequence of an alternate mechanism of fatty acid synthesis. Again the difference must have been due to instrumentation or to an increased utilization of methionine for fat production due to decreased growth and lower energy requirements. Nearly mature larvae appear far less active than growing larvae so that energy requirements as well as protein demands could have decreased.

Because methionine was poorly represented in the total lipid (0.3%) a small increase in its use for fatty acid synthesis would perhaps show up quite easily. Since the mitochondial elongation mechanism was less productive of fatty acids in this study than the cytoplasmic system following Wakil's (1960) theory of fatty acid synthesis, a change in methionine use shortly before pupation would more likely appear in the C-16 acids.

The distribution of radioactivity in the unsaponifiable fractions of the lipids follows a pattern very similar to that reported by Robbins et al. (1960), who studied incorporation of injected acetate into adult

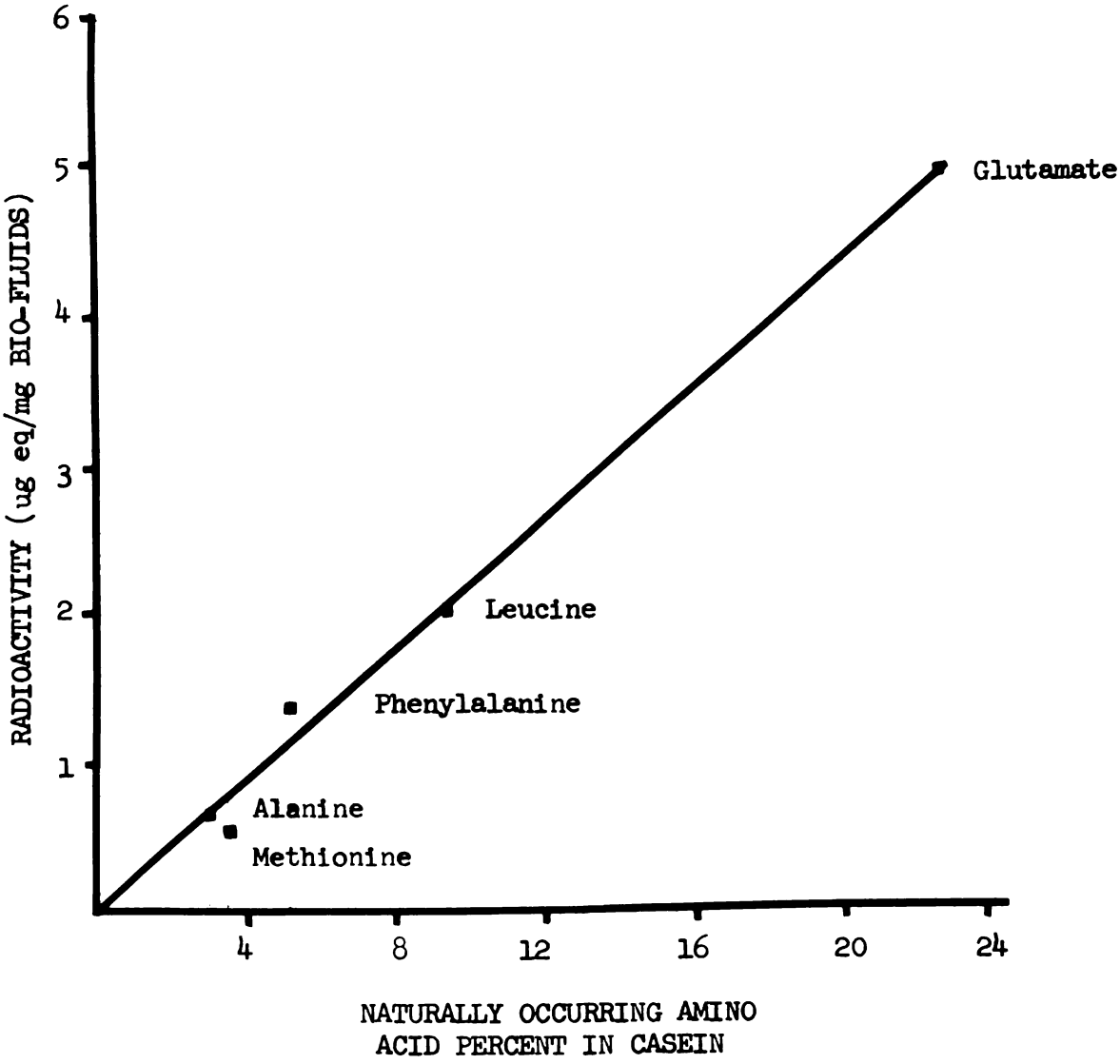
housefly lipids. These workers performed a digitonin precipitation of the sterol fraction and removed all significant activity from the sterols. Since there was low activity in the sterol fraction of this study, no further analysis was made. Because of the poor representation of "methionine-total lipids" and the losses incurred using the alumina columns, no detectable activity was recovered.

Aqueous

The linearity of the data in Figure 2 is very logical if one assumes that the feeding housefly larva tries to maintain an equilibrium between materials entering the bio-fluids and those leaving it. A constant level of metabolites is achieved, and to maintain this equilibrium level, the overall rate of each amino acid's metabolic reactions must be in the same ratio *in vivo* as the ratios of amino acids in the diet. The comparison of ratios below shows that this balance does exist.

Amino Acids	Ratios			
	In casein		In bio-fluids	
	%	μ mol/100 μg	μg eq/mg	μ moles/mg
	I	II	I	II
Glu/Ala	7.4	4.5	7.8	4.7
Glu/Leu	2.4	2.2	2.4	2.2
Glu/Met	6.6	6.6	6.3	6.6

Fig. 2. Relationship between casein composition and radioactivity in the bio-fluids of housefly pupae (under 24 hours old) aseptically reared on a casein diet containing free, radiolabelled amino acids.



Comparisons are between columns marked (I) and between columns marked (II).

Closer consideration of Figure 2 allows additional relationships to be calculated. Glutamate constituted 22.4% of the casein diet used (Hawk et al., 1954). Exact calculation of the slope of the plotted line is done easily using the formula $y = mx + b$ where $b = 0$. From the glutamate treatment:

$$y = mx$$

$$4.92 = m\ (22.4)$$

$$\frac{4.92}{22.4} = m$$

$$m = 0.2196$$

Thus it is reasonable to assume that but for experimental error and biological variation, the slope would be 0.224, numerically equal to the per cent of glutamate in the casein.

Carrying the graph out to its theoretical limit by allowing any amino acid to be 100% of the diet reveals that the sum of individual amino acid contributions to the equilibrium level of amino acid derived metabolites (filterable as per procedure) is 22.4 µg. From the diet used, glutamate would be expected to contribute 22.4% of this total, or 5.0 µg compared to 4.92 µg eq actually measured. The sum of the per cent values for the 5 amino

acids studied is 43%. The sum of their contributions to body aqueous is 9.44 µg or 42.1% of 22.4 µg.

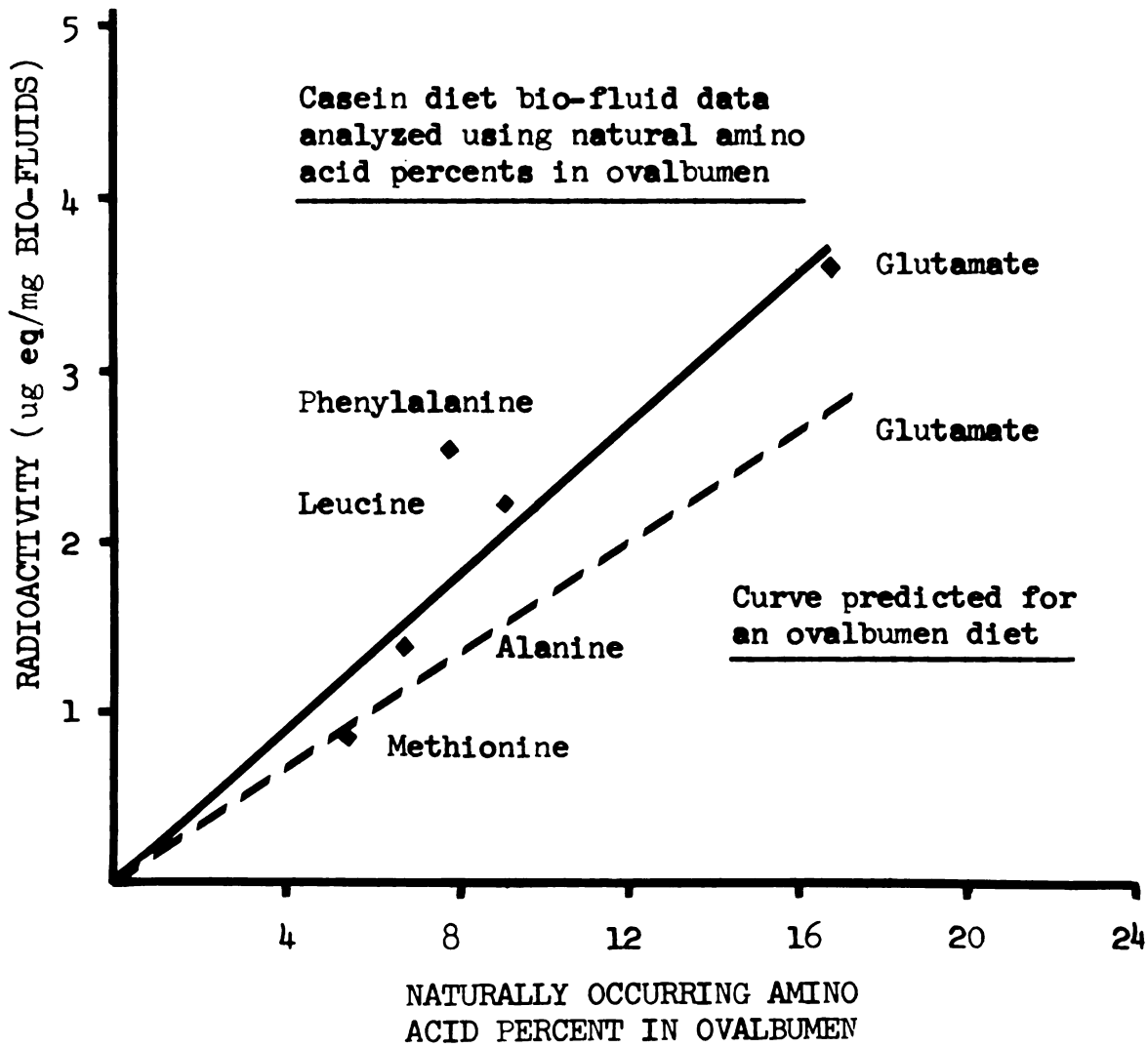
At this point, it seems appropriate to draw these observations together into an hypothesis. This hypothesis applies as yet only to housefly pupae reared under the conditions of this study.

In a biologically balanced diet the per cent of the most abundant amino acid is read by the metabolic systems of the pupae with at least two resulting effects:

1. An equilibrium will be reached in the pupal body such that the sum of all amino acids and their non-protein metabolites (in µg) per mg of bio-fluids equals the dietary per cent of the most abundant amino acid.
2. The contribution from the most abundant amino acid to the equilibrium sum in µg/mg bio-fluids equals the product of its dietary per cent and the same per cent decimally.

Until such time as additional study can be done on this hypothesis, only one additional empirical point can be offered in its support. Figure 3 is a graph made from the bio-fluid data of this study using µg eq values determined as if the diet had been ovalbumen (per cent from Hawk et al., 1954). The theoretical line (broken) is compared to that calculated as just described. The close linearity of the bio-fluid data is lost, but a slope

Fig. 3. Two curves relating dietary amino acid composition to radioactivity in the bio-fluids of housefly pupae (under 24 hours old) aseptically reared on a synthetic diet with free, radiolabelled amino acids added.



identical to that in Figure 2 gives a fair fit. If all points could be plotted, the fit might appear much better. These observations are interpreted to mean that the slope of Figure 2 was independent of the composition per cent used to determine the value of 1 µg eq for each amino acid. If slope were dependent on the per cent used the data would have assumed the slope of the theoretical curve. Extended to its theoretical limit, the bogus graph also shows that the sum of all contributions to the pupal body equals 22.4 µg/mg bio-fluid. This result is inherent in the aqueous fraction radioactivity data and is analogous to saying that a road 100 miles long will always be 100 miles long regardless of its physical path.

For the sake of the present discussion, it will be assumed that the establishment of the equilibrium level (22.4 µg of total non-protein, amino acid-derived metabolite/mg of pupal bio-fluids) occurs very early in larval life and is maintained at least through the first 24 hours of pupal life. It is expected that some level of metabolites would be found in the bio-fluids fraction but why should the particular relationship found in these studies exist?

In theory, the relationship exists because it benefits the larvae. Natural diets for fly larvae probably vary a good deal in amino acid composition, and it would be advantageous if there were an internal mechanism designed

to bring about more favorable amino acid ratios for growth and maintenance. The hypothetical plan outlined below could accomplish this end.

Table 8 shows that amino acids accounted for about 26% of the activity in the bio-fluids of the glutamate treatment. Assuming that *in vivo* this value is 22.4% of the glutamate contribution (determined by the bio-system as it "reads" the incoming nutrients), one arrives at 1.1 ug of free amino acid. Through selection over ages a "best" *in vivo* ratio exists between glutamate and each other amino acid in free form. Acting on only the next most abundant amino acid, or several at once, the 1.1 µg of glutamate-amino acid might induce a "best" ratio by determining the proper activity for specific enzymes removing carbon from the amino acid form within the package contributed by another amino acid to the bio-fluids. At the same time, the enzymes removing metabolites from the equilibrium level also have to alter their activity to maintain the equilibrium at all levels of food intake. The activity of this second group of enzymes might be determined by the changing amount of non-amino acid metabolites in the glutamate contribution to the equilibrium pool, or by the metabolites produced by each amino acid itself.

Thus, there might be a major key effect to nutrition, genetically based on the most abundant amino acid of the

larval diet. Best balance of amino acid ratios and use of metabolites for energy from a biologically acceptible diet results by keying in on the most abundant amino acid.

SUMMARY

A study was made and reported of the lipogenic activity of 5 radioactive amino acids ingested by aseptically reared houseflies, Musca domestica L. In close accord with the per cent of these amino acids in casein, glutamate contributed 22.1% of the total lipid weight, alanine 4.3%, and phenylalanine 4.2%. The contribution from leucine was disproportionately high (25.1%) and that from methionine was low (0.3%).

It was suggested that departures from linearity probably were a factor of abundance versus protein demands (growth and maintenance), intermediary metabolism (leucine going more directly to fat precursors than methionine), and fat precursor formed (leucine to acetoacetate, methionine to acetate). Another factor may have been a differential permeability of fat body membranes favorable to leucine uptake and unfavorable to methionine uptake.

The fatty acid composition of pupae reared on a casein diet was, myristic (1.7%) palmitic (28.7%), palmitoleic (39.7%), stearic (trace), and oleic (29.9%). Where the incorporated activity of a peak differed noticeably from the relative peak area, possible reasons were discussed.

Unsaponifiable lipids distributed the radioactivity in 4 of 5 treatments as follows: hydrocarbons 69-76.6%, high weight alcohols 16.5-29.9%, sterols 0-4.5% (column data, without further analysis), and more polar compounds 1.1-5.4%. No activity was recovered after fractionation of the unsaponifiable lipids from the methionine treatment. The study indicated that of the weight contributed by each amino acid to fatty acids and unsaponifiable lipids, 95% was directed for fatty acid synthesis.

When plotted, the radioactivity in the bio-fluids of the pupae was very linear against the naturally occurring per cent of the donating amino acid in the casein diet. Graphically, it was indicated that a total of 22.4 µg (an equilibrium level) of metabolites from all amino acids were present per mg of bio-fluids. For hypothetical purposes, it was assumed that this level was maintained through larval life.

The most abundant amino acid in the diet was glutamate at approximately 22.4%. An hypothesis was made that the metabolic systems of the larvae read the amount of glutamate present and established an equilibrium level equal in µg per mg of bio-fluids to the per cent of glutamate present. Additional speculation from this hypothetical basis was made.

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