

AN ABSTRACT OF THE THESIS OF

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LARVA OF THE FLESHFLY, SARCOPHAGA BULLATA

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The purpose of this study was to characterize the neutral lipids of the fat body of the larva of the fleshfly Sarcophaga bullata and to examine the means by which these lipids are synthesized.

The weight of the larva, the fat body as well as its lipid content were examined in relationship to the growth of the larva. The average larva attained maximum body weight of about 220 mg in 70 hours. However, fat body lipid continued to increase in weight after the feeding stage of the larva during which time there was a small decrease in total larval wet weight.

The fat body lipid comprised mainly of the neutral lipid and triglyceride formed the major portion of the neutral lipid. Approximately 60% of the total fatty acids analysed was found to be unsaturated and an increase in unsaturated fatty acid content was found in the fat body of the nine day old larvae. The major portion of fatty acids in the fat body as analysed by gas-liquid chromatography were

palmitic, palmitoleic, stearic, oleic and linoleic acids.

Very small amount of acetate-1-C¹⁴ was incorporated into the fat body lipid. Palmitate-1-C¹⁴, on the other hand, was more extensively incorporated. Most of the radioactivity which was incorporated into the neutral lipid was present in the three glyceride fractions, of which the diglycerides showed the highest specific activity. It is suggested that diglyceride could play an important role in lipid transport in the insect system (13, 14).

Incorporation of palmitate-1-C¹⁴ into fat body lipid during different time intervals of incubation indicated that there was a very rapid uptake of the radioactive material in the first 30 minutes. Comparison of palmitate-1-C¹⁴ incorporation between the three day and seven day old larvae showed that larvae at their prepupal stage were still able to incorporate long chain fatty acids but to a somewhat less extent.

Fatty acids of neutral lipid were separated into saturated and unsaturated components on thin-layer plates. After incubation of fat body of three day old larvae with palmitate-1-C¹⁴, the ratio of the distribution of the label between unsaturated and saturated fatty acid was approximately 60-40. This ratio was achieved within ten minutes of incubation. In seven day old larvae, most of the label was found in the saturated fraction.

NEUTRAL LIPID METABOLISM IN THE FAT BODY
OF THE LARVA OF THE FLESHFLY,
SARCOPHAGA BULLATA

by

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A THESIS

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To my husband

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
CoA	coenzyme A
TPNH	reduced nitotinamide adenine dinudeotide phosphate
acetate-1-C ¹⁴	sodium acetate labelled with C ¹⁴ at the carbonyl position
palmitate-1-C ¹⁴	potassium palmitite labelled with C ¹⁴ at the carbonyl position
POPOP	1,4-bis-2-(5-phenyloxazoyl)-benzene
PPO	2,5-diphenyloxazole
TL	total lipid
NL	neutral lipid
FFA	free fatty acids
DEGS	diethylene glycol succinate
C _{6.....22}	fatty acid with the designated carbon chain length
C _{16:1.....2}	unsaturated fatty acid with the designated carbon chain length and degree of unsaturation. Positions of double bonds are not indicated.

C, centigrade; c.p.m., counts per minute; dpm, disintegrations per minute; gm, gram(s); mg, milligram(s); ml, milliliters; sp. act., specific activity; wt., weight; μ m, micromoles; μ c, microcuries; psi, pounds per square inch; N, normal; V, volume, + present; - absent; > greater than; < less than.

NEUTRAL LIPID METABOLISM IN THE FAT BODY
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INTRODUCTION

There has been a growing interest, in recent years, in all aspects of the biochemistry of lipids of insects. This stems from the realization that lipids play an important role in metamorphosis, flight, water retention and resistance to insecticides, and that they are related in some way to the ability of various species to withstand extremes of temperature. The characterization of lipids has been reported for only 60 species of insects. For the most part, these characteristics have been made of total lipid, extracted from whole insects. In no taxonomic group or even single species has an overall study of lipid biochemistry been attempted.

Several species of flies are ideal subjects for biochemical studies. They grow rapidly with many generations the year around and can be reared in large numbers in the laboratory. They can be readily subjected to experimental variation of environment and samples of all developmental stages are readily obtained. One such species, Phormia regina, has been used in this laboratory for many years, in studies of phospholipids. The fleshfly, Sarcophaga bullata, is related to Phormia and many aspects of its physiology

are similar. It has a major advantage in being two to three times the size of Phormia and thus the organs can be more easily obtained.

The present work is an analysis of the neutral lipids of the fat body of S. bullata. The fat body in insects is the primary storage tissue and a major site of metabolic conversions apparently serving the capacities similar to those of the liver and adipose tissues of higher organisms. The neutral lipid and the constituent fatty acids have been characterized. Certain aspects of the synthesis of these components have been studied by measuring the incorporation of palmitate-1-C¹⁴ during incubation with isolated fat bodies. These studies have been made with the developing larva under more or less normal conditions of growth and temperature. Some comparisons have also been made with mature larva.

HISTORICAL REVIEW

The fat body of insect larvae, as described by Wigglesworth (74) consists of a layer composed of a single sheet of flattened cells applied to the surface of the body in each segment, and a visceral layer made up of few small lobes in the prothorax, groups of cells applied to the imaginal disks and a thin membrane covering the gonads.

Study of the lipid content in larvae dates back to 1909 when Weinland (71) noticed that fat amounted to 22% of the dry weight of the blowfly larvae. In 1926, Rudolf's (57) showed that in the tent caterpillar, the amount of fat decreased during incubation of the eggs and increased tremendously during the larval stage. Along with the increase in fat, there was a decrease in water content, especially in the later stage of larval life. Finkel (25) in 1948 worked on the larvae of the meal worm, Tenebrio molitor, and found that the total lipid, neutral fat and fatty acids increased in proportion to wet weight as larval growth progressed; phospholipids decreased very slowly while total cholesterol remained practically constant. He concluded that lipid composition during larval growth of the mealworm was consistent with that known to exist in vertebrate embryos. Levinson and Silverman (42) studied the lipid of housefly Musca vicina (Macq). They noted that lipid content of this species increased

in seven days from 2.5 μ g in the egg to 1225 μ g in late third stage larva. However, within 24 hours of the onset of pupation, the fat content dropped to 880 μ g and only 294 μ g could be recovered from the emergent housefly. In contrast with Rudolf's and others results, Levinson and Silverman found that the water content of the three stages of this housefly species remained much the same.

Pearincott (55) noted that during the larval growth of housefly Musca domestica, the body weight per larva increased to an average of 19.6 mg in the fifth day. It then decreased to an average of 13.4 mg in the sixth day and 12.3 mg immediately following puparium formation. Along with the weight lost, he also noted a decrease in water content in the larvae prior to puparium formation.

In general, most holometabolous insects accumulate large quantities of fat during the larval state at a higher rate than other body materials. However, fat synthesis in some insect species such as Bombyx mori, Paris napi and Hyalophora cecropia follows a different pattern (23). Average fat content in these species decreases steadily during the instars I and II, then remains almost constant during instars III and IV and part of V. In the middle of the fifth instar, lipid synthesis increases sharply so that in the early pupa the relative fat content is two or three times that of instars IV or V. Strogaya (67) claimed that the increase in lipid content in the non-feeding prepupal stage of P. rapae was due to the result of internal

reorganization of dry matter.

Fast (23) made a rather extensive survey of the lipid content of insects. It is evident from the data he listed that information of this type is far from complete. Differences in methodology, age and stage of experimental animals and the lack of modern analytical techniques made the comparisons very difficult and it is even more difficult to draw any sort of generalizations. Besides, many of the old data are found to be inaccurate when repeated by modern methods of lipid analysis. Nevertheless, Fast's collection of data does indicate that three-fourths of the species studied contain less than 10% wet weight as lipids. The mean lipid content for larvae on a dry weight basis is about 30%. In two-thirds of the insects Fast examined, 60% to 80% of the fatty acids are unsaturated. The fatty acids present in insect lipid seem to follow the same pattern as those found in other animals. Barlow (4) concluded that Diptera, as a group, was distinguished by a high percentage of palmitoleic acid both in the glyceridic and phospholipid fractions. The larva of Aedes aegypti was found to contain more than 30% palmitoleic acid (24). Indeed, some insects do have very unusual lipid patterns, such as Tuberolachnus salignus in which more than 80% of the fatty acids are 14-carbon acids. Barlow (4) also found no acids of chain length longer than 14 carbons in Dactynotus ambrosiae.

Examination of the fatty acid composition of the Bombyx mori

larvae during development showed that although the percentage of fat was relatively constant during the larval stages, distinct changes in fatty acid composition had taken place (23). A significant increase in arachidic, stearic, and linoleic acids with a concurrent relative decrease in the quantity of palmitic, oleic and linolenic acids was found at the early stage of the Bombyx mori larvae, but before the fourth moult, a reverse in this lipid pattern was observed which persisted through the prepupal stage. On the other hand, larvae of M. vicina and Galleria mellonella accumulated primarily unsaturated fatty acids in the early stages and saturated ones during later growth (23).

The influence of dietary lipids on the character of the depot fat has been explored by a number of workers, all of whom seemed to conclude that there was a direct correlation between the iodine numbers of the two. Bachstetz and Aragon (2) determined the fatty acid component of the fat of the caterpillar Acentrocne me hesperiaris which was fed on the leaves of Mexican agave, the sap of which contains mostly carbohydrates. The fat, which in this instance was almost certainly synthesized by the insect from carbohydrates, contained 30% palmitic and 61% oleic acids. Analysis of body fat of insects of the Orthoptera (locusts, grasshoppers, etc.) by the same author showed that the major components of these fats were evidently linoleic and oleic while saturated acids, mainly palmitic and stearic, were also prominent. However, the saturated fatty acid contents of

grass leaves which formed the main diet of these Orthopterous insects, were much smaller in percent distribution. The beetle which feeds on the endosperm of the nuts of Manicaria saccifera (Palmae) were found by Collins (19) to contain nearly 50% fat in which lauric acid was 24%, myristic 21%, palmitic 8%, oleic 32%, linoleic 3%, and stearic 12% of the fatty acid content. Comparing these figures with those for the kernel fat of M. saccifera, it appeared that lauric acid was present in the larval fat in only about half the amount of that in the kernel fat, while oleic and linoleic acids appeared in a much higher amount. These results suggested that the insect could derive its fat partly by direct assimilation of the dietary fat, and partly by synthesis from carbohydrates or other non-fatty materials.

Fatty acids are recognized as being of importance to living organisms in two primary roles (14). First, they serve as substrates for oxidative catabolism and production of energy. The high energy equivalence per unit weight of the fatty acids makes them important in the storage of energy. This is especially evident in insect larvae where a large portion of the lipid is stored in the fat body. Secondly, the complex lipids of which the fatty acids are essential components serve as structural components of cells and of subcellular organelles. Their insolubility in aqueous media makes the complex lipids suitable for the function.

Most of the information concerning the biosynthesis of fatty

acids in insects is limited to the formation of fatty acids from acetate (63, 77). This synthesis proceeds by a condensation of acetyl-CoA to malonyl-CoA as it does in higher organisms (39). Nothing is yet known regarding the synthesis of unsaturated fatty acids in insects. Fat transport in insects may be very different from the mammalian system. Recent findings have indicated that lipid transport in insects may be in the form of diglyceride which is released from the fat body and transported to the sites of utilization by the hemolymph protein (13).

Many years ago, people were looking for a carrier system which would transport fat into adipose tissue. Stern and Shapiro (66) were the first to demonstrate the in vitro uptake of lipid materials like monoleate and triolein into the adipose tissue of rats. They found that long chain free fatty acids such as sodium stearate were also incorporated into the system. Shapiro, et al. (61) found that adipose tissue could preferentially absorb fatty acid compounds. When adipose tissue was incubated in serum, some of the neutral lipid of the serum was found in the adipose tissue after the experiment. Incubating with labelled free fatty acid, they found that more than 90% of the free fatty acids taken up by the adipose tissue were found in the triglyceride fraction at the end of the incubation. They also extended the observations and reported that the nutritional state of animals from which tissue was obtained also markedly affected

the quantity of labelled free fatty acid uptake.

Recently, Vaughan (70) proposed the following scheme showing reactions leading to the accumulation or removal of free fatty acids in the adipose tissue. Her scheme showed that free fatty acids in the extra cellular space could equilibrate with that of the free fatty acids in the cytoplasm and a small portion of the cytoplasmic free fatty acids may diffuse into the fat droplet. However, the extra cellular free fatty acids which are incorporated into the cytoplasm of the cells may become acylated. The acyl CoA derivatives may either be oxidized or participate in the synthesis of triglycerides. The excess triglyceride may then be stored in the fat droplet. Her proposed scheme, however, did not explain how triglyceride in the fat droplet could be transported to sites of utilization.

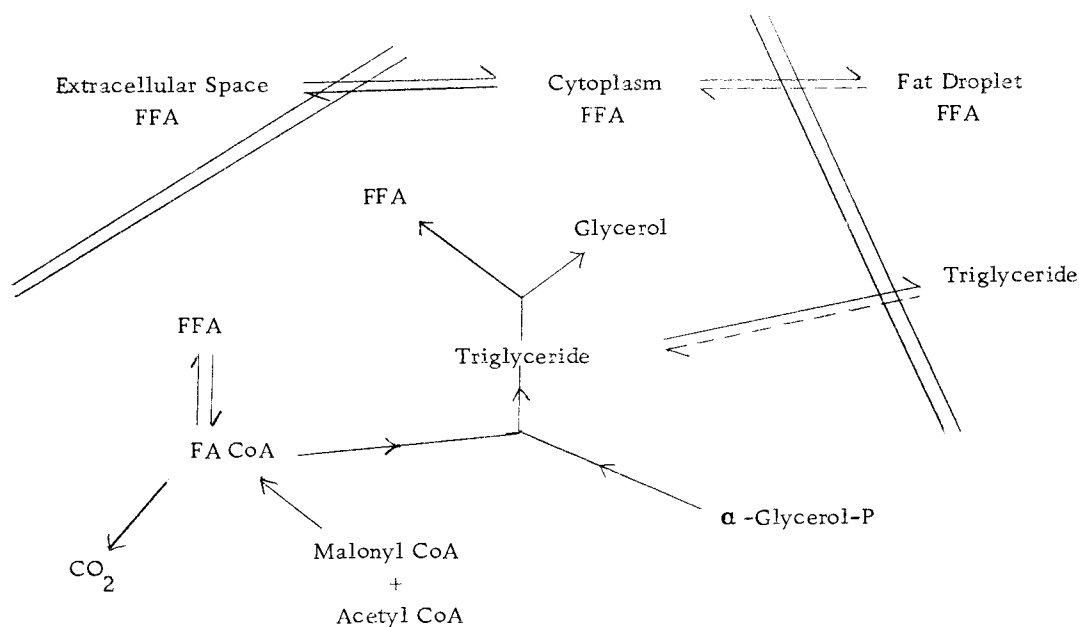


Figure 1. Free fatty acids metabolism in the adipose tissue as proposed by Vaughan (70).

In 1957, Stein, Tietz and Shapiro (65) observed that more radioactivity was incorporated in the neutral lipid than in phospholipid after incubating rat liver mitochondria with labelled free fatty acids for 30 minutes. Neptune et al. (52) also found a more rapid incorporation of labelled palmitate into the neutral lipid than phospholipid of rat diaphragm. But, after prolonged incubation, they actually found an increase in phospholipid activity.

Neptune et al. (53) further studied the incorporation of palmitate into triglyceride of rat diaphragm using prelabeled material. The tissue was first incubated with palmitate-1-C¹⁴ and then it was transferred to a second medium and incubated with unlabelled palmitate. He found that although there was no change in the amount of phospholipid during the second incubation, there was a significant fall in both the amount and specific activity of triglycerides. His experimental result suggested that a two-way shuttle of fatty acids could exist between triglyceride and phospholipid. Palmitate may actually enter into the glyceride first and then be more slowly incorporated into phospholipid.

There has been considerable speculation on the biosynthesis of triglycerides. Borgstrom (9) suspected that the process of triglyceride synthesis in rat lumen may take place by the reversal of lipase reaction. By the action of pancreatic lipase in vitro, he was able to demonstrate that long chain triglycerides were degraded via 1,2-diglyceride to 2-monoglyceride. He believed that the

incorporation of fatty acids into glycerides could be catalyzed by this enzyme. On the other hand, Jedeiken and Weinhouse (33) found that triglyceride synthesis was not dependent upon the active respiration of the tissue. Tietz and Shapiro (69) observed that incorporation of palmitate-1-C¹⁴ into rat liver homogenate required the presence of ATP and the presence of the respiratory system was also not obligatory. Synthesis of triglyceride by the reversal of hydrolytic action, by transpeptidation and by transglycosidation was also suggested (33).

In 1956, Weiss and Kennedy (72) suggested that diglycerides could be a common intermediate involved in the synthesis of triglycerides as well as phospholipids. He proposed the following pathway:

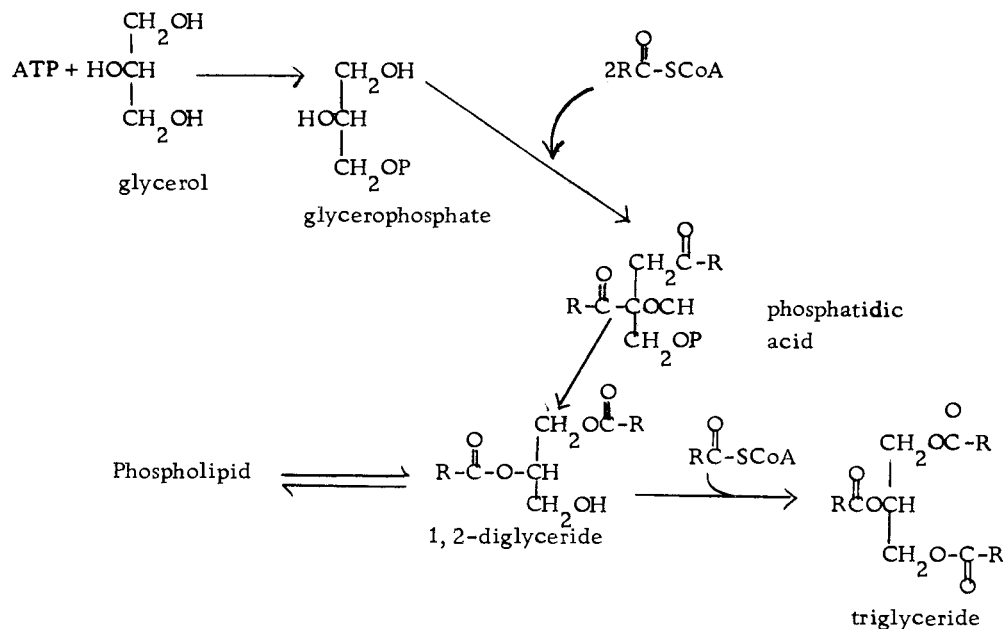


Figure 2. Synthesis of triglycerides and phospholipids as proposed by Weiss and Kennedy (72).

They (36) found that the same chicken liver enzyme preparation which would carry out the synthesis of triglycerides when supplemented with D- α , β -diglyceride and palmityl-S-CoA labeled with palmitate-1-C¹⁴. Extensive incorporation of labelled palmitic acid into the triglyceride fraction was noted with the complete system. This triglyceride synthesized enzymatically was later isolated by Borgstrom (9) on a silicic acid column.

Since 1,2-diglycerides are recognized as intermediates in synthesis of both neutral fat and phospholipids, the importance of these compounds in lipid metabolism is obvious. It is of interest that 1,2-diglycerides are also the primary products of the action of pancreatic lipase on triglycerides. The scheme therefore explains the experimental results which show that diglyceride has the highest specific activity of all the lipid fractions after incubating the tissue with labelled free fatty acids.

However, the possible existence of alternative pathways must also be considered in view of the fact that free glycerol is found to be inert in the living system and is not incorporated into triglycerides to a significant extent (15).

A second pathway for the synthesis of triglycerides termed the monoglyceride pathway was first suggested by Clark and Hübscher (15). After food absorption, the monoglyceride was expected either to be hydrolyzed to glycerol and free fatty acids by an intestinal

monoglyceride lipase, or directly acylated to form lipid of higher glycerides. The biosynthetic pathway in comparison with that proposed by Weiss and Kennedy is shown in the diagram below. The monoglyceride-triglyceride system has been demonstrated both in vitro and in vivo in the mucosa of the small intestine.

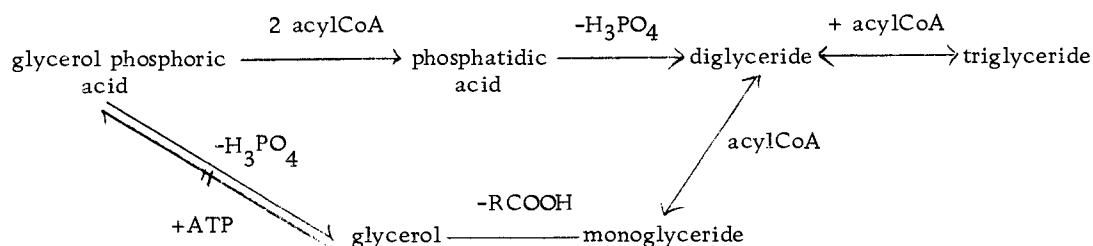


Figure 3. Neutral lipid metabolism in rat mucosal cells by Clark and Hubscher (16).

Although the necessity for complete or partial hydrolysis of ingested triglycerides as a prerequisite for absorption is still uncertain, many researchers have found that in the mammalian system, a significant amount of ingested triglycerides are broken down to fatty acids before absorption (35). Whether the same system exists in the insects is yet to be established. One of the main reasons for triglyceride hydrolysis in the gut of both insect and mammalian systems and subsequent resynthesis is to facilitate absorption of the complex lipid molecules.

From a physiological standpoint, a direct esterification of

monoglyceride, to di- and triglyceride is reasonable. The presence of monoglyceride transacylase in the intestinal mucosa points also to the possible involvement of monoglyceride pathway during intestinal absorption (17, 1). Substantial amounts of monoglyceride are known to occur in the digestive tract during fat absorption and to enter the mucosal cells of the intestine. Therefore, unless the intestinal lipase achieves complete hydrolysis of the dietary monoglycerides, a direct esterification seems to be obligatory. In fact, Kern and Borgstrom (37) found a preference for the synthesis of triglyceride through the monoglyceride pathway by the hamster intestinal mucosa. The biosynthetic pathways of glycerides have not been established in insects. The operation of the above proposed pathways which have been demonstrated in the mammalian system can probably be operative in the insect system. However, further investigations are desirable.

MATERIALS

Sodium-acetate-1-C¹⁴, potassium palmitate-1-C¹⁴ and toluene-1-C¹⁴ were obtained from New England Nuclear Radioactive Chemicals. Non-labelled long chain fatty acid materials were obtained from Eastman Organic Chemicals and Matheson Coleman and Bell Company. Qualitative and quantitative long chain fatty acid standard mixtures and boron-trifluoride-methanol were obtained from Applied Science Laboratory. Silica gel G was obtained from Brinkmann Instruments Incorporated, silicic acid from Mallinkrodt Chemical Works and floridil from Matheson Coleman and Bell Company. PPO and POPOP were from the Packard Instrument Company.

GENERAL METHODOLOGY

Maintenance of Insect Colony

Batches of 100-200 Sarcophaga bullata pupae were placed into a 400 ml beaker containing one inch of dry sawdust at the bottom. Each beaker of pupae was put in a fly-rearing cage which measures 11" × 12" × 15" and these cages were stored in a constant temperature insect rearing room at 30 degrees centigrade. On the emergence of adult flies, a bottle of water and a petri dish full of food were introduced into each cage for the maintenance of the colony. The food was composed of a dry mixture of sucrose, powdered milk and powdered egg (6:6:1 by weight). This would maintain normal healthy colonies.

In order to obtain larvae, small pieces of beef heart were placed inside the cage soon after the emergence of the flies. A piece of damp cotton was placed over the meat to prevent it from drying out and the meat was generally replaced every day. After about a week the females deposited young, live larvae on the meat. The supply of meat was thereafter discontinued unless more larvae were needed. Successive generations of larvae could be produced by the exposure of the mature adults to fresh meat for a short period of time. For the purpose of maintaining stock, fresh meat was

put in the fly-rearing cages for several hours every week and several hundreds of larvae were usually produced by the matured females during this period.

Larvae that were deposited by the flies into the meat were then transferred into 400 ml or 800 ml beakers depending on the quantity obtained. The number of larvae placed in any beaker was only a rough estimate, since the young larvae were very small and difficult to handle. More beef heart was put into the beaker and the meat was covered by approximately one inch of dry sawdust and incubated at 30°C in a constant-temperature room. In later experiments, beef heart was replaced by beef liver. The larvae were maintained under this condition from six to nine days until pupation. Usually, more meat was added once or twice during growth in order to give the larvae an abundant food supply. Prior to pupation, the larvae migrated to the layer of dry sawdust where they pupated. The pupae were then transferred from the sawdust to a clean beaker which was placed in a cage when the first adult appeared.

Fat Body Isolation

Sarcophaga bullata larvae of the desired age were removed from the meat on which they had been growing. The larvae to be dissected were counted, washed with water, and then blotted dry on a paper towel. They were then weighed in a tared beaker and kept

cool in an ice bath. The removal of the larval fat body was done in insect Ringer's solution in a petri dish resting on crushed ice. The Ringer's solution was made up of 128 mM NaCl, 4.7 mM KCl and 1.9 mM CaCl_2 according to the method described by Ephrussi and Beadle (21).

Isolation of the fat body simply involved snipping off the anterior tip of the larva with fine forceps and squeezing out the gut and fat body into Ringer's solution. The fat body was then separated from the digestive tract and trachea. It was necessary to avoid bursting the gut, because this was highly contaminated with bacteria. Sometimes it was more desirable to do the dissection under a low power magnifying glass. The isolated fat bodies were pooled into a small beaker containing cold Ringer's solution.

Lipid Extraction from Isolated Fat Body

Lipid was extracted from the isolated fat body according to the method of Folch, Lees and Sloane (26). Fat body preserved in cold Ringer's solution was transferred to a Dounce homogenizer and homogenized in about a five-fold volume of chloroform-methanol(2:1). The combined chloroform-methanol extracts were washed with 0.2 volume of 0.79% NaCl and then washed with 0.2 volume of 50:50 mixture of 0.79% NaCl and methanol as described by Bieber et al. (6). The washed chloroform-methanol extract containing lipid was

then dried through anhydrous sodium sulfate and the lipid was recovered after evaporating the organic solvent in a vacuum rotatory evaporator at 30° C. The lipid was re-dissolved in a small volume of chloroform-methanol and stored in a refrigerator. The lipid materials were separated into neutral lipid, free fatty acids and phospholipid by silicic acid column chromatography. The neutral lipid was evaporated into its components using a floridil column.

Saponification and Esterification of Fat Body Lipid

Lipid to be saponified was evaporated from its solvent and five volumes of 30% KOH in methanol was added. Saponification took place at 80° C for two hours in a constant-temperature water bath, after which the fatty acids were freed from their salts by acidification with 6N H_2SO_4 to pH 3 or below. The mixture was then extracted three times with n-hexane. Fatty acids in the pooled hexane extracts were washed once with water and dried through anhydrous sodium sulfate. These fatty acids were then ready for esterification. A number of methods have been introduced for fast and easy esterification of fatty acids; of these the method based on BF_3 -methanol reagent was chosen (50). One hundred to 200 mg of fatty acids were placed in a test tube and 3 ml of BF_3 -methanol reagent were added. The mixture was then boiled in a water bath at 80° C for three minutes. The boiled mixture was then transferred to a

separatory funnel with 20 ml of water and 30 ml of petroleum ether. The contents were mixed and allowed to separate. The aqueous layer was extracted two more times with petroleum ether and discarded. The combined organic fractions were washed with water once and dried with anhydrous sodium sulfate. The methyl esters were recovered by evaporating the solvent and were then ready for gas-liquid chromatography and other analyses.

PART ONE: THE DEPOSITION AND CHEMICAL COMPOSITION OF THE LIPIDS OF THE FAT BODY

Deposition of Lipids During Larval Growth

The purpose of the first series of experiments was to compare the growth of the fat body, particularly with respect to the lipid, with the growth of the larvae and to determine the major classes of lipids and their constituent fatty acids.

Larvae obtained from three female flies were placed in a 600 ml beaker and reared according to the method described on page 16. At various time intervals, 20 larvae were removed from the beaker, washed clean, blotted dry with paper towels and weighed. The larvae were dissected and the fat bodies were placed directly into chloroform-methanol and homogenized in a Dounce homogenizer. The homogenate was filtered on tared filter paper and the precipitate was washed with more chloroform-methanol. The combined lipid extracts were washed and dried to constant weights according to the method described on page 18. The residue remaining on the filter paper was also dried to constant weight. The weight of the lipid together with the weight of the dry residue is considered to be the total dry weight of the fat body.

The increases in weight of the larvae, fat body and lipid during the later stages of growth were measured in three separate

experiments and are illustrated in Figures 4 and 5. The results indicated that larvae attained maximum size at about 65 to 70 hours of age. The weights then became more or less constant for the remaining period of observation. The individual larva had an average weight of 52 mg at 35 hours, 221 mg at 70 hours, 208 mg at 90 hours and 210 mg shortly before pupation and during the early stages of metamorphosis.

The dry weight of the fat body was about 2.6 mg in a single larva at 45 hours, 13.2 mg at 75 hours and 23.6 mg at 115 hours. The weight increased ten fold during the 70 hour period and doubled after the larvae had ceased to increase in body weight.

A single fat body contained approximately 1 mg of lipid at 45 hours and it increased steadily to 14 mg at 115 hours. At 115 hours the fat body lipid corresponded to 6.4% of the total weight of the larva. The increase in lipid was roughly parallel to the increase in the dry weight of the fat body, but as shown in Figure 5, the percentage of lipid in the fat body did not attain a constant value until the larva was 80 hours of age.

Fractionation of Neutral Lipids

Isolation of the Neutral Lipid Fraction

The development of new chromatographic methods in recent

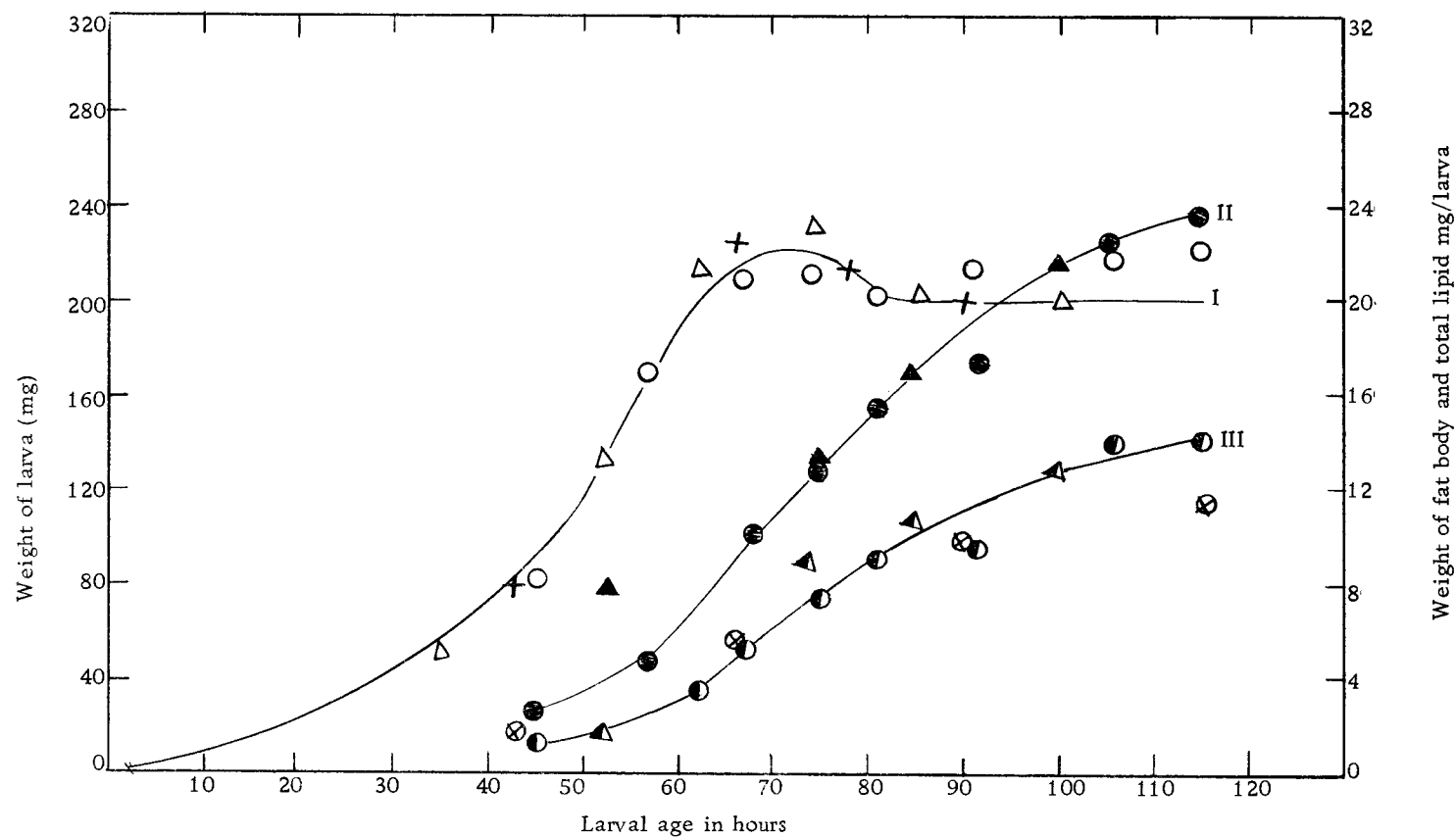


Figure 4. Weight of larva, fat body and lipid during larval growth. Experimental details are described in the text.

- \triangle \times \circ Body weight of larva for different growth intervals in three separate experiments. Each point is the average result of three weighings and 20 larvae were used in each weighing (I)
- \blacktriangle \bullet Dry weight of fat body (II)
- \blacktriangle \bullet \otimes Weight of total lipid extracted from larval fat body (III). II and III are referred to scale on the right.

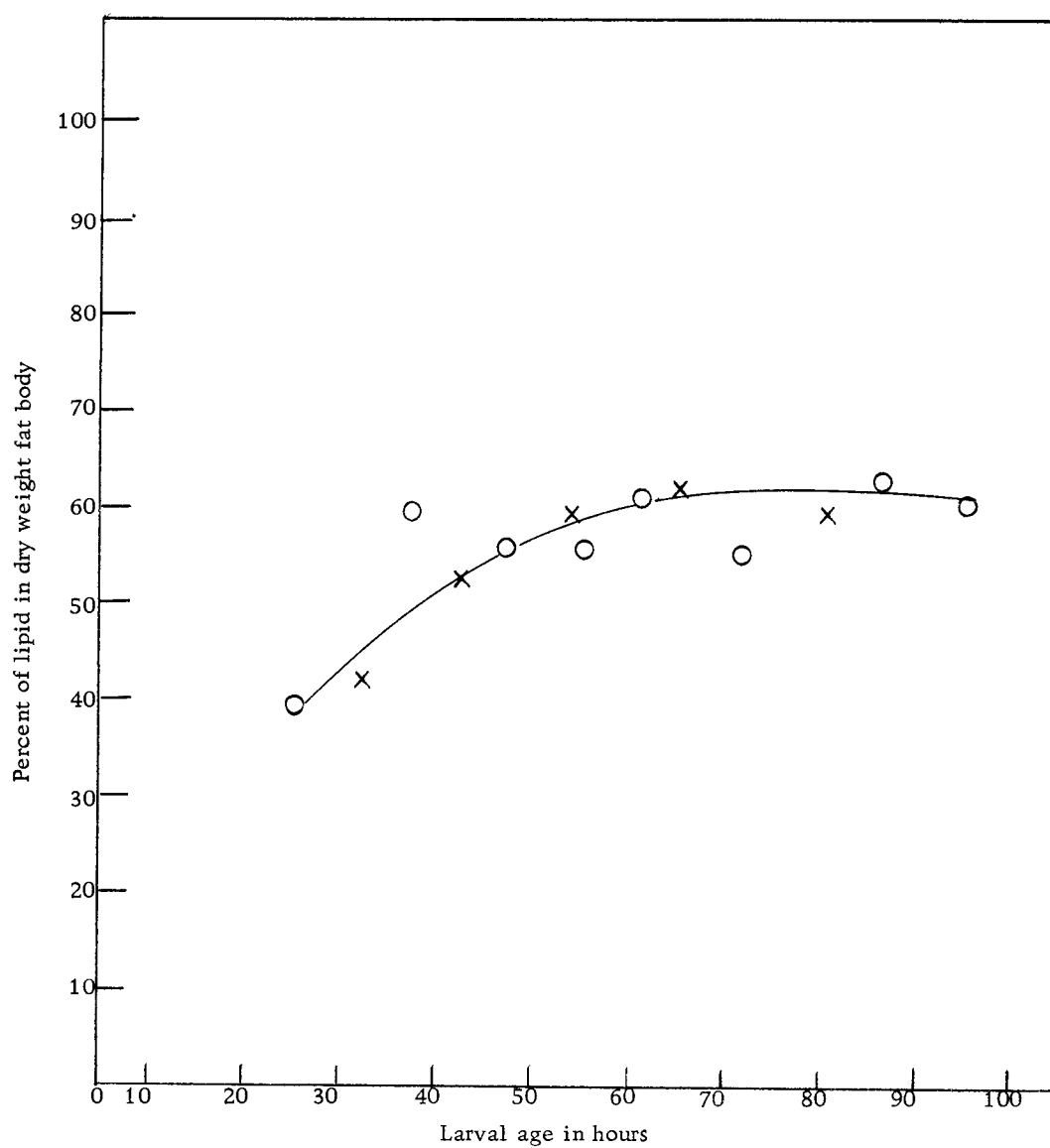


Figure 5. Percent lipid in fat body during growth of the larvae.

years has enabled the separation of the major classes of lipids as well as the components in each class. Since later experiments involved the investigation of the glycerides contained in the neutral lipid fraction, it was first necessary to separate the neutral lipid from other lipid classes.

The method described by McCarthy and Duthie (48) was used for the separation of neutral lipid from free fatty acids and phospholipids. Separation involved the use of a silicic acid column coated with isopropanol-KOH. One hundred grams of silicic acid was suspended in 400 ml of methanol and the fine particles which were not settled were decanted after five minutes. This was repeated once with methanol and once with 400 ml of acetone. The silicic acid was then rinsed with ethyl ether and dried in air. Five grams of this treated silicic acid were weighed into a small beaker and 10 ml of isopropanol-KOH and 30 ml of ethyl ether were added. The isopropanol-KOH solution contained approximately 50 mg of KOH per ml. The silicic acid was added as a slurry to a glass column followed by 100 ml of ethyl ether. Lipid extracted by chloroform-methanol was evaporated to dryness and redissolved in a small quantity of ethyl ether. It was then transferred into the column with two or three rinsings. Neutral lipid which contains cholesterol, cholesterol esters, mono-, di- and triglycerides, was eluted in one fraction from the column with 100 to 150 ml of ethyl ether. These were

usually collected in one fraction in a 250 ml Erlenmeyer flask. The free fatty acids and phospholipids were retained on the column.

Free fatty acids, however, could be eluted by 50 ml of 2% formic acid in ethyl ether, followed by 75 to 100 ml of ethyl ether.

A mixture of weighed tripalmitin, trilinolein, cholesterol and palmitic acid were taken as standards and the separation showed more than 95% recovery of the neutral lipid and the free fatty acids. Since this column separation was used as a method of purification of the neutral lipid from free fatty acids and phospholipids, no further attempt was made for the recovery of phospholipids which remained in the column.

Fractionation of the Neutral Lipids on Floricil

A number of methods have recently been developed for the separation of neutral lipid into its components. The three kinds of materials most commonly used for column separation of lipid classes are silicic acid, uricil and floricil. The method of Carroll (12) using floricil was found to be most suitable for the separation of neutral lipid isolated in our laboratory.

Floricil consists of hard, porous, white granules and has the following composition: magnesium oxide $15.5 \pm 0.5\%$, silicone dioxide $84.0\% \pm 0.5\%$ and sodium sulfate 0.5% . It has been noted by Carroll that the adsorption strength of floricil varied with the degree

of activation, and that this variation could affect the incomplete separation of sterol and di-glyceride fractions. The best separation could be achieved when floricil was deactivated by the addition of 7% by weight of water.

Twelve grams of floricil were added to 50 ml of hexane in a slurry to a column approximately 2 cm by 60 cm. The lipid to be separated was added to the column with a small volume of hexane. An automatic fraction collector was used and fractions of 6.5 ml were collected. The elution pattern was according to that of Carroll and is shown in Table 1.

The distribution of lipid in the collected fractions was determined gravimetrically after the lipid from the individual fraction was transferred to a tared planchet with a minimum amount of solvent. Weights were measured to 0.1 mg. The separation of neutral lipid on floricil was tested with a known mixture of squalene, cholesterol acetate, tripalmitin, trilinolein, cholesterol and monopalmitin. The lipid material from each fraction was recovered and identified by thin layer chromatography. The separation was satisfactory.

Separation of Neutral Lipid in the Fat Body

An experiment was designed for the analysis of neutral lipid components in the fat body and to compare the possible changes of

Table 1. Elution pattern of neutral lipid components on a floricil column as described by Carroll (12).

Class of eluent	Eluting solvent	ml
Hydrocarbon	Hexane	20
Sterol ester	5% ether in Hexane	50
Triglyceride	15% ether in Hexane	75
Free sterol	25% ether in Hexane	60
Diglyceride	50% ether in Hexane	60
Monoglyceride	2% methanol in ether	75
Free fatty acids	4% acetic acid in ether	75

these components with the age of the larvae. The amount of triglyceride present in the fat body was of special interest because it was found to be the major component of the adipose tissue.

Fat body lipid obtained from four different larval ages was subjected to a KOH-silicic acid column for the isolation of neutral lipid and free fatty acids. The percent neutral lipid ranged from 88.2 to 93.6 of the total for the four different larval ages under study as shown in Table 2. Free fatty acid fractions from these different larval ages were also collected. The amount represented in percentages is also shown in Table 2. The results showed that there was a small increase in neutral lipid with a corresponding decrease in free fatty acids toward the end of the larval stage. Free fatty acids decreased from 3% of the total lipid at 74 hours of age to about 1% at 100 hours. However, the 2% difference was in the range of experimental error.

Neutral lipid was separated into the following components from the floricil column: hydrocarbon, sterol esters, triglycerides, free sterols, diglycerides and monoglycerides. Figure 6 shows a typical pattern of separation of the neutral lipid obtained from the fat body of 72 hour old larvae.

Triglycerides constituted the major component of the neutral lipid and the percent composition of this fraction for several larval ages is shown in Table 6. In the 40 hour old larvae, the concentration

Table 2. KOH-silicic acid column separation of total lipid components from the fat body of Sarcophaga bullata larvae.

Age of larvae in hours	Percent neutral lipid	Percent free fatty acids
74	88.2	2.92
85	89.8	2.70
100	90.0	0.93
140	93.6	1.01

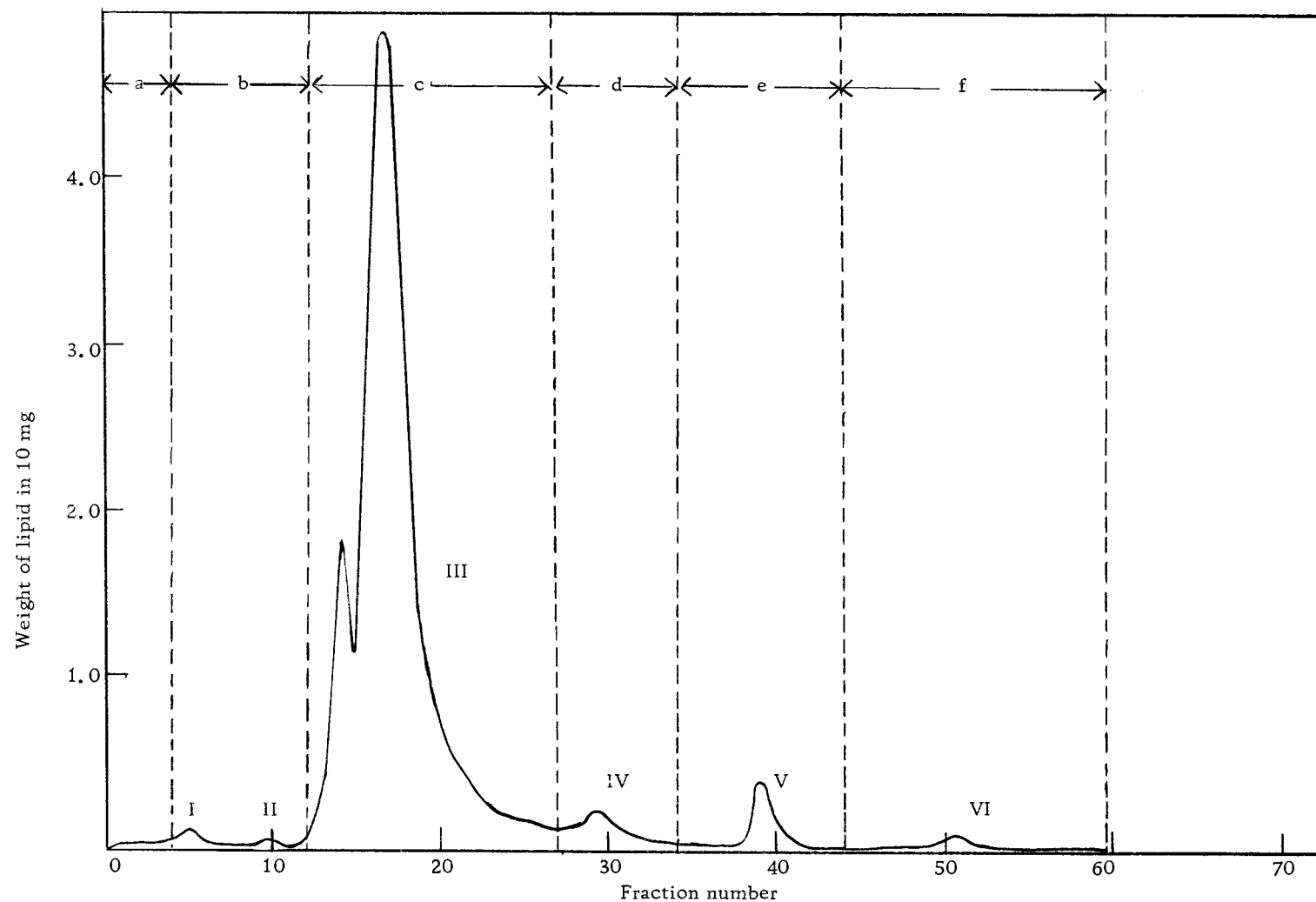


Figure 6. Column separation of neutral lipids from fat body of 3 day old larvae. 12 gm floricil was used for column material. Solvents for elution in increasing amount of ethyl ether in hexane were (a) 0% (b) 5% (c) 15% (d) 25% (e) 50% (f) 2% methanol in ether. Neutral lipid components in order of elution: I hydrocarbon, II sterol ester, III triglyceride, IV free sterol, V diglyceride, VI monoglyceride.

Table 3. Separation of neutral lipid components by floricil column.

No. of experi- ments	Age of larvae in hours	Percent distribution of neutral lipid components					
		hydro- carbons	sterol esters	triglycer- ides	free sterols	diglycer- ides	monoglycer- ides
1	40	1.04	1.37	85.0	2.41	5.83	4.03
2	67	< 1	< 1	92.8	< 2	< 4	< 2
3	72	< 1	< 1	90.1	< 2	< 4	< 2
4	90	< 1	< 1	91.2	< 2	< 4	< 2
5	114	< 1	< 1	94.0	< 2	< 4	< 2
6	216	< 1	< 1	94.8	< 2	< 4	< 2

of all the components except the triglyceride fraction amounted to 15% of the neutral lipid. The amounts of this lipid emerging from the floridil column were such as to make accurate weighing of these components impossible. The results indicated a slight increase in the percentage of triglyceride fraction of the fat body toward pupation. However, the small differences shown may also come within the realm of experimental error.

Analysis of Fatty Acids by Gas-Liquid Chromatography

The Methodology of Gas-Liquid Chromatography

Gas-liquid chromatography has proven to be a most efficient method in the separation and identification of fatty acids. Nogare (54) has given a thorough review of gas-liquid chromatography and Horning et al. (30, 31) have discussed the problems involved in quantitative and qualitative analysis of fatty acids by gas-liquid chromatography and the use of fatty acid standards.

In the first part of the gas-liquid chromatography analysis described here, a Beckman model GC-2 with a thermal conductivity detection system was used. Only semi-quantitative analyses were performed with this instrument. Later, an F & M model 700-12 with a hydrogen flame detector was employed to complete the quantitative determination.

The thermal conductivity detector system was less sensitive than the flame ionization detector, but it was more suitable when fractions were to be collected because thick film columns could be used. The load limit of the thermal conductivity detector was at least a hundred fold larger than the hydrogen flame detector.

With the Beckman gas chromatograph, the separation was very satisfactory on a 12' by $\frac{1}{4}$ " aluminum column packed with 14% DEGS (diethylene glycol succinate) on a solid support of chromosorb P. These polymers had relatively low thermal stability, but they were entirely satisfactory for use at temperatures around 200° C which is the usual operating temperature of the gas-liquid chromatography. With the hydrogen flame detector system in the F & M model, a 6' by $\frac{1}{8}$ " column packed with 15% DEGS coated on Chromosorb P also gave good separation.

Horning et al. (31) stated that satisfactory application of gas-liquid chromatography to fatty acids analysis depended on the precision with which temperatures were controlled around the column and detector, at the point of sample application, at the column head and also at the bridge between column and detector. Ideally the operating temperature should be held the same throughout the column length and the detector temperature should be a few degrees higher in order to minimize condensation of column residue material in the detector. With the Beckman gas-liquid chromatograph, preliminary

experiments showed that methyl esters of the long chain fatty acids could not be completely volatilized at the head of the column. Therefore, a flash heater was later connected at the column head to insure instantaneous volatilization of the sample injected.

Sample injection of the F & M model was according to the method devised by Lowry (43) in which a measured volume of the dissolved sample was fed into a specially designed spoon. The solvent was then allowed to dry in air and the sample was introduced into the gas chromatograph in solid form. The operating conditions of the two detector systems are shown in Table 4.

Qualitative Identification of the Fatty Acids of the Fat Body

Identification of fatty acid peaks from the gas chromatogram was done by means of two methods. The first method was based on the use of known methyl ester standards. Individual peaks were identified by matching retention time with that of the known compounds and by coding the uncertain peaks with the standards. However, with the complex mixture of fatty acids occurring in the fat body, it was not possible to identify all the peaks simply by coding with the limited kinds of standards available. The second method of identification was based on the relationship between the semi-log retention time and carbon number (75). The retention time of each

Table 4. Operating conditions and column specifications of the Beckman GC-2 and F & M 700-12 gas-liquid chromatographs.

Operating conditions and column specifications	Beckman GC-2	F & M 700-12
Type of detector	thermal conductivity	hydrogen flame ionization
Column length	12 ft	6 ft
Column I. D.	1/4 in	1/8 in
Coating material	diethylene glycol succinate	ethylene glycol succinate
Wt % of coating material	14%	15%
Solid support	Chromosorb P	Chromosorb P
Mesh	60/80	60/80
Carrier gas	Helium	Helium
Inlet pressure	50 psi	20 psi
Column temperature	220° C	190° C
Sample size	1-3 μ l	0.03 μ l
Chart speed	.20 in/min	0.20 in/min

fatty acid methyl ester appearing in the chromatograph was a function of its molecular weight. When these were plotted on semi-log graph paper, i. e., semi-log retention time versus carbon number of the fatty acids, a straight line joining all of the saturated fatty acids would be constructed. A value corresponding to the retention time of any other peak can be read from the graph and can give the chain length of the hypothetical ester. The retention times of the mono-unsaturated acids and the polyunsaturated ones would describe linear functions parallel to the saturated one. Hence by plotting graphs from the known peaks in the chromatogram, the unknown peaks might be deduced. This method, however, was limited somewhat due to the fact that retention time of the same material might vary with each experiment depending upon the size and composition of the sample to be analyzed. Actually one must be very cautious in regard to the practice of identifying specific fatty acids through gas-liquid chromatography alone. Analysis by this method can indicate only tentatively what structures can be assigned to individual peaks. Hydroxy and branched-chain fatty acids and other components such as hydrocarbons, alcohols, acetals etc., may prove to be very confusing indeed for they may have very similar retention times as the methyl esters of corresponding chain length. Preparation of very pure samples helps to eliminate part of this problem. Identification through different lengths of column and under different operating

conditions also helps to eliminate part of the error.

Figure 7 showed a gas chromatogram of a qualitative mixture which contained methyl esters of saturated fatty chain lengths C_6 , C_8 , C_{10} , C_{12} , C_{14} , C_{16} , C_{18} , C_{20} , and C_{22} . The chromatogram indicated a satisfactory separation between all the components in the mixture. Methyl esters of very long chain lengths were represented by peaks of very broad base lines, and identification becomes more difficult when chain length increased. Figure 8 shows a typical gas-liquid chromatogram of the fatty acid methyl esters isolated from fat body of Sarcophaga bullata. It is evident that the composition of fatty acids found in the insect lipid is very different from the standard mixture shown in Figure 7. Most of the fatty acids in the fat body lipid were of the palmityl and stearyl series with a high degree of unsaturation. The presence of negligible amount of short chain fatty acids is also evident from this chromatogram. Therefore, more standard mixtures were obtained for the identification. The three groups of standard mixtures were I: C_6 , C_8 , C_9 , C_{10} , C_{11} ; II: C_{15} , C_{16} , C_{17} , C_{18} , C_{19} ; III: C_{18} , $C_{18:2}$, $C_{18:3}$. They were separately coded to the insect sample, and identification of fatty acid methyl esters was done by comparing the retention time and increase in peak height of the insect sample with the standards.

An example of the gas-liquid chromatogram showing fatty acid methyl esters of insect fat body mixed with a standard mixture

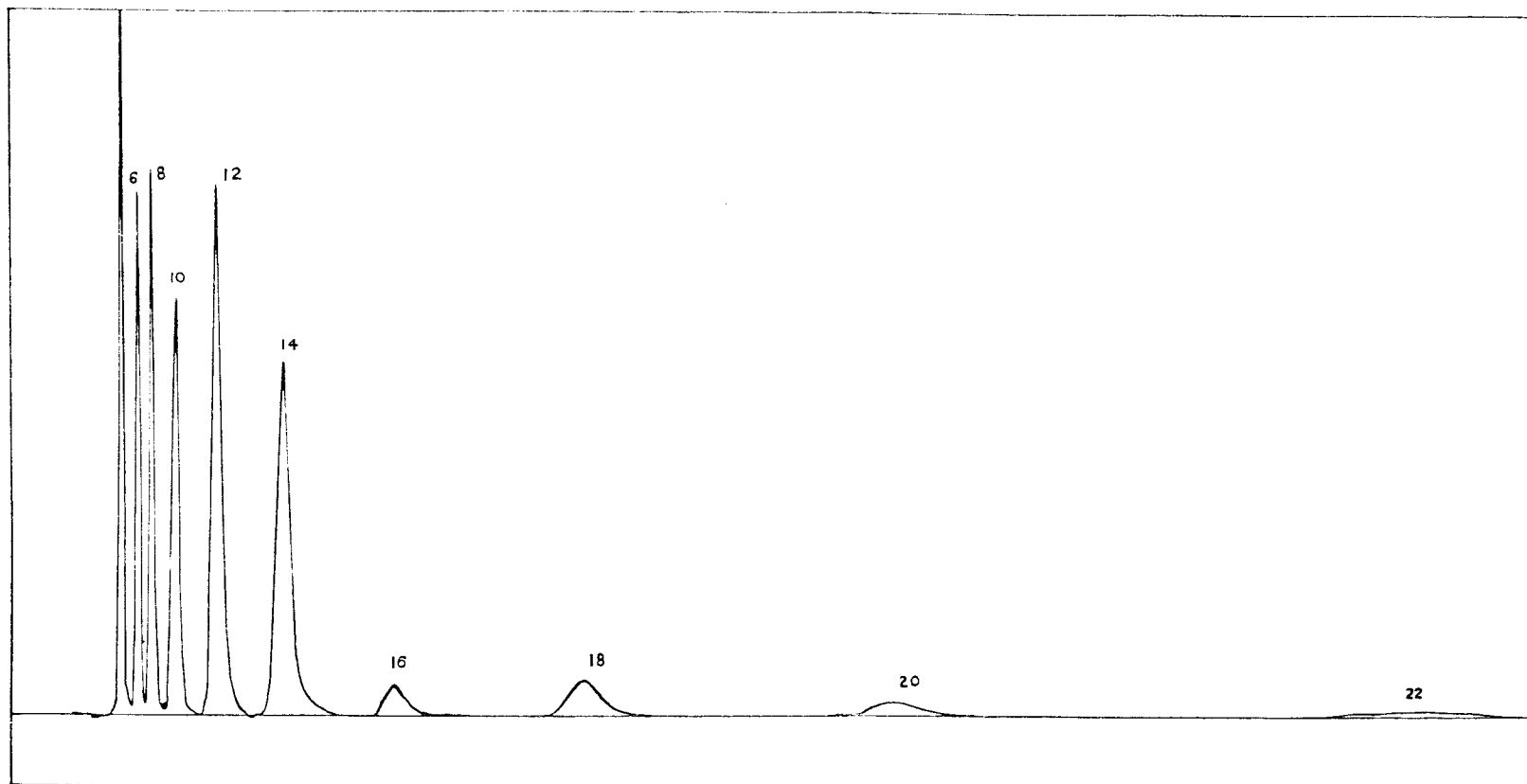


Figure 7. Gas-liquid chromatographic separation of fatty acid standards: C_6 , C_8 , C_{10} , C_{12} , C_{14} , C_{16} , C_{18} , C_{20} and C_{22} . Operating conditions of all gas-liquid-chromatographic separations by the Beckman model are described in Table 4.

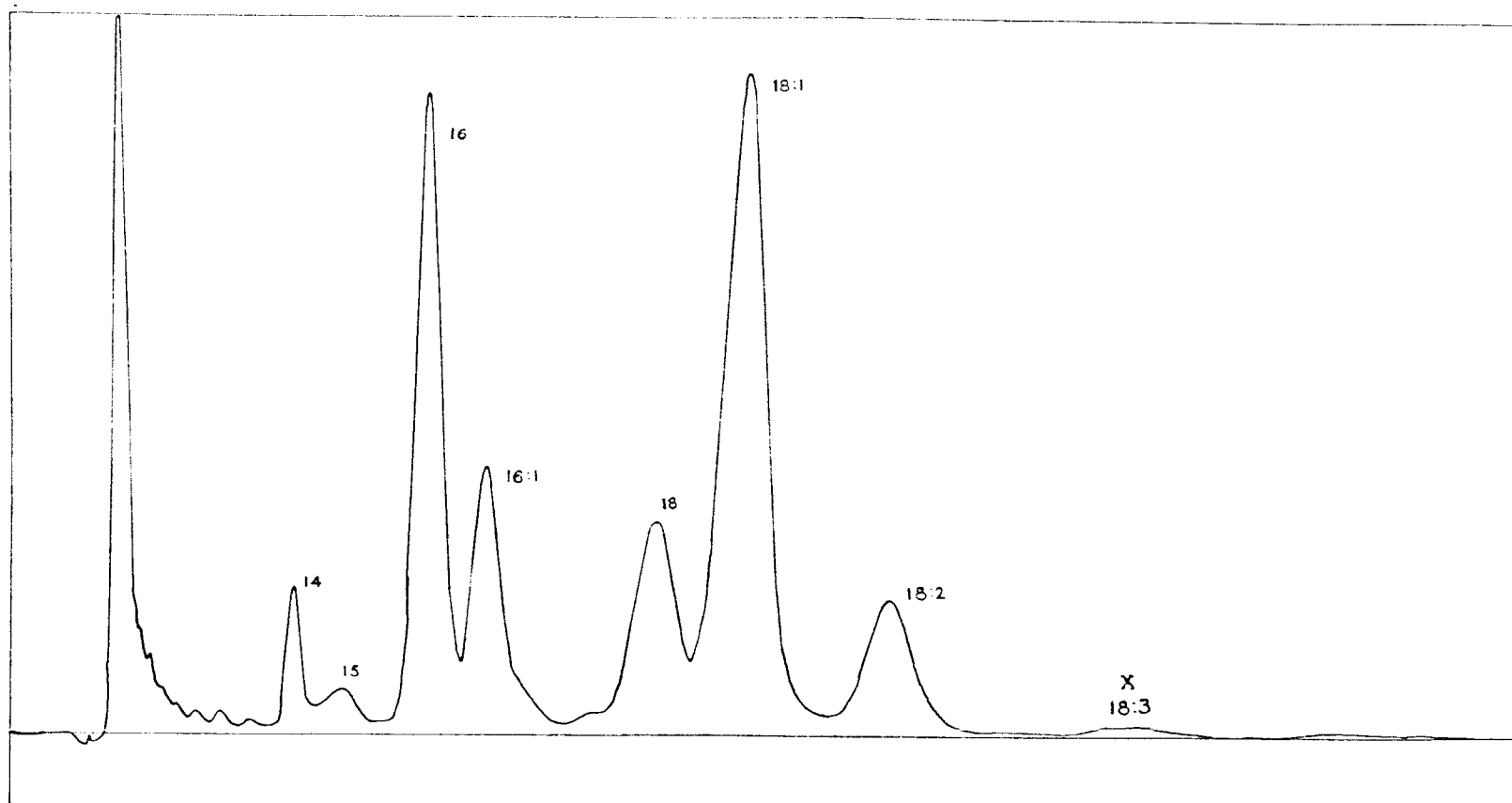


Figure 8. Gas-liquid chromatographic separation of lipid extracted from fat body of Sarcophaga bullata larvae. Operating conditions of all gas-liquid chromatographic separations are described in Table 4.

containing C_{15} , C_{16} , C_{17} , C_{18} , and C_{19} fatty acids is shown in Figure 9. From this chromatogram, the presence of C_{15} and C_{17} fatty acids could be assured. Also the peak which appeared after oleic acid in the chromatogram of the insect fat body was found to be different from the C_{19} standard indicating the absence of C_{19} fatty acid. Using the other groups of standard mixtures, this peak was confirmed to be linoleic acid. Linolenic acid was also present in small amount. In like fashion, the fatty acid composition in the fat body was identified to contain the following major components: C_{14} , C_{15} (trace), C_{16} , $C_{16:1}$, C_{17} (trace), $C_{16:2}$ (trace), C_{18} , $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$. Fatty acids which may be present but have retention values longer than that of linolenic acid were not resolved by this gas-liquid chromatograph and therefore were not observed. Fatty acids with chain length shorter than C_{14} were only present in trace amounts and were therefore not taken into account.

Identification of fatty acids by plotting retention time versus carbon number on a semi-log paper can only serve as a supplementary support to confirm some of the uncertain compounds. The graph in Figure 10 shows the linear relationship given by the standards, C_6 through C_{22} , whose chromatogram is shown in Figure 7. Results obtained from the insect system were plotted in Figure 11. The two lines parallel to the first one indicated the presence of mono- and polyunsaturated fatty acids.

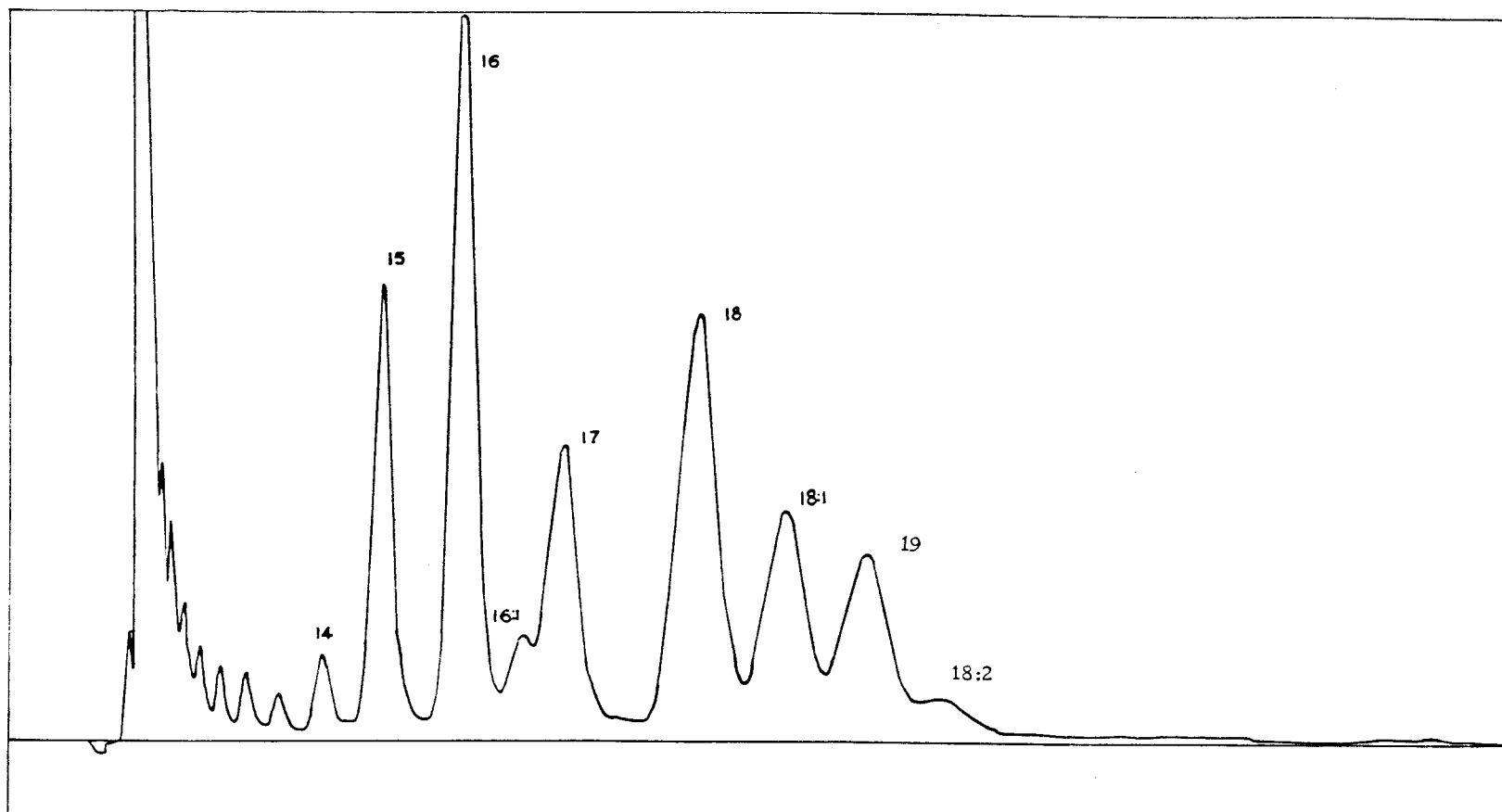


Figure 9. Gas-liquid chromatographic separation of fat body lipid from *Sarcophaga bullata* larvae coded with qualitative standards, C_{15} , C_{16} , C_{17} , C_{18} and C_{19} . Operating conditions of gas-liquid-chromatographic separations are described in Table 4.

Some of the fatty acids that were confirmed by this method were palmitlinoleic acid, heptadecanoic and pentadecanoic acids.

It appeared from the above results that unsaturated fatty acids comprised a large part of the total composition of the fat body. Portions of the samples were hydrogenated with a low pressure hydrogenation apparatus using platinum as catalyst in order to confirm the identification of these unsaturated fatty acids. The hydrogenated methyl esters were chromatographed as shown in Figure 12. The hydrogenated chromatogram showed an increase in the palmitic and stearic acid content with the relative disappearance of palmitoleic, oleic, linoleic and linolenic acids. Also the presence of arachidic and behenic acids became more evident after hydrogenation, indicating that trace amounts of the unsaturated derivatives of arachidic and behenic acids must be present in the fat body. However, on the contrary, the absence of C_{19} fatty acid after hydrogenation indicates the absence of unsaturated fatty acids with 19 carbon atoms.

Later an F & M gas-liquid chromatograph was available for more detailed identification and quantitative determination. Figure 13 showed a typical separation of the fatty acid methyl esters by the F & M chromatograph. The chromatogram showed a similar distribution pattern when compared to the ones produced by the Beckman instrument. Consequently, with a hundred fold more

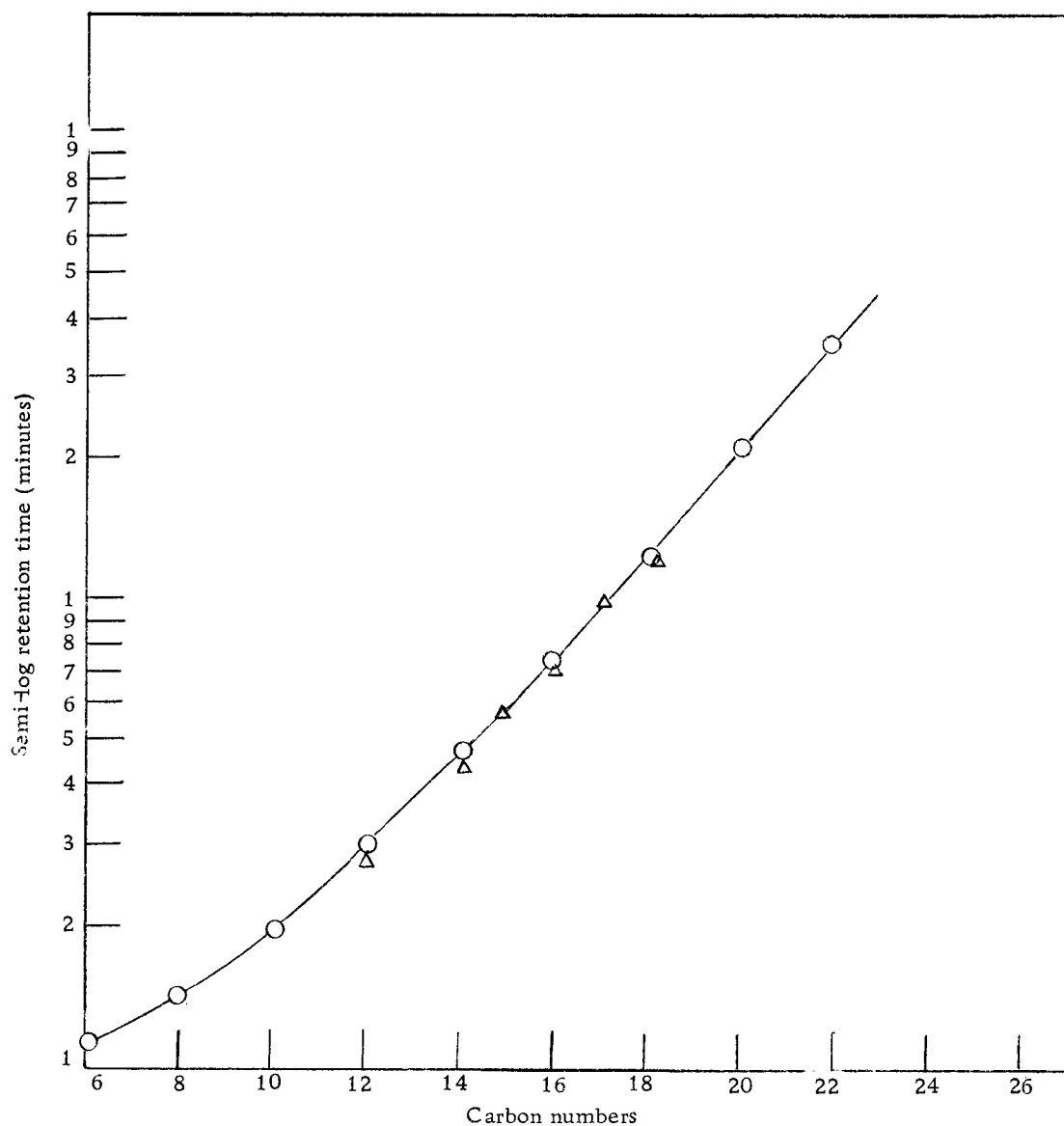


Figure 10. Identification of long chain fat acids by graphic method.

- Fatty acid standards C_6 , C_8 , C_{10} , C_{12} , C_{14} , C_{16} , C_{18} , C_{20} , C_{22} which correspond to gas-liquid chromatography in Figure 7
- △ Hydrogenated fatty acids from fat body of Sarcophaga bullata larvae which correspond to gas-liquid chromatography in Figure 9

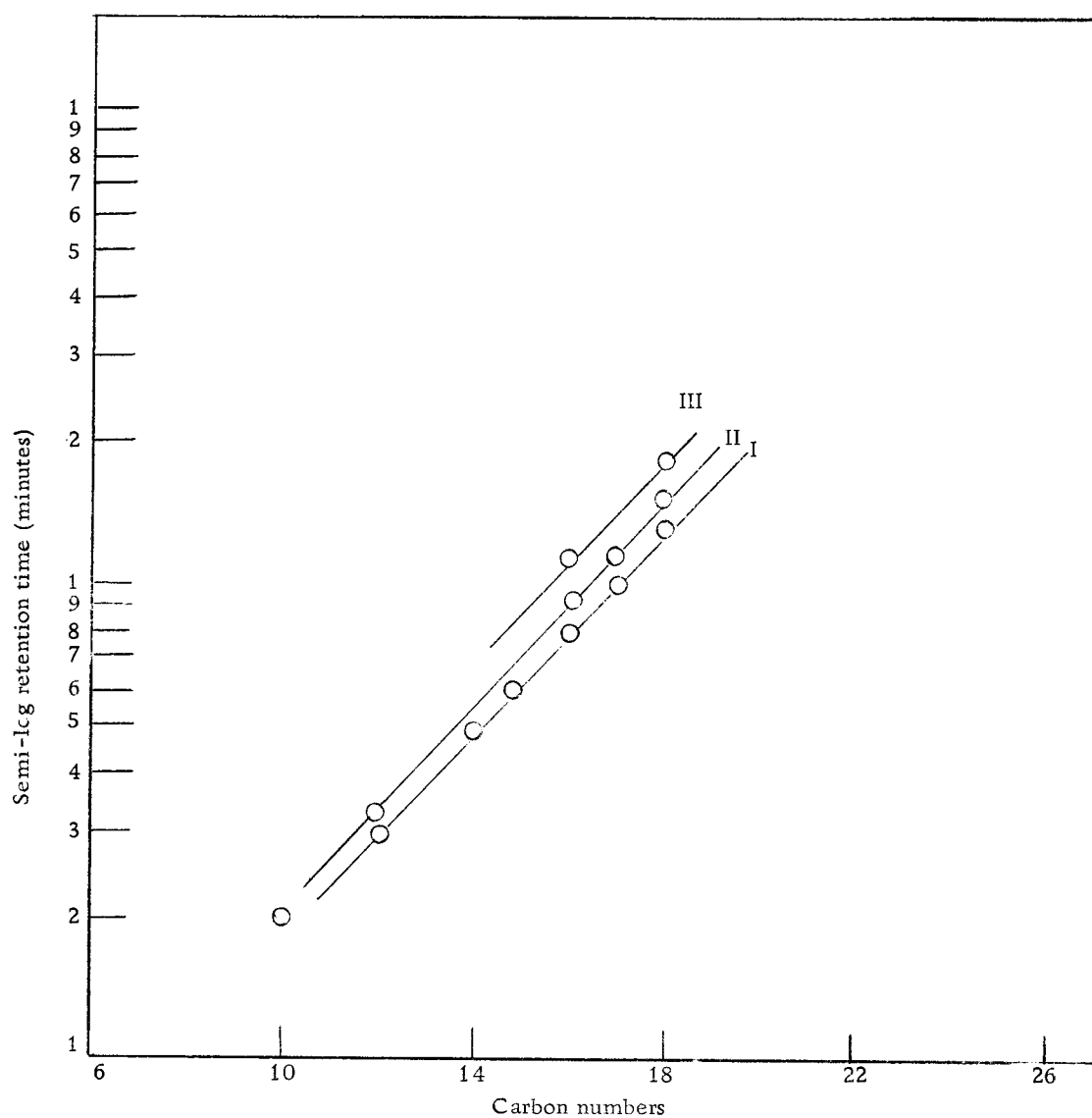


Figure 11. Graphic identification of fatty acid methyl esters from fat body of *Sarcophaga bullata* larvae.

- I Saturated fatty acids
- II Mono-unsaturated fatty acids
- III Di-unsaturated fatty acids

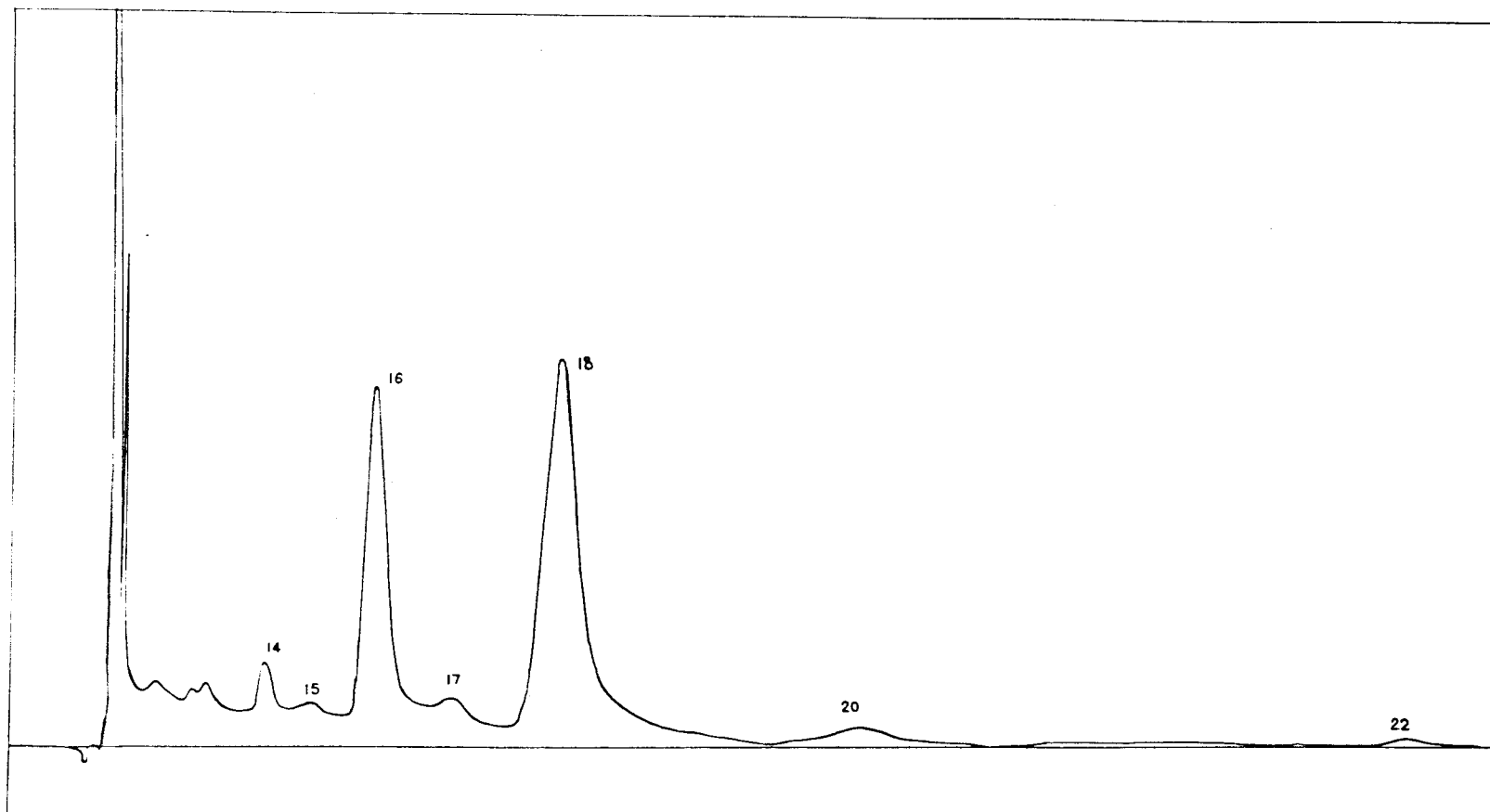


Figure 12. Gas-liquid chromatographic separation of hydrogenated fatty acid methyl esters from fat body of Sarcophaga bullata larvae. Operating conditions of gas-liquid-chromatographic separations are described in Table 4.

sensitive detector, a much better resolution of the peaks was obtained. For example, the C_{17} and $C_{16:2}$ peaks which were not resolved by the Beckman instrument became two distinct peaks in this chromatogram. Also, the peak corresponding to linolenic acid was more clearly defined in this chromatogram. Many small peaks corresponding to a very long retention time were also observed. Therefore, more standards were obtained in an attempt to identify some of these long chain fatty compounds. With the help of the graphic method and the limited number of standards available, these peaks were tentatively identified as I: $C_{20:1}$, II: $C_{20:3}$, III: $C_{20:4}$, IV: C_{22} , V: $C_{22:1}$ in order of appearance as indicated in Figure 13.

The larvae of Sarcophaga bullata were reared on beef heart or beef liver. However, another species of fly larvae was also reared in our laboratory. Phormia regina can be reared aseptically on a synthetic caesin diet (48).

Fatty acids from Phormia regina were also chromatographed and the differences in the fatty acid pattern existing between these two closely related species were compared. The chromatogram of the Phormia regina fat body lipid is shown in Figure 14. The fatty acid patterns of the two closely related species were similar in certain respects--in spite of the differences in diet composition. Both species have large amounts of palmitic, palmitoleic, oleic, and stearic acid. Phormia, however, have relatively small amount of

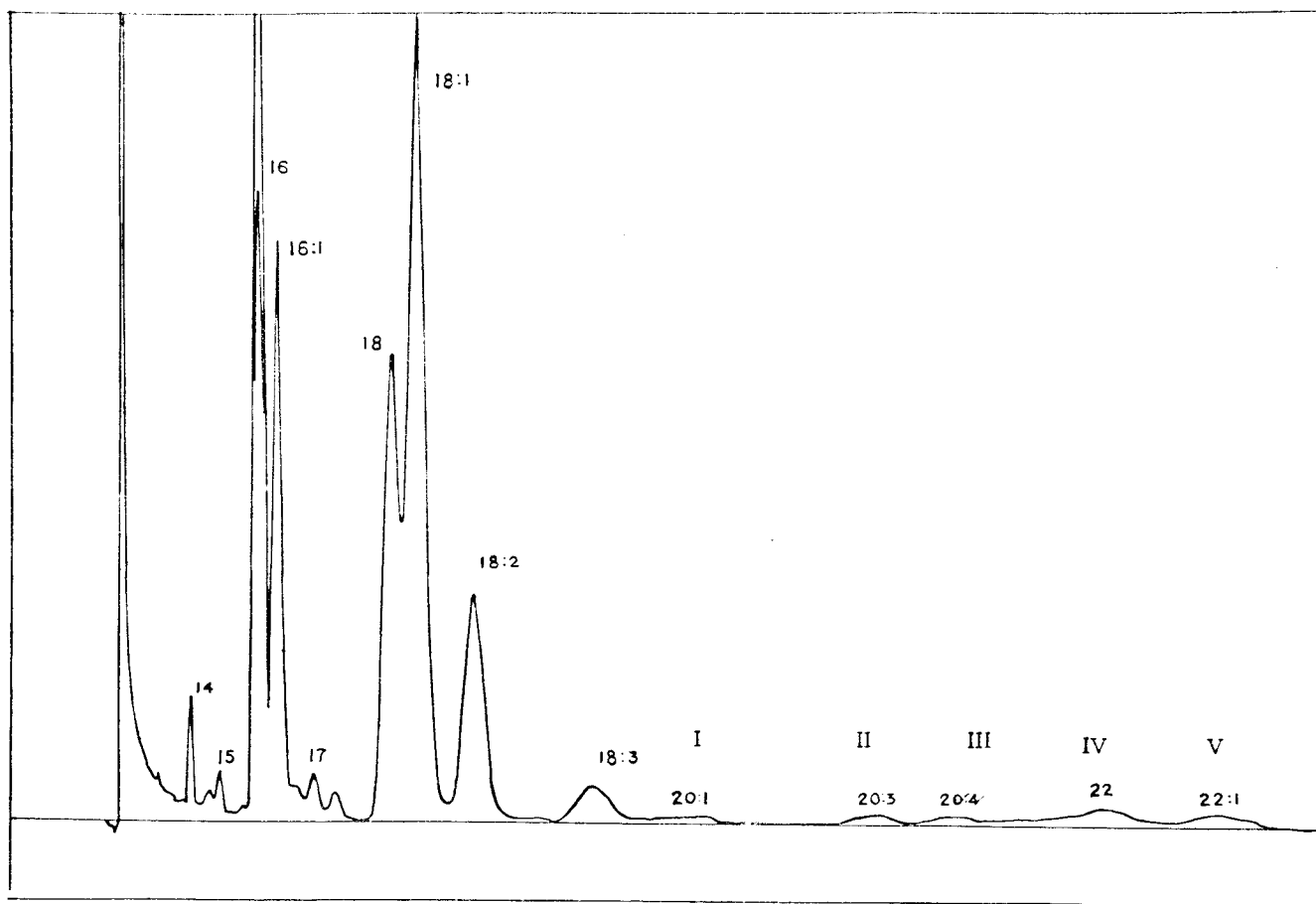


Figure 13. Gas-liquid chromatographic separation of lipid extracted from fat body of *Sarcophaga bullata* larvae using a hydrogen flame detector. Operation conditions of the F & M gas-liquid chromatograph are described in Table 4.

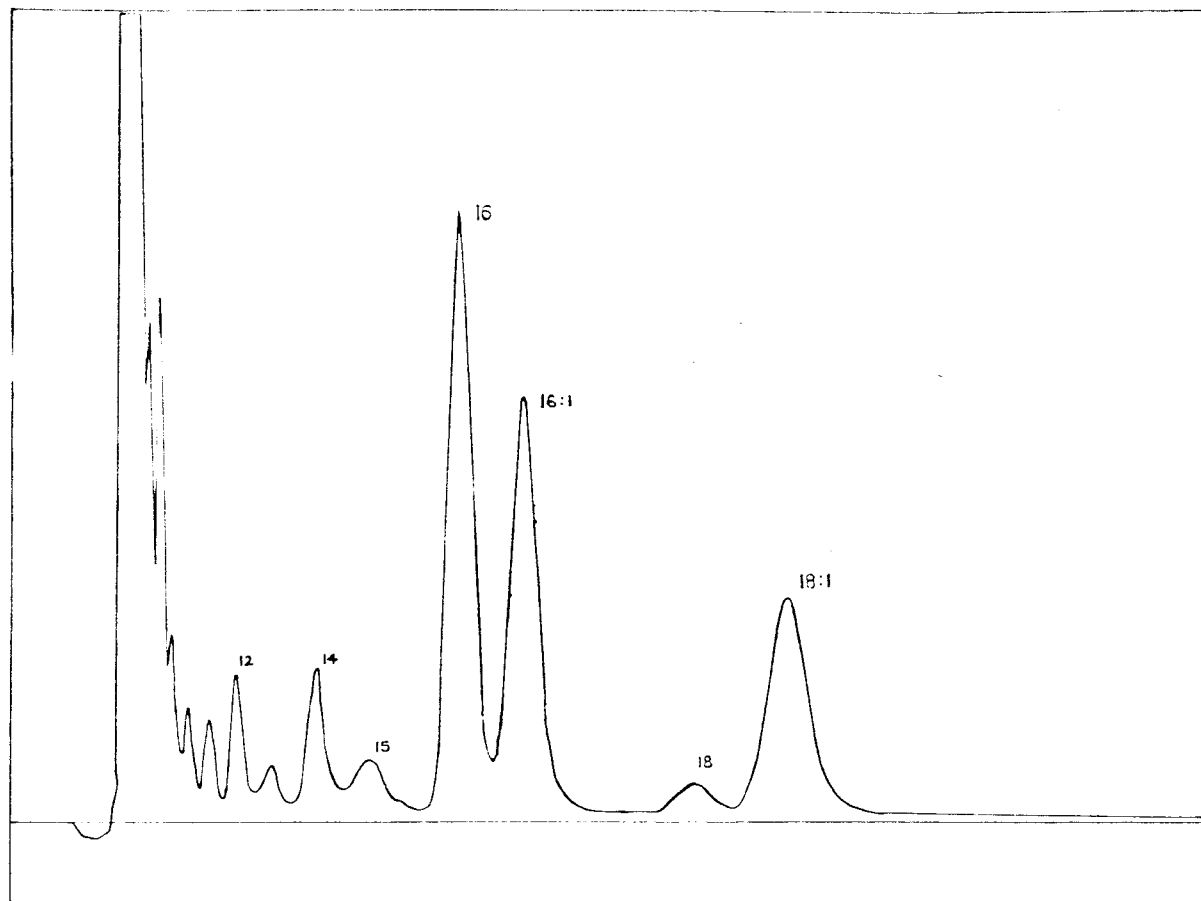


Figure 14. Gas-liquid chromatographic separation of fatty acid methyl esters from fat body of Phormia regina larvae. Operating conditions of gas-liquid-chromatographic separations are described in Table 4.

linoleic acid which is a prominent fatty acid in the fat body of Sarcophaga bullata. Linoleic acid is in fact not observed in the Phormia fat body, but the species appears to have relatively higher amounts of fatty acids with chain length shorter than C_{14} .

Quantitative Determination of Fatty Acids of the Fat Body

Percentage composition calculations are carried out commonly. For this purpose, it is necessary to obtain an area measurement associated with each component. Many different procedures have been proposed for area calculations. Methods in use include: (a) multiplication of peak height by width at half-height, (b) multiplication of peak height by one-half base width, (c) multiplication of peak height by adjusted retention time, (d) measurement of peak area by planimetry, (e) measurement of peak area by weighing the cut out peak and (f) measurement of peak area by integrations. When employing the first four methods, one must recognize that serious errors may be introduced in measuring the widths and heights of very narrow or very broad peaks.

The two methods employed here for the calculation of percentage compositions are (1) multiplication of peak height by adjusted retention time, and (2) the integrator method when using the Beckman GC-2 model. The first method was initially proposed by

Bartlet and Smith (5) and modified by Carroll (11). It is based on the evidence that the peaks obtained with a well designed chromatograph can be closely adapted to a normal or Gaussian distribution curve. The area under the curve may be calculated by multiplying the peak height and a standard deviation which is a linear function of the retention time. In applying this method to peaks which are only partially resolved, Bartlet and Smith noted that the measured peak height will be higher than the true peak height by amounts which are determined by the degree of overlap and by the relative amounts of the components. It is possible to make a mathematical correction; however, in practice, the correction factors are very small, and are thus neglected.

Quantitative standard mixtures were used to determine the percentage composition of the fatty acids present in the fat bodies of Sarcophaga bullata. Gas-liquid chromatography involved in the following experiments was with an F & M model with a hydrogen flame ionization detector which has a hundred fold higher sensitivity than the thermal conductivity detector. Sample sizes were thus reduced from the usual 3 μ l to 0.01 to 0.03 μ l. The peak area determination was according to the method given by Bartlet and Smith (5). Three groups of quantitative standards were tested on the chromatograph and they showed satisfactory separation. The percent composition obtained from these chromatograms was compared with

the standard values given by the company from which these standards were obtained. The comparison is shown in Table 5. It is evident that some of the results deviate somewhat from the standard values. The shorter chain length methyl esters tended to have a peak area less than the standard value and deviation increased as the chain length decreased. However, deviation became very small among the longer chain length fatty acids such as palmitic and stearic acids.

The percent distribution of fatty acids from three day old larval fat body is shown in Table 6. The percent composition of the major components is as follows: 21.1% palmitic, 11.4% palmitoleic, 16.0% stearic, 30.2% oleic and 10.1% linoleic. Oleic acid occupied one-third of the total composition and among these major components, the three unsaturated fatty acids, palmitoleic, oleic, and linoleic comprised 51.6% of the total composition. The arachidic and behenic acids comprised about 5% of the total composition. An experiment was performed in which the fatty acid distribution was compared between the total lipid, neutral lipid and triglyceride fraction. Neutral lipid was obtained by fractionating the total lipid through a KOH-silicic acid column. Portions of the neutral lipid were further separated through a floricil column in order to isolate the triglyceride fraction. The lipid obtained was saponified, neutralized and methylated for gas-liquid chromatographic analysis.

Table 5. Percent composition of quantitative standard mixtures obtained by peak area method.

Carbon no.	Percent composition of standards					
	Group I		Group II		Group III	
	1 ^a	2 ^b	1	2	1	2
8:0	1.5	0.4				
10:0	3.0	1.7				
12:0	6.0	4.7				
14:0	12.0	11.2	11.8	10.5		
16:0	19.4	20.1	23.6	22.0		
16:1			6.9	6.7		
17:0					10.0	9.7
18:0	24.9	27.0	13.1	14.2	9.8	10.4
18:1			44.6	45.7		
19:0					20.0	20.3
20:0	33.2	34.6			35.0	35.9
21:0					25.1	23.8

^a Standard percent composition obtained from Applied Science Science Laboratories, Incorporated.

^b Percent composition obtained by peak area determination.

Table 6. Percent composition of fatty acids from insect fat body analyzed by gas-liquid chromatography.

54

Carbon no.	Three day old larvae			Nine day old larvae		
	Total lipid	Neutral lipid	Triglyceride fraction	Total lipid	Neutral lipid	Triglyceride fraction
C ₁₄	1.01	1.02	1.01	0.81	0.85	1.05
C _{14:1}	0.27	0.21	0.22	0.23	0.25	0.30
C ₁₅	0.60	0.53	0.50	0.43	0.46	0.46
C ₁₆	21.12	21.13	20.14	15.40	15.87	20.80
C _{16:1}	11.42	11.59	11.60	13.77	14.94	16.10
C ₁₇	1.14	1.14	0.99	0.74	0.83	1.18
C _{16:2}	0.72	0.74	0.82	1.41	1.29	1.20
C ₁₈	16.03	16.64	15.36	9.30	9.59	13.50
C _{18:1}	30.21	30.31	28.61	32.00	33.83	37.70
C _{18:2}	10.13	10.97	11.86	13.56	13.65	5.97
C _x ^a	-	0.40	0.61	1.27	1.04	1.71
C _{18:3}	2.00	1.86	0.78	1.07	1.26	
C _{20:1}	-	-	-	0.62	0.74	
C _{20:}	0.90	1.30	2.45	1.17	0.90	
C _{20:}	0.78	-	3.78	4.19	1.99	
C ₂₂	2.10	0.99	-	-	-	2.48
C _{22:1}	1.49	1.09	1.19	4.28	2.48	

Gas-liquid chromatography were performed with F & M hydrogen flame detector.

^aC_x is unidentified peak.

This experiment was repeated with the three day old and nine day old larvae. The results obtained from this experiment are shown in Table 6 also.

A comparison between the percent fatty acid composition of total lipid, neutral lipid and triglyceride fraction showed that the over-all percent composition was very much the same. However, a comparison between the three day old and the nine day old larval fatty acids showed an increase in the degree unsaturation and a corresponding decrease in saturated fatty acids. In the fat body of the three day old larvae, 51.6% of the fatty acids which comprised the lipid was found to be unsaturated. These unsaturated fatty acids comprised 60% of the total in the nine day old larvae. The neutral lipid and the triglycerides also reflect similar changes.

DISCUSSION

The larvae of Sarcophaga bullata grew rapidly between 40 and 70 hours. During this period weight increased from less than 80 mg per larva to 200 mg or more in a rather linear fashion. The weight of the larva attained a maximum after 70 hours of age and then there was a small decrease for the next ten hours or so. It was observed that the larvae stopped consuming food on about the third day and started to migrate to a dryer place for pupation. Pupation, however, did not occur until after the sixth day depending upon the condition of the environment.

The results were in accord with the ones given by Pearincott (55) who reported that the larvae of Musca domestica increased in weight to an average of 19.6 mg in the fifth day, then decreased to 13.4 mg in the sixth day, and 12.3 mg immediately following puparium formation. The decrease of the larval body weight shown in Sarcophaga bullata as well as the other species may be due to a loss in water content. This explanation is supported by many other workers who also measured the moisture content in insects. Rainey (56) showed that the moisture content of the sheep blowfly larvae decreased 3% from hatching to the middle of the third instar, and decreased as much as 10% during the prepupal stage. The author suggested that this final rapid fall in moisture content and hence,

decrease in body weight was probably associated with the fact that after feeding ceased the larvae left the meat and migrated to a much drier surrounding for pupation.

Fast (23) cited evidence indicating that most holometabolous insects accumulated large quantities of fat during the larval stage and accumulation proceeded at a somewhat higher rate than that of other body materials. He also made a survey of the lipid content of some 100 insects and claimed that 75% of these insects contained less than ten percent wet weight as lipids. The results on lipid content of Sarcophaga larvae were in general agreement with his; a total of 6.4% lipid was found in Sarcophaga bullata larvae.

Levinson and Silverman (42) found that the lipid content of the housefly Musca vicina dropped one third of the maximum weight within 24 hours of the onset of pupation. Lipid content of Sarcophaga bullata, however, continued to rise after 72 hours of age and prior to the puparium stage. Fat synthesis in Bombyx mori and Pieris napi followed still a different pattern. Average fat content in these two species decreased steadily during the first two instars, then remained almost constant during the II, III, IV and part of the V instar. In the middle of the fifth instar lipid content increased sharply so that in the early pupae the relative fat content was two or even three times that of instar IV. The increase in lipid content during the non-feeding prepupal stage was explained by Strogaya (53)

as the result of internal reorganization of dry matter. This explanation may also be applicable to Sarcophaga bullata larvae but it is more likely that this increase in lipid content is brought about by assimilation of the food material which remains in the crop after feeding has ceased.

Neutral lipid constituted nearly 90% of the total lipid in the fat body. Free fatty acids, on the other hand, comprised less than 3% of the total. No apparent change was observed in the neutral lipid and free fatty acid composition up to 100 hours of larval age.

Separation by floricil column showed that 90% of the neutral lipid was made up of triglycerides, while diglyceride and monoglyceride fractions together occupied only 5%. There was a very small amount of sterol and sterol esters in the neutral lipid, a total of less than 3%.

The analysis of fatty acid composition of Sarcophaga bullata larval fat body showed that this species contained a high percentage of unsaturated fatty acids. The major components of the fatty acids were C_{16} , $C_{16:1}$, C_{18} , $C_{18:1}$ and $C_{18:2}$. There were small amounts of C_{14} , C_{15} , C_{17} , $C_{16:2}$, $C_{18:3}$ and some with chain lengths longer than 20 carbon atoms. The two odd carbon chain fatty acids, C_{15} and C_{17} , although only present in trace amounts, were found in all analyses made. Hydrogenation of the insect lipid sample helped to confirm the position of the unsaturated fatty acids

in the chromatogram. The appearance of arachidic and behenic acids after hydrogenation indicated that trace amounts of C_{20} and C_{22} unsaturated fatty acids were present but they were not detected by the chromatograph. These were observed in the hydrogen flame detector and identified as $C_{20:1}$, $C_{20:3}$, $C_{20:4}$, C_{22} , and $C_{22:1}$ fatty acids in the later studies. Fifty-five percent of the total fatty acids were unsaturated. The major fatty acids were found to be oleic, linoleic, palmitoleic, palmitic and stearic comprising 30, 10, 11, 20 and 10% of the total respectively.

Two-thirds of the insect species listed in Fast's review showed unsaturation amounting to 60-80% of the total fatty acids, indicating that high degree of unsaturation may be a characteristic of the insects (23).

Wigglesworth (74) reported that when the mosquito larvae were fed on casein alone, the reserves in the fat body and elsewhere did not differ from those formed in larvae feeding on the normal diet of micro-organisms. Here, in spite of the diet difference between the Phormia regina and Sarcophaga bullata, the pattern of fatty acid distribution of these two closely related species was quite similar.

The data on fatty acid composition of the beef liver and beef heart is collected in Table 7. A comparison of the fatty acid distribution in the diet food and the insect shows similarity in that both are highly unsaturated and both contain around 10% palmitoleic and

Table 7. Fatty acids of ox liver and ox heart muscle.

	Source of animal organs	% Saturated fatty acids				% Unsaturated fatty acids				
		C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂
1 ^a	ox liver glycerides	--	25	20	--	--	9	37 (-2.6 H) ^c	8 (-5.0 H)	1 (-7.5 H)
2 ^b	ox liver glycerides	1.4	30.4	6.6	--	1.1	9.9	40.3 (-8.0 H)	8.5 (-6.9 H)	1.8 (-6.9 H)
3 ^a	ox heart muscle neutral lipid	--	22	20	--	--	1.2	45	--	--

^aAnalysis obtained from Klenk, F. and O. V. Schoenebeck (40).

^bAnalysis obtained from Hilditch and Shorland (29).

^cProbable amount of unsaturation.

around 20 to 25% palmitic acids. However, although stearic acid represented 20% of the fatty acids in the diet food, it comprised only 10% of the fatty acids of the insect fat body. Similarities and differences indicate that insects in the larval as well as mature state lay down fats very similar in type to those produced by mammals and that they assimilate fats present in their diet as well as synthesize them from the constituents of food (23). Sarcophaga bullata proved to be another example in this respect.

The results of the experiment analyzing the percent composition of the total lipid, neutral lipid and the triglyceride fractions indicated that all three fractions had a very similar fatty acid content. Age and stage of development are also known to influence the composition of fats of some insects. The effects of age and stage were found to cause little change in the general composition of the fats of Agria affinis and Galleria mellonella (23). On the other hand, extensive change was observed in fatty acid composition during the spinning and prepupal periods of the silkworm Bombyx mori. The relative quantities of stearic and linoleic acids decreased sharply with a concurrent two-fold increase in the relative quantity of palmitic acid (23). In Sarcophaga bullata larvae, small increase in unsaturation was found towards the prepupal stage. A similar change was also observed in the neutral lipid and triglyceride fractions. Evans (22) found that for Lucilia, a related species, the amount of

fat was greatly decreased during metamorphosis and that the loss was chiefly from the more reactive unsaturated acids while the amount of saturated acids remained nearly constant. The increase in unsaturation during prepupal stage of Sarcophaga bullata may be a preparative step towards metamorphosis.

PART TWO : INCORPORATION OF PALMITATE-1-C¹⁴
INTO LARVAL FAT BODY OF SARCOPHAGA BULLATA

Incubation of Fat Body of *Sarcophaga bullata*
Larvae with Palmitate-1-C¹⁴

The object of the experiments in this section was to observe the metabolism of palmitate-1-C¹⁴ in the intact fat body of *Sarcophaga bullata* larvae. The experimental animals used in most of these experiments were either three days or seven days old unless stated otherwise. The larvae were reared by the conventional method as described on page 16. Fat body was taken from the larvae carefully so that it would remain intact for the rest of the experiment. The fat body was then placed into a 5 ml beaker containing 3 ml of 0.05 M potassium phosphate-Ringer solution. Fat bodies from ten larvae were grouped into each flask in order to allow uniformity in the amount of material used in each incubation flask.

The incubation flasks were of the type with a center well and the mouths of the flasks were fitted with rubber serum stoppers through which a syringe needle could be inserted. One ml of the 0.05 M phosphate-Ringer solution (0.1 M sodium phosphate diluted with one volume of insect Ringer solution and adjusted to pH 7.0), fat body from ten larvae and 0.1 ml of sodium palmitate-1-C¹⁴ containing 0.078 μ mole (1 μ curie) were placed into each reaction flask.

In the experiment where the metabolism of palmitate was compared to that of the acetate, 0.1 ml of sodium acetate- 1-C^{14} containing 1.27 μmoles (1.14 μcuries) was used instead. Incubation medium was placed in the outer well of the incubation flask. The flasks were then placed in a Dubnoff constant-temperature water bath and incubation was carried out for the desired time interval at 30°C with shaking.

In experiments involving the collection of C^{14}O_2 , 1 ml of 1 N NaOH was injected into the center well after incubation. The flasks were then incubated for 10 more minutes in order to convert the CO_2 in the reaction flasks to Na_2CO_3 . The flasks were then taken out of the water bath and the center well solutions transferred to graduated test tubes with several rinsings of water. Aliquots were taken for the measurement of radioactivity.

After incubation, the fat bodies remaining in the incubation flasks were filtered and washed repeatedly with cold phosphate-Ringer solution in order to remove the exogenous labelled material that was attached to the surface of the fat body. Total lipid from the fat body was extracted with chloroform-methanol (2:1 v/v) following the same extraction and purification procedure as described on page 18.

A KOH-silicic acid column as described on page 22 was used for the separation of lipid extracted from the incubated fat body.

The neutral lipid fraction was then separated into its components by a floricil column. One mililiter aliquot from each fraction was used for the measurement of radioactivity. The remaining solvent in each test tube was transferred to a pre-weighed planchet and the weight of the lipid material was obtained after evaporation of the solvent. In many experiments, where weighing information of each individual fraction was not necessary, the fractions were combined and a gross weight was obtained for each lipid class.

Measurement of Radioactivity

Aliquots of radioactive lipid material dissolved in ether or hexane were placed in scintillation vials containing 15 ml of scintillation liquid. Scintillation liquid for the counting of organic solvents was made up of 6 gm of PPO and 0.150 gm of POPOP in one liter of toluene. The counting of the aqueous system, including $C^{14}O_2$ dissolved in NaOH, were performed in scintillation fluid containing the following components:

naphthalene	60 gm
PPO	4 gm
POPOP	0.20 gm
ethanol	100 ml
ethylene glycol	20 ml
p- dioxane	to one liter

The dioxane system as described by Bray(10) can hold as much as 1 ml of aqueous system and has proven to be suitable for the counting of $C^{14}O_2$ dissolved in NaOH. Later in the experiments, it was found that the dioxane system was actually very suitable for both the aqueous and nonaqueous measurement of radioactivity and so the system was used in both kinds of counting.

In the first experiment, in which the metabolism of acetate- $1-C^{14}$ and palmitate- $1-C^{14}$ was compared, the measurement of radioactivity was performed in a Tricarb two channel liquid scintillation counter, at a high voltage setting of 1550 volts and a gain of 9. A known amount of toluene- $1-C^{14}$ was added as an internal standard and the samples were counted again to determine the efficiency of the counting system.

$$\frac{a - b}{c} \times 100 = \% \text{ efficiency}$$

a - observed counts per minute (CPM) due to unknown and

internal standard

b - observed CPM due to unknown sample

c - known disintegrations per minute (DPM) of internal sample

For the other experiments, the measurements of radioactivity were performed in a Tri-Carb three channel liquid scintillation counter equipped with an automatic external standardization device.

The toluene system was counted at a gain of 9.5 and the dioxane system was counted at a gain of 18. In the determination of efficiency by the use of an external standard, it is necessary to perform two counting operations on the sample. The first operation being that of the sample and the second one that of the sample and the standard which can be automatically brought into position. In practice, one needs to relate the net external standard count to the counting efficiency of the sample. In order to obtain the net external standard count, discriminator settings of the third channel were adjusted so that the external count excludes any sample contribution. A discriminator setting of 30-70 was used in the dioxane system. To generate a useful correlation curve, a series of samples were prepared, each containing a known amount of toluene- l -C¹⁴. Each sample also contained a different amount of quenching additive which was chloroform so that the series would range from unquenched to nearly completely quenched. The efficiencies of these samples were plotted on a graph against their respective net external standard counts as shown in Figure 15. After the establishment of this curve, samples of the same composition and volume of scintillation fluid could be counted in similar way and the efficiency of the sample read out from the curve. The measurement of radioactivity is represented as dpm/mg of counting material.

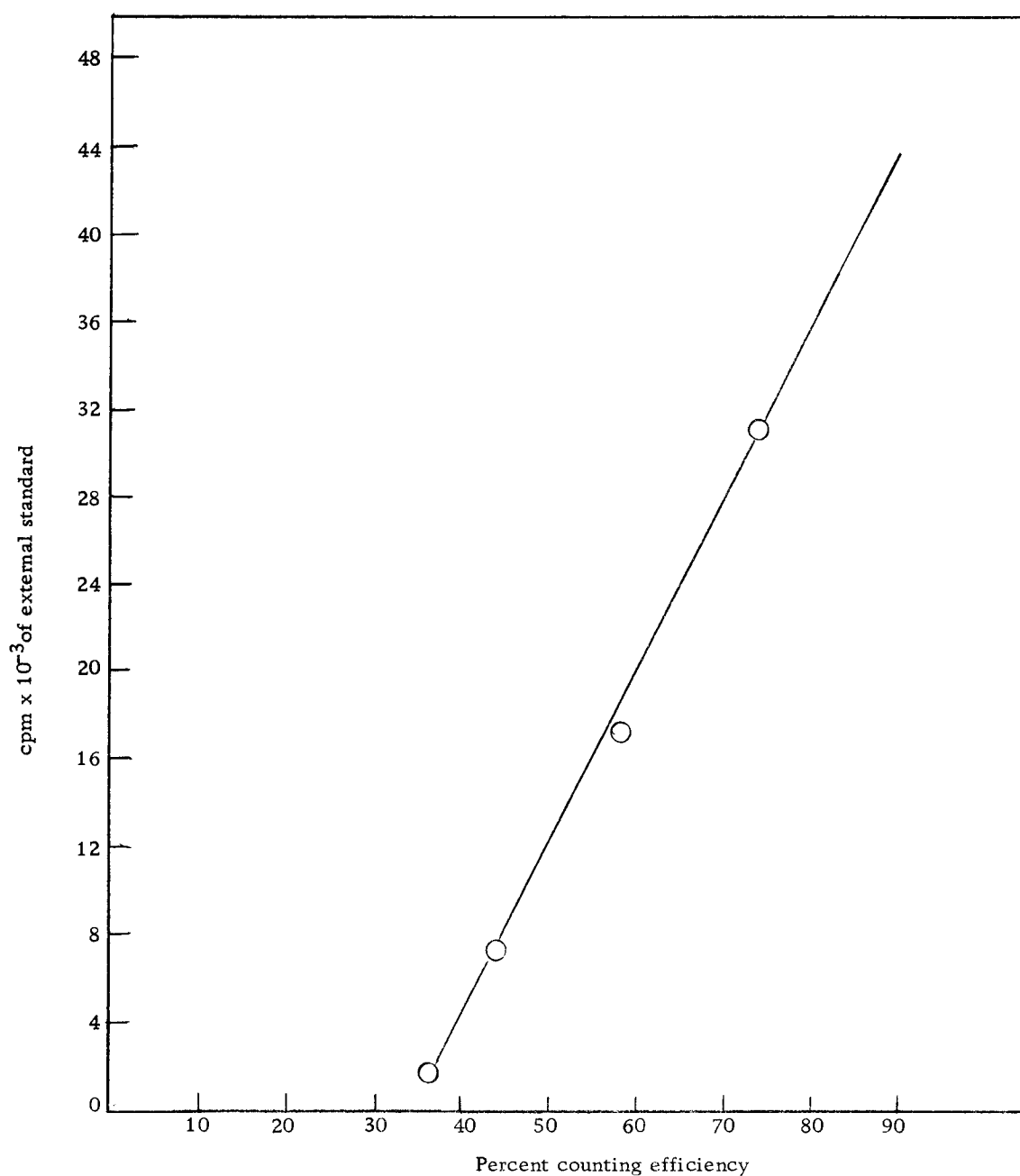


Figure 15. Determination of counting efficiency by external standardization method using the dioxane scintillation system.

Dioxane scintillation system is described on page 65.

Scintillation counter settings are described on page 67.

Thin-Layer Chromatography Separation of Saturated and Unsaturated Fatty Acids

Portions of the neutral lipid were saponified to free the fatty acid moiety. Methods for saponification and methylation were described on page 19. Two kinds of thin-layer chromatographic methods were used for the separation of saturated and unsaturated fatty acid methyl esters. The procedures recommended by Jantzen and Andreas (32) for the preparation of the acetoxymercuri-methoxy derivatives of methyl esters of unsaturated fatty acids were used initially. The reagent is a solution of mercuric acetate (14 gm) in methanol (250 ml), 2.5 ml of water and 1 ml of glacial acetic acid. About 25 ml of this solution was added to 1 gm of the esters and allowed to react in a stoppered flask in the dark, at room temperature. With the small quantity of esters obtained from our experiments, only three ml of the solution was used. After 24 hours, the methanol was evaporated at less than 30° C by a rotatory evaporator and the dry residue dissolved in chloroform (10 ml). The chloroform solution was washed with 25 ml of water to remove excess mercuric acetate and then dried with anhydrous Na_2SO_4 .

Thin-layers, 275 μ thick, of silicic acid on 20 by 20 or 20 by 10 cm glass plates were prepared according to Stahl (64) using commercial grade silica gel G (100-200 mesh). The chromatoplates

were heated to 110° C for one hour before use.

The samples were applied in chloroform solution along one side of the plates, 2 cm from the edge. The solvent used for the separation was a mixture of petroleum ether (b. p. 60-70° C) and diethyl ether in a ratio of 4:1 v/v. The saturated methyl esters with an R_f of 0.9 were separated from the acetoxymurcuric-methoxy-derivatives with an R_f of less than 0.1 within 1.5 to 2 hours, during which time the solvent front reached a height of 15 to 18 cm. After drying the plates in air, the acetoxymurcuri-methoxy-compounds as well as the saturated fatty acids were detected in an iodine vapor tank. They appeared as yellow brown spots and their locations were marked with a sharp pointer.

The silicic acid containing the derivatives of unsaturated esters was scraped off the plate into an Erlenmeyer flask. The adducts were then shaken with 10 ml of methanol and 0.5 ml of concentrated HCl. After most of the adsorbent had settled, the supernatant was decanted and filtered. The residual silicic acid was treated again with methanol-HCl. The combined filtrates of the two extractions were diluted with water (25 ml) and extracted with 10 ml of diethyl ether three times. The combined ether extracts were washed with water, dried with anhydrous Na_2SO_4 and aliquots taken for measurement of radioactivity. The saturated fatty acids that appeared near the solvent front were scraped off the plate

and extracted with diethyl ether. The ether extracts were washed with water, dried and aliquots taken for the measurement of radioactivity. These fatty acid methyl esters recovered from the thin-layer plates were also suitable for gas-liquid chromatography.

The mercury adduct method of separating unsaturated fatty acids from saturated fatty acids was a rather time consuming method. It was also found that varying amounts of the unsaturated fatty acids were converted to adducts in each experiment. The percent conversion, however, increased with time of reaction. Due to this incomplete conversion of unsaturated fatty acids to their adducts, the remaining free unsaturated fatty acids would appear in a different position (R_f 0.7) in the thin-layer plates. This could easily be demonstrated by experiments with an unsaturated fatty acid standard such as oleic-1- C^{14} .

The second method, which was found to be a more convenient one, involved the use of $AgNO_3$ impregnated silicic acid thin-layer chromatoplates (44). Six hundred twenty-five grams of $AgNO_3$ was dissolved in 50 ml of water and 25 gm of Silica Gel G was added to the silver nitrate solution just before application. The chromatoplates were 275 μ thick and were heated to 110° C for one hour before use. The chromatoplates were used shortly after preparation due to the presence of $AgNO_3$. Methyl esters to be separated were applied along one side of the plate and the chromatoplate was

developed in a solvent system composed of 20% ether in hexane. It was more suitable to perform the experiment in a semi-dark room. After two hours, the saturated fatty acid methyl esters with an R_f value more than 0.9 were separated from that of the unsaturated ones which appeared at various positions with R_f of less than 0.7. The esters were detected as yellow spots under a fluorescent lamp after spraying the chromatoplate with a 0.2% solution of 2, 7-dichlorofluorescein in methanol. The esters were scraped off the plate and recovered by ether extraction. The combined extracts were washed, dried and aliquots taken for measurement of radioactivity.

Incorporation of Acetate-1-C¹⁴ and Palmitate-1-C¹⁴
into Fat Body of *Sarcophaga Bullata* Larvae

Acetate-1-C¹⁴ and palmitate-1-C¹⁴ were incubated separately with the fat body of three day old larvae according to the method described above. Incubation was carried out for one hour at 30° C and C¹⁴O₂ was collected from each incubation flask at the end of the incubation. The amount of C¹⁴O₂ recovered is shown in Table 8. Total lipid was extracted from the fat body and subsequently separated into neutral lipid and free fatty acids. Aliquots of these fractions were also taken for the measurement of radioactivity and the results obtained are shown in Table 8.

Table 8. Incorporation of Acetate-1-C¹⁴ and Palmitate-1-C¹⁴ into the fat body lipid of the larva of *Sarcophaga bullata*.

Substrate	Flask no.	CO ₂		TL			NL			FFA			Radioactivity recovered in fatty acid moiety of NL
		DPM	%	DPM	Sp. Act.	%	DPM	Sp. Act.	%	DPM	Sp. Act.	%	
Acetate-1-C ¹⁴													
	1	8.32x10 ⁵	54	1.19x10 ⁴	232	0.477	6430	-	0.42	2740	392	0.18	
	2	1.01x10 ⁶	64	1.50x10 ⁴	235	0.97	8480	224	0.55	5350	690	0.35	24%
	3	7.8x10 ⁵	51	1.50x10 ⁴	250	0.80	7280	228	0.47	3060	525	0.20	
Palmitate-1-C ¹⁴													
	4	1.99x10 ⁵	9.8	1.02x10 ⁶	1.82x10 ⁴	50.0	6.35x10 ⁵	1.68x10 ⁴	31.4	3.12x10 ⁵	8.17x10 ⁴	15.4	
	5	2.12x10 ⁵	10.2	1.15x10 ⁶	1.85x10 ⁴	56.5	6.35x10 ⁵	1.65x10 ⁴	31.4	3.60x10 ⁵	5.8x10 ⁴	17.7	91%
	6	1.72x10 ⁵	8.5	1.03x10 ⁶	1.61x10 ⁴	50.5	5.90x10 ⁵	1.43x10 ⁴	29.0	3.58x10 ⁵	6.15x10 ⁴	17.6	

Experimental conditions are described in Figure 16. TL = Total Lipid NL = Neutral Lipid FFA = Free fatty acid
Specific Activity is expressed as dpm/mg

Results showed that 50% of the total radioactivity was recovered as $C^{14}O_2$ in the experiment with acetate-1- C^{14} whereas less than 10% was recovered when palmitate-1- C^{14} was the substrate. Results also showed that as high as 50% of the radioactivity from palmitate-1- C^{14} was incorporated into total lipid of the fat body whereas only 0.8 to 0.9% was incorporated when acetate-1- C^{14} was the substrate. The difference was similarly reflected in the specific activity of the lipid.

Neutral lipid from fat body incubated with palmitate-1- C^{14} showed a specific activity of 1.6×10^4 dpm/mg whereas that from acetate-1- C^{14} had only 2.2×10^2 dpm/mg. A hundred fold difference in specific activity was seen between the two kinds of incubation. The free fatty acid fraction was also collected from the KOH-silicic acid column and its radioactivity measured. Seventeen percent of the total activity was found in the free fatty acid fraction when palmitate-1- C^{14} was used as substrate whereas only a trace amount of the activity was recovered in the acetate-1- C^{14} experiment. The relatively large amount of radioactivity recovered from the palmitate-1- C^{14} incubated experiment may have come from the exchange of exogenous labeled fatty acids in the incubation medium with the free fatty acid pool in the fat body.

Portions of the neutral lipid from the two sets of experiments using different substrates were saponified and the fatty acid moiety

of the glycerides was extracted with hexane. Aliquots of the saponified fatty acids were taken for the measurement of radioactivity. Results of the measurement showed that most of the radioactivity from palmitate-1-C¹⁴ was recovered in the fatty acid moiety of neutral lipid fraction whereas only 24% of the total activity of this fraction was recovered from acetate-1-C¹⁴.

Incorporation of Palmitate-1-C¹⁴ into Neutral Lipid Components of the Fat Body

Results from the previous experiments indicated that a large amount of the palmitate-1-C¹⁴ activity could be incorporated into the fat body lipid of three day old Sarcophaga bullata larvae. Also, a large portion of the radioactivity in the lipid was found in the neutral lipid fraction.

Neutral lipid was separated on a 12 gm floricil column as described on page 22. As a preliminary trial, portions of the neutral lipid from the previous experiment were taken for the separation. The eluted lipid material in each fraction was then transferred to the scintillation vial for the measurement of radioactivity. The pattern of radioactivity distributed among the neutral lipid components is shown in Figure 16 and the amount of the distribution is shown in Table 9. Results showed that radioactivity was distributed among all the neutral lipid components. However, less than 1% of

Each incubation flask contained 1 ml of 0.05 M sodium phosphate-Ringer solution (pH 7.2) 0.1 ml of 0.078 μ mole palmitate-1- C^{14} (1 μ curie) and fat body from ten three day old larvae. Incubation was carried out at 30° for one hour. Neutral lipid was separated on 12 gm floricil column. Radioactivity measurement was made on 1 ml aliquot of each fraction. Solvents for elution in increasing percent ethyl ether in hexane (a) hexane (b) 5% (c) 15% (d) 25% (e) 50% (f) 2% methanol in ether. Neutral lipid components are: I hydrocarbons, II sterol esters, III triglycerides, IV free sterol and undetermined material, V diglycerides, VI monoglycerides.

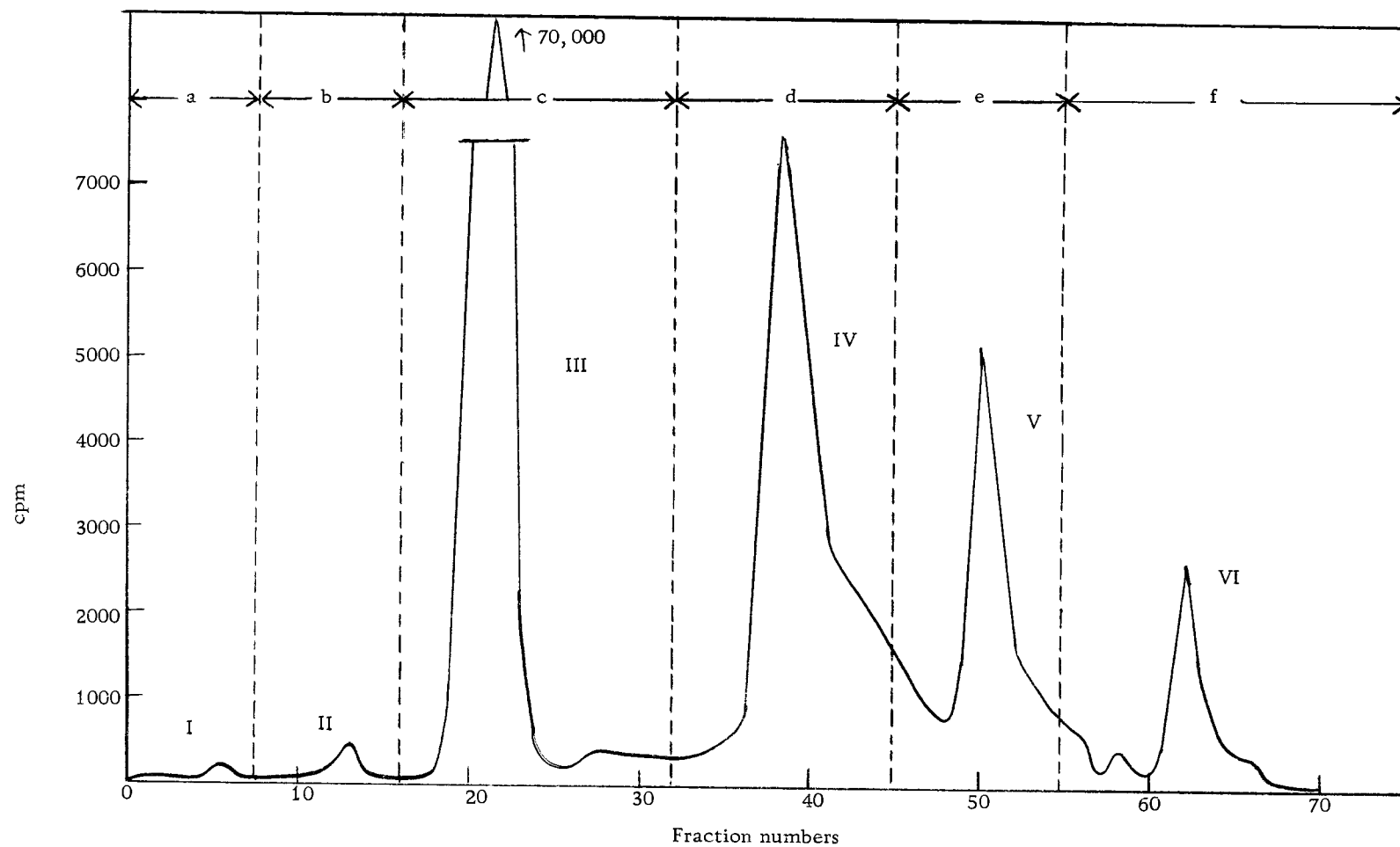


Figure 16. Distribution of radioactivity among neutral lipid components of larval fat body after incubating with palmitate-1- C^{14} .

Table 9. Distribution of radioactivity among neutral lipid components of larval fat body after incubation with palmitate-1-C¹⁴.

Peak no. ^a	Neutral lipid components	Amount of radioactivity cpm	Percent distribution of radioactivity
I	hydrocarbons	154	0.09
II	sterol esters	768	0.45
III	triglycerides	105777	62.50
IV	free sterols and diglycerides	39863	23.60
V	diglycerides	15488	9.15
VI	monoglycerides	6759	4.00

^aPeak numbers are the same as the ones in Figure 16.
Experimental conditions are described in Figure 16

radioactivity was found in the hydrocarbon and the sterol ester fractions while the triglyceride fraction comprised 62.5% of the total. Diglyceride fraction comprised 9% of the total, monoglyceride 4%, and 23.5% of the total radioactivity was found in the free sterol fraction. Since insects are believed not to have the capacity to synthesize sterols, therefore activity in the free sterol fraction was unexpected and further investigation was made to characterize the labelled component.

Neutral lipid from more experiments was similarly separated from the free fatty acids and other components and subsequently separated into various components. Specific activity of each major lipid fraction was determined in this separation. The specific activity of these neutral lipid fractions was found to be as follows: I and II (hydrocarbons and sterol esters) 262 cpm/mg; III (triglycerides) 4710 cpm/mg; IV (free sterols and undetermined material) 6850 cpm/mg; V (diglycerides) 4950 cpm/mg; VI (monoglycerides) 912 cpm/mg. The specific activity was highest in the free sterol and undetermined material fraction, whereas triglyceride and diglyceride fractions had specific activities of similar magnitude.

Fractions III and IV were pooled and the lipid material was re-separated on a floricil column with a slightly modified elution pattern. Results showed that modification of elution pattern could not separate the unknown material from the free sterol and the

triglyceride fractions. Therefore, more of the pooled material was obtained and the thin-layer chromatographic method was attempted for their separation. The solvent system used for the separation of neutral lipid components was petroleum ether, ethyl ether and acetic acid in the proportion of 90:10:1 by volume. The spots were developed by iodine vapour and compared to known standards. Results of the thin-layer chromatographic separation of fraction IV revealed the presence of diglyceride and cholesterol as major components with only a trace of triglyceride. The lipid material from these spots was consequently recovered and radioactivity measured. Radio-activity measurement showed that most of the activity was distributed between the diglyceride and the cholesterol fractions.

Thin-layer chromatography also did not give a well defined separation between the cholesterol and diglyceride. Therefore, digitonin precipitation of cholesterol was applied in an attempt to isolate the unidentified material. Portions of more lipid material from fraction IV were treated with digitonin solution for the precipitation of cholesterol. The procedure was that of Sperry and Webb (62). After precipitation of cholesterol, lipid material was recovered from the supernatant by evaporation and further separated on a thin-layer plate. Results showed that after the precipitation of cholesterol material, most of the radioactivity from fraction IV was recovered

as diglycerides. Therefore most of the radioactivity found in this fraction consisted of diglycerides. Carroll (12) in his experiments also discussed the possible overlapping of diglyceride material with the free sterol fraction when the floril material was hydrated with more than 6% water. The batch of floril material employed for these experiments, therefore, may have been over hydrated. When neutral lipid components were later separated with a new batch of floril without prior hydration, the emergence of fraction IV with the diglyceride fraction resulted; indicating that the degree of hydration of the floril material could actually alter the position of elution of the diglyceride fraction. Figure 17 shows a typical separation of neutral lipid with this newly obtained floril material without treatment with 7% water. Diglyceride fraction of this particular separation constituted 26% of the total radioactivity and had a specific activity higher than the triglyceride fraction.

Oxidation of Palmitate-1-C¹⁴ to C¹⁴O₂.

Time Course Experiment

The oxidation of labelled palmitate in the fat body was measured at various intervals during incubation in two experiments. Although the amount of C¹⁴O₂ obtained from these two experiments varied somewhat. A general increase in C¹⁴O₂ production was observed with time of incubation. Carbon dioxide activity tended to

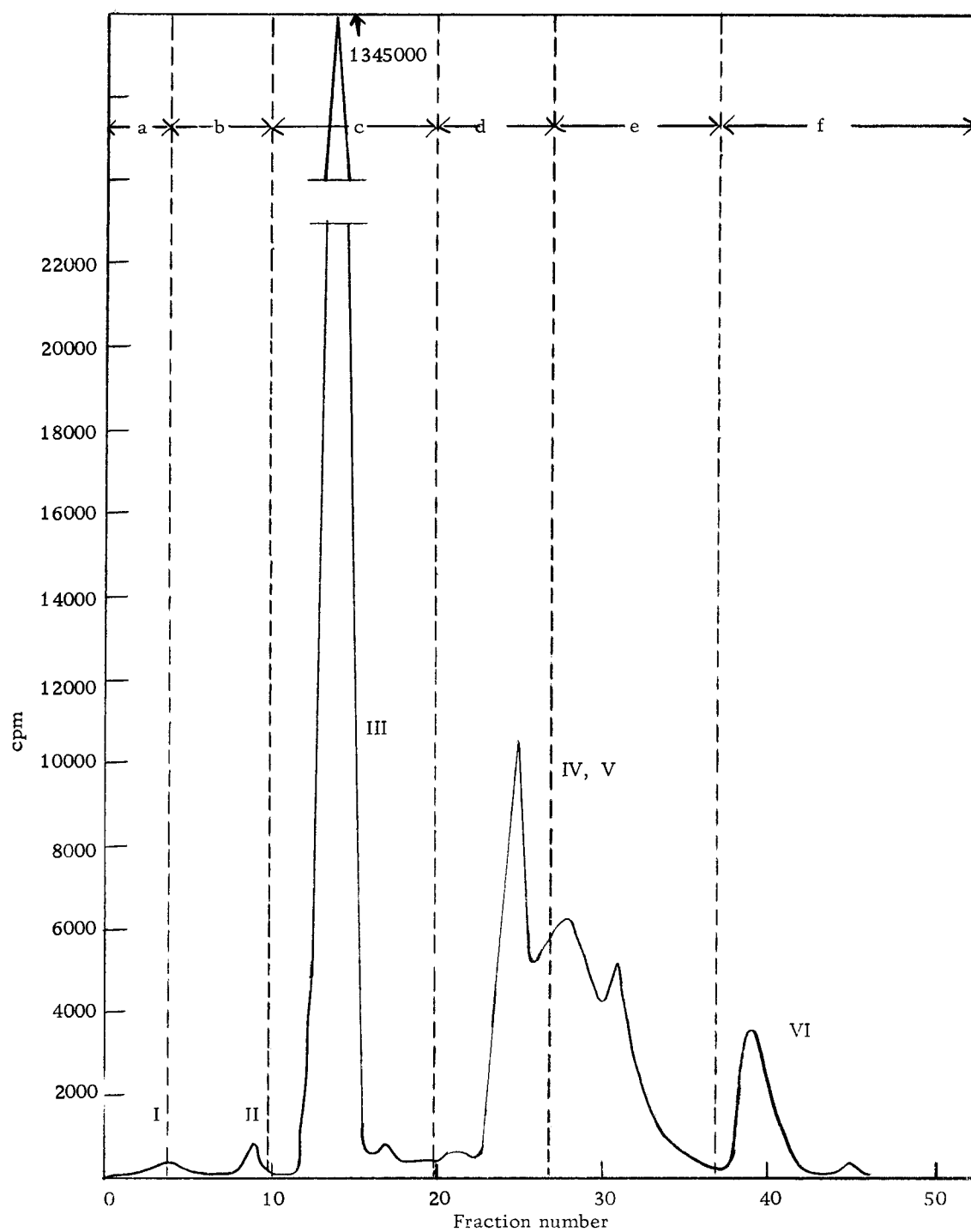


Figure 17. Distribution of radioactivity among neutral lipid components of larval fat body after incubation with palmitate-1- C^{14} . Experimental conditions are the same as described in Figure 16 except that the floril material was not hydrated with 7% of water. Solvents for elution are also the same as described in Figure 16.

become constant after one to one and a half hours of incubation as was shown in Figure 18. The difference in amount of palmitate-1-C¹⁴ oxidized to C¹⁴O₂ was probably due to changes in physiological conditions taking place during larval growth.

Incorporation of Palmitate-1-C¹⁴ into Lipid
Components of the Fat Body. Time Course Experiments

Results of the incorporation of palmitate-1-C¹⁴ into the total lipid and neutral lipid fractions were obtained from two time course experiments. Specific activity of the total lipid for these two experiments is represented in Figure 19. It was evident that palmitate-1-C¹⁴ was rapidly incorporated into lipid of the fat body and that incorporation reached the maximum within 30 minutes of incubation time for both experiments. The maximum incorporation into fat body lipid was 1.35×10^6 dpm at 27 minutes and 1.32×10^6 dpm at 30 minutes respectively for the two experiments.

Figure 19 indicates that there were two peaks of incorporation during the first 60 minutes of incubation of fat body with palmitate-1-C¹⁴, after which the specific activity decreased to a value which then remained constant during the remainder of the incubation period. This biphasic nature of incorporation was not seen in the neutral lipid, which suggests that it was brought about in part by the oxidation of palmitate and by equilibration between the tissue pool and the

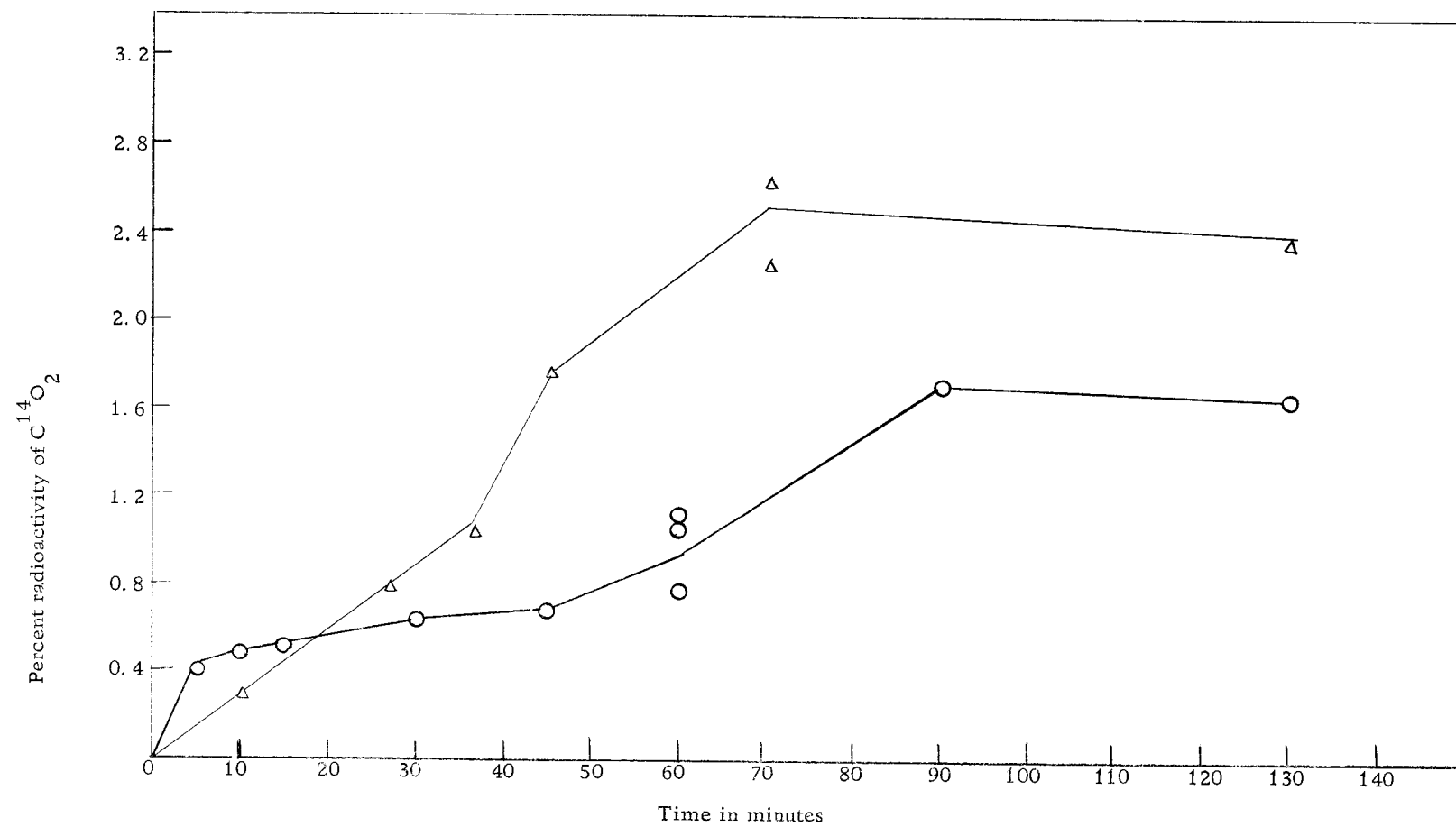


Figure 18. Oxidation of palmitate-1- C^{14} to $C^{14}O_2$ in the larval fat body.

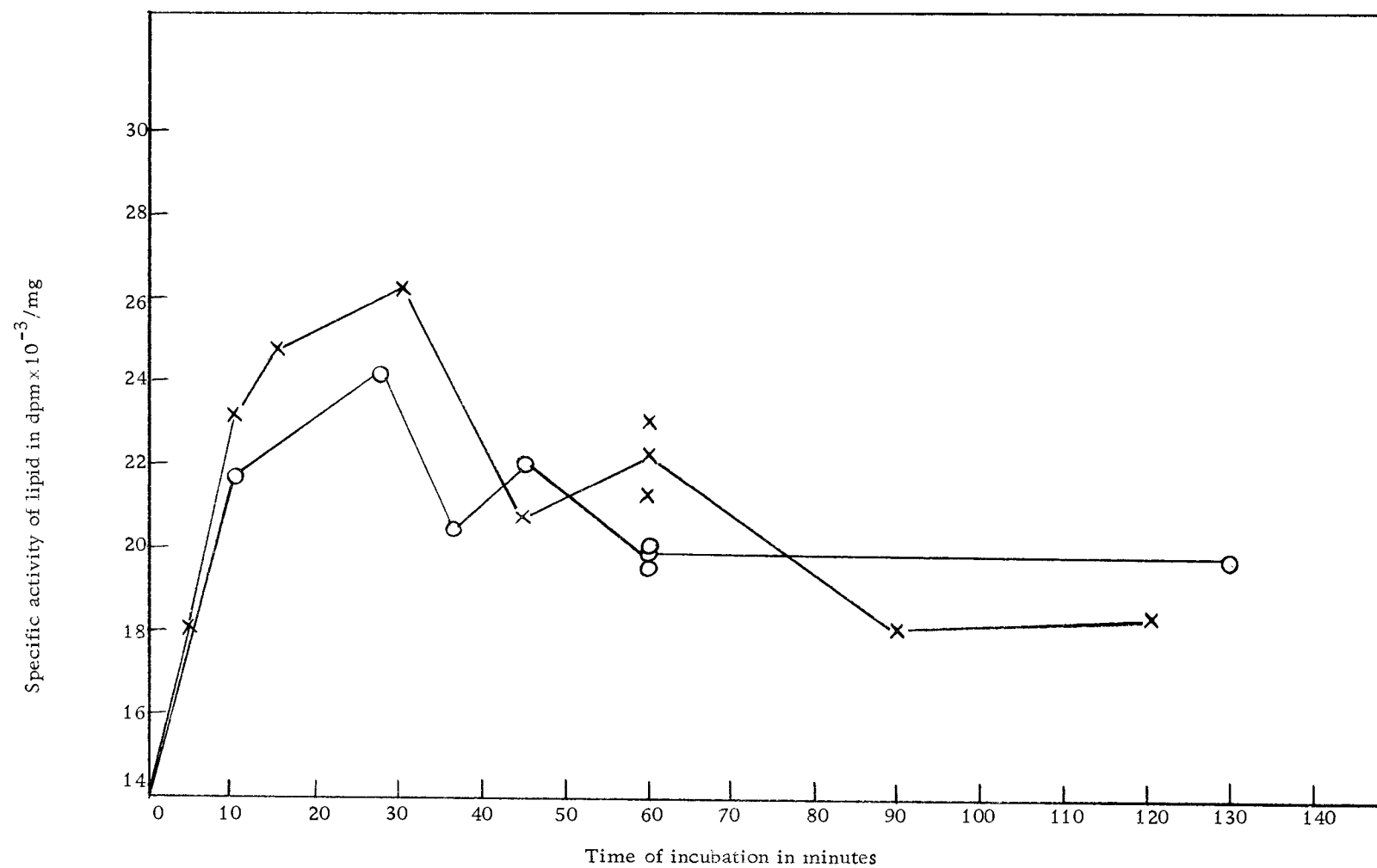


Figure 19. Incorporation of palmitate-1- C^{14} into total lipid of the larval fat body.

medium. The specific activity of neutral lipid reached a maximum within 30 minutes of incubation and thereafter was constant (Figure 20). The difference in specific activity between the total lipid and neutral lipid is due to the free fatty acid fraction which was removed in the preparation of neutral lipid.

Neutral lipid from one of the time course experiments was subsequently separated into its glyceride components by floricil column chromatography. In these separations, the weight and radioactivity of each glyceride fraction were obtained with respect to time of incubation. The distribution of specific activity among the triglyceride, diglyceride and monoglyceride fractions during different incubation intervals is shown in Table 10. Specific activity of the triglyceride fraction with time of incubation is also shown in Figure 20. The specific activity of triglyceride fraction appeared to follow a course similar to that of the neutral lipid. Maximum incorporation occurred in all the glyceride fractions within 30 to 45 minutes of incubation followed by some reduction of activity during prolonged incubation. Specific activity of the diglyceride fraction was the highest of the three glyceride fractions. Due to the difficulty in obtaining precise weight of the diglyceride and monoglyceride fractions, the specific activity of these fractions could not be interpreted meaningfully according to time of incubation in this series of experiments.

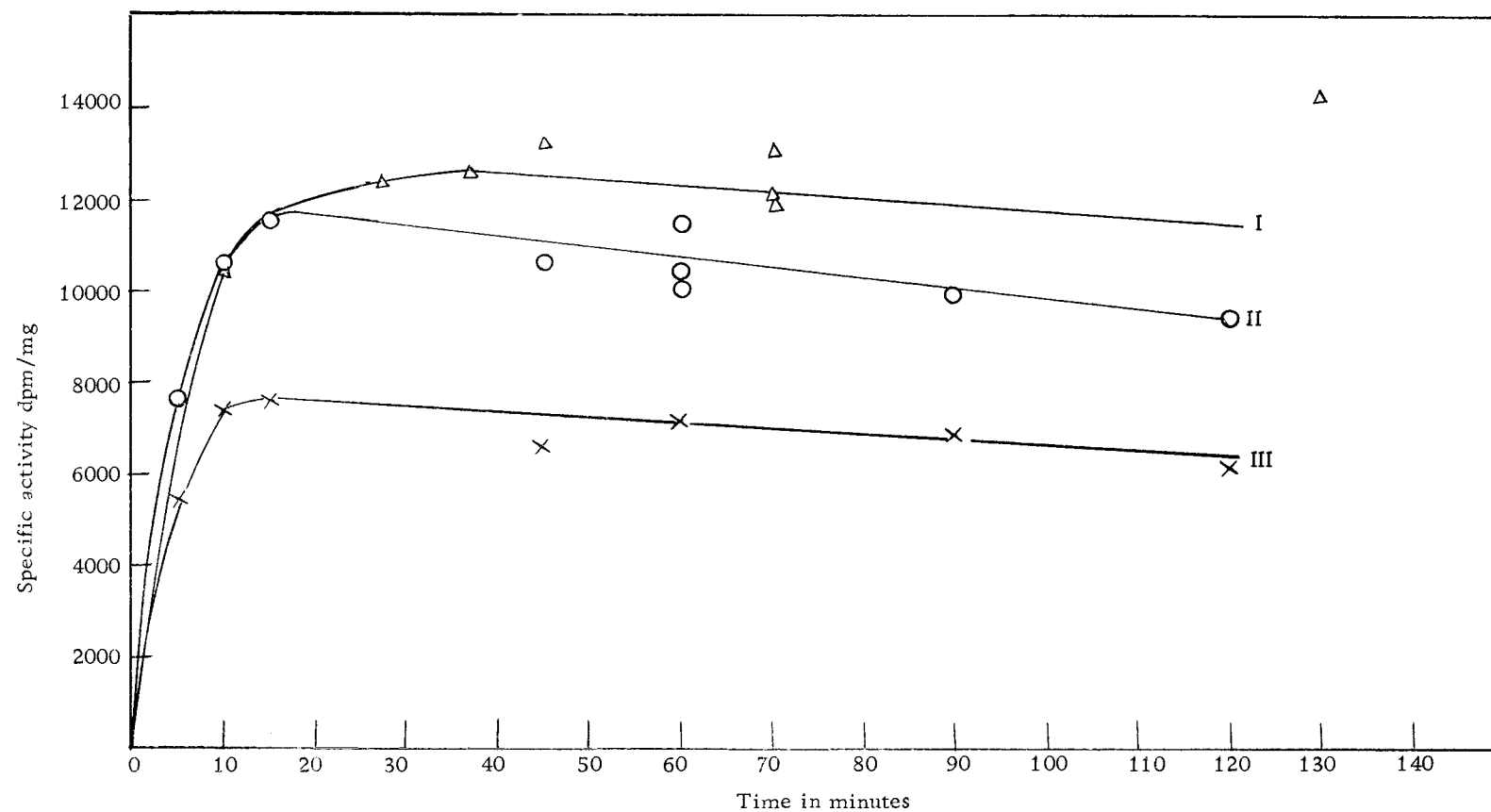


Figure 20. Incorporation of palmitate-1- C^{14} into neutral lipid and the triglyceride fraction of the larval fat body.
Time course experiment.

I and II: Specific activity of neutral lipid in two separate time course experiments.

III: Specific activity of triglyceride fraction which corresponds to II.

Experimental conditions are described in Figure 16.

Table 10. Incorporation of palmitate-1-C¹⁴ into triglyceride, diglyceride and monoglyceride fractions of the larval fat body.

Time of incubation	Neutral lipid								
	Triglyceride			Diglyceride ^a			Monoglyceride		
	Weight in mg	cpm	Sp. act. cpm/mg	Weight in mg	cpm	Sp. act. cpm/mg	Weight in mg	cpm	Sp. act. cpm/mg
5 minutes	14.5	80871	5575	6.73	64222	9530	3.55	12829	3580
10 minutes	12.1	89913	7470	6.03	73234	12120	2.63	14427	5480
15 minutes	14.5	110611	7630	8.55	86003	10050	3.04	15741	5180
45 minutes	22.3	149786	6720	4.08	59556	14600	2.44	9338	3820
60 minutes	21.7	157242	7250	7.06	60644	8580	3.06	10776	3520
90 minutes	21.7	151185	6970	6.22	78766	12480	2.81	10419	3710
120 minutes	19.5	121339	6220	6.70	67593	10420	3.54	10168	3060

^aSpecific activity of the diglyceride is the combination of fraction IV and V in Figure 16.

It was further observed that fractionation of the neutral lipid from fat body incubated with palmitate-1-C¹⁴ seemed to show a different pattern of glyceride distribution when compared to the neutral lipid of fat body without incubation. Results of the column separations of neutral lipid from both incubated and un-incubated fat body are represented in Table 11. Neutral lipid extracted from fat body which had not been incubated was found to contain more than 80% triglyceride while the neutral lipid isolated after incubation with palmitate-1-C¹⁴ was found to contain only 60 to 65% of triglyceride with a corresponding increase in diglyceride and monoglyceride components. Results of column separation of the unincubated material were in good agreement with results given in Part 1, page 32.

Incorporation of Palmitate-1-C¹⁴ into Fat Body
of Larvae from Two Different Ages

Since previous experiments involved only the three day old larvae, it was of interest to observe the metabolism of palmitate-1-C¹⁴ in the prepupal larvae. Two groups of larvae, one of three day and the other of seven day old, were taken for this incubation experiment. The fat bodies of these larvae were incubated for one hour, C¹⁴O₂ collected and total lipid extracted according to the methods described previously. Neutral lipid obtained from the KOH-silicic acid column was separated into its components. Results of

Table 11. Percent weight distribution of neutral lipid components in experiments with and without incubation.

Age of larvae	Incubation time	Percent of neutral lipid			
		Hydrocarbon and sterol ester	Triglyceride	Diglyceride	Monoglyceride
3 days	27 min	2.0	58.7	29.7	9.6
3 days	1 hr	5.56	63.0	20.6	11.1
3 days	1 hr	3.84	62.6	26.0	7.18
7 days	1 hr	3.85	43.0	37.5	15.5
4 days	no incubation	--	97.5	0.65	0.28
6 days	no incubation	1.14	88.5	3.66	1.19
9 days ^a	no incubation	--	94.5	3.24	0.72

^aPercent of neutral lipid was based on the three glyceride fractions only.

the experiment were summarized in Table 12.

Table 12 shows that in the seven-day old larval fat body only 0.8% of the palmitate-1-C¹⁴ was oxidized to C¹⁴O₂ as compared to 2.6 to 2.2% in the three-day old larval fat body. Nevertheless larval fat body towards the prepupal stage was also able to incorporate a considerable amount of palmitate-1-C¹⁴ into the lipid fractions. Specific activity of the total lipid for the seven day old was 11030 and 8630 dpm/mg as compared to 20410 and 19860 dpm/mg of the three day old ones. The two fold difference in specific activity was mostly due to the larger amount of lipid in the seven day old larval fat body. The amount of radioactivity incorporated into neutral lipid of the seven day old larval fat body was 2.63×10^5 dpm and 3.08×10^5 dpm compared to 4.29×10^5 dpm and 4.58×10^5 dpm in the three day old.

When neutral lipid was separated into its components, it was found that percent distribution of radioactivity among these neutral lipid fractions in the young and the prepupal larvae was similar. About 50% of the radioactivity in neutral lipid was incorporated into the triglyceride fraction and approximately 40% was in the diglyceride fraction. The remaining 10% was distributed among the monoglyceride, hydrocarbon and sterol ester fractions. In fact, hydrocarbon and sterol ester fractions together comprised less than 1% of the total radioactivity. However, in spite of the fact that the percent

Table 12. Incorporation of palmitate-1-C¹⁴ into lipids of fat body of three day and seven day old larvae.

Experiment no.	3 day old		7 day old	
	1	2	1	2
Percent C ¹⁴ O ₂	2.60	2.22	0.86	
Amount of radioactivity incorporated into total lipid	1.22x10 ⁶ dpm	1.19x10 ⁶ dpm	0.95x10 ⁶ dpm	1.0x10 ⁶ dpm
Specific activity of total lipid	20410 dpm/mg	19860 dpm/mg	11030 dpm/mg	8630 dpm/mg
Amount of radioactivity incorporated into neutral lipid	4.29x10 ⁵ dpm	4.58x10 ⁵ dpm	2.63x10 ⁵ dpm	3.08x10 ⁵ dpm
Specific activity of neutral lipid	12250 dpm/mg	12170 dpm/mg	6830 dpm/mg	6610 dpm/mg

	N. L. components 3 day old			N. L. components 7 day old		
	Sp. act. dpm/mg	C ¹⁴ incor- poration %	Weight %	Sp. act. dpm/mg	C ¹⁴ incor- poration %	Weight %
Hydrocarbons	-	<0.1	<2.6	-	<0.1	<2.3
Sterol esters	-	<0.6	<1.3	-	<0.3	<1.5
Triglycerides	8880	55.4	62.6	6530	50.8	43.0
Free sterols and diglycerides	16000	28.3	17.8	7320	31.2	23.5
Diglycerides	10940	9.0	8.21	4210	10.7	14.0
Monoglycerides	9650	6.9	7.18	2400	6.8	15.5

distribution of radioactivity among these neutral lipid fractions was similar in the young and prepupal larval fat body, a decrease in weight percent of the triglyceride fraction in the seven day old larval fat body was observed. The triglyceride fraction of the prepupal larval fat body comprised only 43% in weight of the total as compared to 63% in the three day old. Consequently, a relative increase in weight percent in all the other fractions was observed.

Evidence of Synthesis of Unsaturated Fatty Acids in
Fat Body When Incubated with Palmitate-1-C¹⁴

The object of this series of experiments was to examine for the presence of radioactive unsaturated fatty acids in the neutral lipids of the fat body of the three day old Sarcophaga bullata larvae after incubating with palmitate-1-C¹⁴ for one hour at 30° C.

Neutral lipid was extracted, saponified, the fatty acids recovered and methylated according to methods described on page 22. The fatty acid methyl esters were then converted to their mercury derivatives and the unsaturated derivatives were subsequently separated from the saturated ones by thin-layer chromatography. The method was described on page 69. The radioactivity of the unsaturated fatty acids plus that of the saturated ones was considered to be the total radioactivity applied to the chromatoplates, since only two components were resolved. The percent of radioactivity distribution of

Table 13. Synthesis of unsaturated fatty acids from palmitate-1-C¹⁴ in the larval fat body of Sarcophaga bullata.

	Age of larvae	Incubation time	Unsaturated fatty acids		Saturated fatty acids	
			Palmitate-1-C ¹⁴ incorporated	%	Palmitate-1-C ¹⁴ incorporated	%
1	3 day	10 min	42200	56.2	32700	43.5
2	3 day	37 min	55600	69.2	24500	30.5
3	3 day	1 hr 10 min	65000	64.7	35000	35.3
4	3 day	2 hr 10 min	73800	64.3	41000	35.7
5	7 day	1 hour	7940	9.2	78000	91.8
6	3 day	1 hour	2920 ^a	60.0	1925	40.0
7	3 day	1 hour	4575 ^a	61.7	2815	38.3
8 ^b	3 day	1 hour	23590	59.2	16310	40.8
9	Standard	oleic-1-C ¹⁴	133000 dpm	99.2	1128	0.8

^aData based on aliquots of free fatty acid fractions.

^bExperiments performed with AgNO₃ impregnated plates.

these two components is represented in Table 13. In general, 60 to 65% of the labelled fatty acids saponified from the neutral lipid were found to be unsaturated. The above results were in agreement with the results obtained by a second method of separation in which silver nitrate impregnated chromatoplates were used.

The amount of radioactivity being incorporated into unsaturated fatty acids of the fat body during different intervals of incubation are also shown in Table 13. Results indicated that incorporation increased with time of incubation and proceeded at a very rapid rate in the first 15 minutes of incubation.

The amount of unsaturated fatty acids synthesized in the three day larvae was also compared to that of the seven day old larvae. Only 9.2% of the total palmitate-1-C¹⁴ activity was incorporated into the unsaturated fatty acid fraction in the seven day larvae compared to a much higher amount in the three day larval fat body.

DISCUSSIONS

In the experiment comparing the incorporation of palmitate-1-C¹⁴ into the fat body of *Sarcophaga* larvae with that of acetate-1-C¹⁴, it was found that the oxidation of acetate-1-C¹⁴ to carbon dioxide was greater than that of palmitate-1-C¹⁴. However, in spite of the high rate of acetate-1-C¹⁴ being metabolized during the incubation, less than one percent of the radioactivity was actually incorporated into the lipid part of the fat body. Since the fat body not only serves as a fat storage organ but is also the site of many other metabolic pathways, it is expected that acetate-1-C¹⁴ is utilized for the metabolism and biosynthesis of other non-lipid materials. In fact, with an adequate supply of lipid material in the larval diet, the fat body may preferentially utilize the dietary lipid material instead of synthesizing fatty acids from the two carbon units. In the mammalian system, Marinetti, Griffith and Smith (45) found that only 0.002% and 0.009% of the acetate-1-C¹⁴ was being incorporated into lipid of rat heart and rat liver homogenate respectively.

Palmitate-1-C¹⁴, on the other hand, was rapidly incorporated into lipid of the fat body. Only a small amount of the labelled long chain fatty acid was oxidized to carbon dioxide. Most of the radioactivity from the palmitate-1-C¹⁴ was incorporated in the neutral lipid and the free fatty acid fractions. The high amount of

radioactivity found in the free fatty acid fraction is most probably due to the exchange of the free fatty acids in the fat body with that of the labeled palmitate in the incubation medium. The presence of a free fatty acid pool in adipose tissue of the rat has been discussed by Gorin and Shafrir (28), who found that the release of free fatty acids from the tissue was less than that of glycerol when compared on an equivalent basis. In the study of the oxidation of fatty acids to carbon dioxide in rat diaphragm, Neptune et al. (53) also mentioned the possibility of the presence of an endogenous free fatty acid pool, although the fatty acids were present in low concentration and were turning over rapidly. There was only a small amount of radioactivity present in the free fatty acid fraction when acetate- 1-C^{14} was used as a substrate.

With palmitate- 1-C^{14} as substrate, most of the radioactivity in the neutral lipid fraction could be recovered in the fatty acid moiety of the glycerides. However, upon saponification of the neutral lipid from the experiment using acetate- 1-C^{14} as substrate, only 24% of the total radioactivity that was incorporated into neutral lipid was recovered in the fatty acid moiety.

Among the different neutral lipid components, palmitate- 1-C^{14} activity was mainly distributed over the triglyceride and diglyceride fractions with monoglyceride, sterol, sterol esters and hydrocarbon occupying less than 5% of the total. In many of these incubation

experiments, we noticed that a large fraction of the radioactivity appeared in the free sterol position. Digitonin precipitation and thin-layer chromatographic separation showed that the fraction was comprised of a mixture of free sterol and diglyceride materials. Since insect larvae are not able to synthesize sterol in the body and results of these incubation experiments did show an increase in percent weight of diglyceride material, the extra diglyceride fraction which seemed to appear after incubation could have a different structure than the one separated apart from the steroid fraction. In fact, the physical properties of 1, 2- and 1, 3-diglycerides are different and they also have different polarities (47). Consequently the two kinds of diglyceride, if they exist, may be expected to be eluted in slightly different positions from the column. However the fact that in vitro incubation of palmitate-1-C¹⁴ with larval fat body may give rise to two different kinds of diglycerides with different specific activity would require further investigations.

Although triglyceride fraction comprised 60% of the total radioactivity incorporated, diglyceride fraction showed the highest specific activity. In Chino and Gilbert's experiments (13, 14), where they injected palmitate-1-C¹⁴ into adult moths and pupae of the American silkworm, diglyceride fraction was also found to have the highest specific activity. In fact, the diglyceride fraction they isolated was more than a thousand times the specific activity

of the triglyceride fraction. They found that triglyceride in the fat body of the adult moth was more than 97% of the total composition while diglyceride fraction only comprised less than one percent. In the in vitro system of our experiments, we noticed a relative weight increase of the diglyceride fraction during incubation, from the usual 3% to 20% as shown in Table 11. The considerably lower specific activity of the diglyceride fraction observed in our experiments was most probably due to this increase in weight and this was not observed in the in vivo experiments described by Gilbert and Chino (13, 14). It is very probable that there was a rapid degradation of triglycerides into diglycerides and monoglycerides during the incubation due to the increase in hydrolytic enzyme activity.

Recently, the same authors demonstrated that extremely high specific activity was rapidly and specifically released from the insect's fat body into the hemolymph and that very little or no triglyceride release occurred. Diglyceride release also occurred in vivo as well as in vitro and in some other insect species. Therefore they suggested that the diglyceride release phenomenon could be common to many or all insects. If their suggestion was right, the increase in weight percent of the diglyceride fraction in our experiments could be explained by the fact that diglyceride was being accumulated in the fat body during incubation due to the absence of hemolymph as a transporting medium. The transport of lipid in the insect system

in the form of diglyceride release is then very different from the mammalian system where triglyceride is found the common means of transport.

Incubating palmitate-1-C¹⁴ with fat body at different time intervals indicated that a large portion of the radioactivity was incorporated into the lipid components within 30 minutes. Colodzin, Neptune and Sudduth (20) incubated rat diaphragm with palmitate-1-C¹⁴ and they also found that radioactivity was distributed in all three fractions of the neutral lipid after ten minutes. The experiment also showed that specific activity of diglyceride fraction exceeded that of other fractions at every incubation time. Specific activity of total lipid in the fat body increased twice within the first hour of incubation and no change in activity was observed after this time. This double peak feature in specific activity was probably due to the equilibration of the palmitate-1-C¹⁴ with the free fatty acid pool and its oxidation to carbon dioxide. This decrease in activity was not seen in the neutral lipid fraction. In fact, it would be of interest for later investigations, to examine the amount of incorporation in the free fatty fraction with time of incubation. Specific activity of the triglyceride fraction during the time course experiment showed a very similar pattern with the corresponding neutral lipid while specific activity of the diglyceride fraction remained highest of the three glyceride fractions throughout the entire incubation period. This

high specific activity found in the diglyceride fraction was in accord with the scheme for the synthesis of triglyceride proposed by Kennedy (36) where diglyceride served as the common intermediate for both the synthesis of triglyceride and phospholipid. However, this assumption certainly does not negate the presence of the monoglyceride pathway in the system as suggested by the other authors because palmitate-1-C¹⁴ was also incorporated into monoglyceride to a small extent.

Palmitate-1-C¹⁴ was also incorporated into the fat body of the seven-day old larvae but to a smaller degree. The ability of the prepupal fat body to incorporate palmitate-1-C¹⁴ into lipid was in agreement with the results shown in the first part which indicated that lipid content of the fat body continued to increase even after the larval feeding stage. However, the more apparent decrease in triglyceride with the corresponding increase of diglyceride in the neutral lipid after one hour incubation showed that the seven-day old larval fat body appeared to be more susceptible to hydrolytic enzyme degradation.

Thin-layer separation of the fatty acids indicated that part of palmitate-1-C¹⁴ was incorporated into the unsaturated fatty acids. From the results showing the amount of palmitate-1-C¹⁴ incorporated into unsaturated fatty acids with time of incubation, a large portion of the unsaturated fatty acids seemed to appear in the first ten minutes

of incubation. Therefore the process of conversion from palmitate-1-C¹⁴ to unsaturated fatty acids took place at a very fast rate. Results also showed that palmitate-1-C¹⁴ was incorporated to a much less extent in the unsaturated fatty acids of the fat body of the seven day old larvae in comparison with the three day old ones, in spite of the fact that a considerable amount of radioactivity was incorporated into the total fatty acids. In the chromatographic results given in Part I, an increase in unsaturation in the prepupal fat body was shown. The deposition of unsaturated fatty acids in the fat body must have proceeded at a greater rate before the prepupal stage.

The mechanism for the formation of unsaturated fatty acids in insects is not known. In other organisms, unsaturation takes place before the fatty acid is complexed with the glyceride moiety. The direct origin of unsaturated fatty acids from saturated precursors was first demonstrated by the classical work of Schoenheimer and Rittenberg (59). Bloomfield and Bloch (7) also worked out the requirements for the enzymatic conversion of palmitate to palmitoleate in yeast. Molecular oxygen, TPNH, and two enzyme fractions, one soluble and the other bound to particles were involved. He also found that the activation of the free fatty acids to the acyl-CoA derivatives was the first step in this process and then the activated molecule was subsequently desaturated in the presence of a particulate enzyme. Savary, Constantin and Desneulle (58) claimed that the

triglycerides in plants seemed to bind exclusively unsaturated chains to the internal glyceride carbon. The conversion of α -glycerophosphate to phosphatidic acid in Kennedy's scheme would then involve the action of two enzymes. The first one, acting closely with the phosphoryl radical, would bind the internal chain and would have, in plants, a very strict specificity for unsaturated chains. The second one, probably less specific, would bind the external chains. Mattson and Volpenhein (46) also found an exclusive esterification of unsaturated fatty acids such as oleic, linoleic and linolenic in the center position of the glyceride molecule in vegetable fat. Sedee (60) found a greater portion of the acetate- 1-C^{14} activity incorporated into unsaturated fatty acids of the blowfly larvae. He suggested that the biosynthesis of the saturated and unsaturated fatty acids took place by different mechanism, and not as the result of inter-conversion by dehydrogenation. Sridgara and Bhat (63) observed that most of the acetate- 1-C^{14} activity was incorporated into palmitate, stearate and oleic acids of the silkworm but not in the polyunsaturated fatty acids. The specific activity of oleic acid was lower than that of the saturated fatty acids.

The present work demonstrates the synthesis of unsaturated fatty acids from palmitic acid but the nature of the fatty acids formed remain to be elucidated. The fact that the ratio of the distribution of the label from palmitate between saturated and

unsaturated fatty acids is roughly the same as the percent distribution of these two types of fatty acids suggests that the major unsaturated fatty acids of the fat body of Sarcophaga bullata such as palmitoleic, oleic and linoleic acids are synthesized by dehydrogenation and elongation of the chain. No information is yet available on mechanism for the construction of the complex glyceride.

SUMMARY

During growth period the body weight of larva of Sarcophaga bullata increased in a rather linear fashion and the insect larvae accumulated large quantities of fat. The increase in lipid content even extended to the non-feeding prepupal stage. This is probably due to the result of internal reorganization of dry matter or due to the assimilation of the food material remaining in the crop after feeding has ceased.

Analyses of lipid content of fat body of Sarcophaga bullata larvae have shown that neutral lipid constituted roughly 90% of the total lipid and 90% of the neutral lipid was made up of triglycerides, while diglycerides and monoglycerides together occupied only 5%. There was a very small amount of sterols and sterol esters in the neutral lipid, a total of less than 3%.

High percentage of unsaturated fatty acids has been shown in the analysis of fatty acid composition of the larval fat body. The major fatty acids were found to be oleic, linoleic, palmitoleic, palmitic and stearic acids comprising 30, 10, 11, 20 and 10% of the total respectively. Fifty-five percent of the total fatty acids were unsaturated. This, in agreement with other's findings, indicated that high degree of unsaturation may be a characteristic of the insect lipid.

A comparison of the fatty acid distribution in the dietary food and the larval fat body showed similarity in that both were highly unsaturated and both contained about 10% palmitoleic and 25% palmitic acid; but there was a 10% difference in stearic acid between the dietary food and insect fat body. These similarities and differences indicated that insect fat body can assimilate fats present in their diet as well as synthesize them from the constituents of food.

Age and stage of the larval development of Sarcophaga bullata were found to cause a small change in the general composition of the fats such as an increase in unsaturation towards the prepupal stage. This increase in unsaturation during prepupal stage may be a preparative step towards metamorphosis.

Studies on the incorporation of palmitate-1-C¹⁴ and acetate-1-C¹⁴ with the fat body of Sarcophaga larva have shown that palmitate-1-C¹⁴ was rapidly incorporated into lipid of the fat body whereas most of the acetate-1-C¹⁴ was oxidized to carbon dioxide with less than one percent incorporation. In fact, with an adequate supply of lipid material in the larval diet, the fat body may preferentially utilize the dietary lipid material instead of synthesizing fatty acids from the two carbon units.

Most of the radioactivity from the palmitate-1-C¹⁴ was incorporated into the neutral lipid and free fatty acid fractions. Of the neutral lipid, triglyceride fraction comprised 60% of the total

radioactivity incorporated while diglyceride, on the other hand, showed the highest specific activity. This high specific activity found in the diglyceride fraction indicates that diglyceride may play an important role in lipid transport of the insect system.

Thin-layer separation of the fatty acids indicated that part of palmitate-1-C¹⁴ was incorporated into the unsaturated fatty acids and unsaturation seemed to take place at a very fast rate. The nature of the unsaturated fatty acids formed and the mechanism of this interconversion remain to be elucidated.

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