STUDIES ON THE BIOCHEMISTRY OF JUVENILE HORMONE AND OTHER INSECT LIPIDS

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ABSTRACT

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AND OTHER INSECT LIPIDS

By

Mark Allan Bieber

The aims of this research were to develop an accurate and reliable method for the quantitative determination of C_{18}-cecropia juvenile hormone, methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate, and conduct studies on its biosynthetic origin.

A reverse isotope dilution/carrier technique was devised for the quantitative determination of juvenile hormone and the occurence and levels of this molecule were studied in a variety of insects. After addition of a deuterium-labeled form of the juvenile hormone to an insect extract, the mixture of deuterium and protium forms were purified and the resultant fraction was analyzed by combined gas chromatography-mass spectrometry. The deuterium/protium ratios were obtained by multiple ion detection using the accelerating voltage alternator accessory of an LKB 9000 gas chromatograph-mass spectrometer. Levels of hormone in abdomina of both adult male and female cecropia, cynthia, and promethea moths, as well as in male and female housecrickets, worker honeybees, mixed male and female cereal leaf beetles, and housefly larvae were determined. The results confirmed the level of juvenile hormone previously reported for the male cecropia moth (approximately 1.6 μg per organism) and indicated sexual dimorphism. Female moths

contained less juvenile hormone than their male counterparts; female crickets, however, contained much more hormone than male crickets. When the results are presented on the basis of the amount of lipid extracted, housefly larvae and cereal leaf beetles stand out for their relatively low and high levels, respectively. As a sidelight, the elution profile of five classes of neutral lipids on Sephadex LH-20 in benzene-methanol (1:1) was determined by use of thin-layer chromatography and direct probe mass spectrometry of selected fractions.

Since the juvenile hormones elucidated are methyl esters, it was of interest to determine if other naturally occurring methyl esters were present in insects. A variety of naturally occurring fatty acid methyl and ethyl esters was found in late third instar housefly larvae reared aspetically and axenically. The fatty acid esters were identified by combined gas chromatography-mass spectrometry and their levels quantitatively determined by computer-controlled mass fragmentography. The total levels of methyl and ethyl esters found were 5.2 and 13.1 nmole per gram wet weight, respectively. This represented approximately 0.007% and 0.019% of the total lipid extracted. Levels of these esters in the synthetic diet were 3.6 and 0.2 nmole per gram, respectively. The esters were not localized to any extent in the insect cuticle and the possibility that they arose as artifacts of extraction or purification was ruled out by control experiments.

Attempts to elucidate the pathway of juvenile hormone formation in housefly larvae, grown aseptically and axenically on diets containing DL-[2-^{14}C]mevalonic acid and L-[*Me*-^{14}C]methionine, proved negative in

that no radiolabeled juvenile hormone was found at the levels of detection used. A thin-layer radiochromatogram of a portion of the lipid extracted after growth on labeled mevlaonic acid revealed at least five radioactive bands, three of which co-migrated with authentic neutral lipid standards (free fatty acid, triglyceride, and sterol ester). This finding would be consistent with a hypothesis that the mevalonate had been incorporated into a fatty acid that was present in these three fractions. The triglyceride fatty acids were therefore studied in detail after enzymatic hydrolysis by hog pancreatic lipase. The acids were esterified with diazomethane and subjected to analysis by gas-liquid radiochromatography. These analyses indicated that a radioactive fatty acid methyl ester was present in the region of the C_{17} methyl esters. Preparative gas-liquid chromatography was carried out to enrich this radioactive component. Through mass spectral analysis and comparative gas chromatographic data the radioactive unknown was tentatively identified as methyl 15-methyl hexadecanoate. It had a specific activity of 7.1 mCi/mmole which is consistent with the incorporation of one mole of mevalonic acid (specific activity=7.2 mCi/mmole). Three other gas chromatographic peaks eluting in the same area were analyzed by mass spectrometric techniques and tentatively identified as a mixture of methyl 14-methyl hexadecanoate and methyl 2,3-methylene-hexadecanoate, methyl heptadecanoate, and methyl 9,10-heptadecenoate. A possible biosynthetic scheme for the formation of the iso fatty acid arising from mevalonic acid is presented.

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LIST OF ABBREVIATIONS

AVA	Accelerating Voltage Alternator
GC-MS	Gas Chromatography-Mass Spectrometry
GLC	Gas-Liquid Chromatography
GLRC	Gas-Liquid Radiochromatography
JH	Juvenile Hormone*
POPOP	1,4-bis-[2-(5-Phenyloxazolyl)]-Benzene
PPO	2,5-Diphenyloxazole
ppt	parts per thousand
TII	Total Ion Intensity
TLC	Thin-Layer Chromatography
TLRC	Thin-Layer Radiochromatography

* Unless specifically noted, juvenile hormone (JH) connotes the C_{18} molecule isolated from cecropia, methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate.

INTRODUCTION

After many years of work in the isolation and partial characterization of the insect juvenile hormone from the giant silkmoth, *Hyalophora cecropia*, its structure was elucidated in 1967 by a research group composed of H. Roller, K. Dahm, C.C. Sweeley, and B.M. Trost (1). The structure of the insect juvenile hormone of the male *cecropia* moth is methyl *cis*-10,11-epoxy-7-ethyl-3,11-dimethyl-*trans*,*trans*-2,6-trideca-dienoate. It was characterized by use of nuclear magnetic resonance spectroscopy and combined gas chromatography-mass spectrometry of the natural hormone and its catalytic hydrogenation product. This structure was received with doubts by some members of the scientific community (2) since it was a methyl ester of an unusual terpenoid-like fatty acid never before encountered in a biological system. The doubts were soon removed when total chemical synthesis was achieved (3) and the biological activity of the synthetic product proved that the structure was correct. A second research group, headed by A. Meyer, confirmed the structure and found a second juvenile hormone in male cecropia the 7-desmethyl homolog, methyl *cis*-10,11-epoxy-3,7,11-trimethyl-*trans*,*trans*-2,6-trideca-dienoate (4,5) which accounted for 13-20% of the hormonal activity observed. A report was recently published in *Chemical and Engineering News* that methyl 10,11-epoxy farnesoate is the juvenile hormone of tobacco hornworm, however no other reports of this have appeared. These confirmations left all doubts behind and speculation that hormonal control

of insects could become the "third generation" of pesticides soon became apparent (6) since application of this developmentally important hormone during a vulnerable time in the insect's life cycle (directly after the last larval or nymphal molt) disrupts metamorphosis radically and creates a supernumerary larva or nymph unable to successfully complete life and become a functional and fecund adult.

Since the structure(s) of all these juvenile hormones resemble the terpenoid alcohol farnesol in carbon skeleton and positions of unsaturation, and insects have been reported to have the capacity for *de novo* synthesis of farnesol (7), it seemed as if the biosynthetic pathway of juvenile hormone formation in insects could be discovered without the long and tedious years of research that has been the case in the elucidation of many biosynthetic pathways now well characterized. Farnesol could be methylated, oxidized, and epoxidized to obtain juvenile hormone (JH). The use of new instrumental techniques such as combined gas chromatography-mass spectrometry (GC-MS), the growing use of computer applications in the biochemical field with these new instruments, and the perfection of classical methods on a micro scale made this possibility seem realistic. The original aims of this research, therefore, included studies on characterization of the biosynthesis of JH by use of *in vivo* radioisotope administration using several substrates that seemed to be likely precursors such as mevalonic acid, homomevalonic acid, methionine, acetate, propionate, leucine, or isoleucine.

It soon became apparent to the investigators in this field that the problem of JH biosynthesis would be much more difficult and elusive than expected (8). A major problem was that methods for purification and

quantitative determination were time consuming, difficult and somewhat inaccurate since much of the hormone was lost during manipulative procedures and biological assay was relied upon heavily for quantitation. The male cecropia moth, which contains more juvenile hormone than any other insect studied to date (9), has at most one or two micrograms per organism and therefore many insects would have to be studied. A primary objective of this research project therefore was to develop a reliable and accurate method for determination of JH in an insect extract, hopefully by techniques that would not involve the use of a biological assay since these have been proven to be inaccurate (10) and open to subjectivity and would require expertise not at hand. After a method had then been devised, biosynthetic experiments using the appropriately labeled precursors could be undertaken.

While the methodology for determination of JH was being developed by use of a reverse isotope dilution/carrier technique, reports that most of the obvious precursors for JH were not incorporated into the molecule in newly emerged male cecropia moths started to appear (11). Even though it therefore seemed that the biosynthetic experiments planned were not likely to succeed, attempts were made using mevalonic acid and methionine added to synthetic diets of housefly larvae reared aseptically. An interesting observation was seen upon thin-layer chromatographic analysis of the neutral lipid fraction that had become labeled after growth of housefly larvae on DL-[2-^{14}C]mevalonic acid. At this point in time, research emphasis was switched to attempt at least partial identification of some of the lipids that had become labeled through mevalonate.

Most of the accepted dogma concerning mevalonic acid has been that it is the first "totally dedicated" molecule in the biosynthesis of terpenes and sterols in mammals and plants (12) and the observation that several neutral lipid fractions became labeled through mevalonate became an interesting problem. Since it is well characterized that insects cannot make sterols *de novo* (13,14), characterization of the radiolabeled lipid could possibly lead to new metabolic fates of mevalonic acid or even to a possible cryptic precursor of the juvenile hormone that is biosynthesized and stored during the larval phase of growth.

Since the naturally occurring juvenile hormones are methyl esters, it was also of interest to determine whether insects (in this case, housefly larvae reared aseptically and axenically) contained other natural fatty acid methyl esters. In these investigations, the unexpected result of finding naturally occurring fatty acid ethyl esters prompted some studies on the characterization, localization, and levels of these fatty acid methyl and ethyl esters in this insect system.

In order to carry out much of the planned research, extensive use of combined GC-MS and multiple ion monitoring was found to be necessary. Efforts were therefore also directed into this area. These efforts, including a collaborative effort with Dr. Jack Holland and Richard Teets to computerize the process of multiple ion detection using the accelerating voltage alternator accessory of an LKB 9000 combined GC-MS, will not be presented as part of this dissertation.

LITERATURE REVIEW

Hormonal control of insect growth and metamorphosis was first postulated by Kopec in 1917 (15) from studies with the gypsy moth (*Porthertia dispar*) in which he removed the brains from young and mature larvae and observed that they continued to grow or pupate. Summaries of many of the early biological experiments done to determine that insect development was under hormonal control have been published in a volume by Wigglesworth (16). Since the observations were made that insect development was under hormonal control, this area of research grew to be of major importance not only to the developmental biologist but also to the biochemist and environmentally oriented entomologist since proper use of these hormones could lead to a highly effective approach to insect control.

Hormonal Control of Growth and Development

There are three basic hormonal systems operating in an insect that control development and these are, in turn, controlled by a myriad of factors. The three hormones are brain hormone; ecdysone, or molting hormone; and juvenile hormone, or *status quo* hormone. Extensive reviews of insect growth and development can be found in the following books and will not be specifically covered in this review. These include: The Control of Growth and Form (16), The Physiology of Insect Metamorphosis (17), Insect Hormones (18), and The Principles of Insect Physiology (19) all by Wigglesworth; The Physiology of Insecta

edited by Rockstein (20); and The Metabolism of Insects by Gilmour (21). Excellent review articles have also been written by Berkhoff (21), El-Ibrashy (22), Gilbert and Schneiderman (23), Siddall (24), and Williams (25,26). A brief review of an insect's endocrine system is included to give the reader an insight into the pertinent physiological relationships.

The brain, or supraesophageal ganglion, is divided into three sections--the protocerebrum, the deutocerebrum, and the tritocerebrum. In the anterior dorsomedial region of the protocerebrum are located the pars intercerebralis, where the medial neurosecretory cells are found. From the pars interecerebralis, axons leave to form bilaterally symmetric tracts that cross and then lead to the posterior edge of the brain. This pair of nerves, the nervus corpus cardiacus I, leaves the brain to terminate in the corpus cardiacum, a paired but often fused endocrine organ whose chief responsibility is storage and release of brain hormone produced in the neurosecretory cells. Lateral neurosecretory cells are located on either side of the protocerebrum and are also connected to the corpus cardiacum by axons. Directly underneath the corpora cardiaca are the corpora allata--paired, non-neural epithelial glands that are responsible for the synthesis and secretion of the juvenile hormone. The other main endocrine gland in the insects is the prothoracic gland, located in the prothorax in most species, sometimes called the ecdysial gland since it is responsible for synthesis and release of ecdysone, the molting hormone. The interaction of the hormones secreted by each organ are quite extraordinary. Brain hormone stimulates the prothoracic gland to secrete ecdysone, which, in turn,

acts at the cellular level and causes molting. Meanwhile, the corpora allata secrete juvenile hormone, determining whether the larval or nymphal molt will become another larva or nymph, or molt into a pupa or adult. The corpora allata radically increase in size at the time of juvenile hormone secretion (23) and the hormone has been chemically identified from *in vitro* organ culture (27). The juvenile hormone therefore permits growth but prevents maturation. These hormones seem to be synergistic and injection of one into the animal will induce the secretion of others (18).

The chemistry of brain hormone has remained elusive since it is present in very small quantities and difficult to bioassay (28). It was first thought that brain hormone was cholesterol (28); however, this has been proven to be incorrect (29) and it is now thought that brain hormone is a heat-stable non-dialyzable protein or polypeptide of molecular weight around 10,000 (29). It has not been purified to homogeneity and therefore nothing is known of its chemical composition. Secretion of brain hormone involves external nerve stimulation as in the case of molting in *Rhodnius prolixus*, a blood-sucking hemipteran. After ingestion of a blood meal, the movement of the stretch receptors tirggers nerve impulses which in turn stimulate the corpora cardiaca and then release of brain hormone, which triggers the release of ecdysone.

Ecdysone, the molting hormone, has been shown to be a water-soluble, hexahydroxy steroid having the structure 2β,3β,14α,22*R*,25-pentahydroxy-5-β-cholest-7-en-6-one. The elucidation of its structure by Butenandt and Karlson (30) by X-ray chrystallographic methods marked a milestone after years of research toward this goal. Since the structure

of ecdysone has been elucidated, at least five other similar insect and arthropod molting hormones have been discovered in many plants (24,31) and the biosynthetic origin of ecdysone from cholesterol (24) has been characterized. An excellent review of the chemistry and biochemistry of the ecdysones has been presented by Rees (129). The mode of action of ecdysone has been explored by many research groups, especially those of Gilbert, Laufer, and Clever. They have found that addition of ecdysone to the giant salivary gland polytene chromosomes of several species, especially *Chironomus tentans*, causes specific chromosomal puffing patterns (32,33,34). Through the use of differential staining, it has been determined that these puffs represent synthesis of RNA and DNA and therefore protein synthesis. These effects will be discussed in greater detail in later sections.

Juvenile Hormone

Overview: Comprehensive reviews of the history of juvenile hormone (JH) research have been published. The biology of the hormone and its function in relationship to the other insect hormones was discussed by Schneiderman and Gilbert in 1964 (28) and chemical aspects of the hormone have been reviewed by Roller and Dahm (8), Pfiffner (35), and Trost (36). Research interest in JH and JH mimics or analogs has grown over the past years to the point that it has merited no less than three major international symposia and the proceedings of these have been published (37,38,39). The reader is referred to any of these references for additional information not presented in this review.

Discovery: The existence of a *status quo* or juvenile hormone that would determine the fate of the subsequent molt was first postulated by

Wigglesworth in the late 1940's (16). It was not until 1956, however, that Carroll Williams at Harvard discovered a rich source of JH activity in an ethereal extract of male cecropia moth abdomina (40). He termed this extract "golden oil" due to its striking color and proceeded to test its juvenilizing effects on a variety of insects (130). He also found JH activity in a variety of sources including lipid extracts of mammalian organs such as thymus and placenta (41). These unusual findings and the need for a quantitative measure of JH in lipid extracts prompted the development of many biological assays for JH. Juvenile hormone bioassay has classically involved topical application or injection of a hormonal agent in an appropriate solvent at the proper stage in the insect's life cycle with subsequent scoring of the next molt by well established and sometimes highly sophistocated criteria. Several of the most accurate and popular bioassays are the *Galleria* wax test (42), the *Tenebrio* assay (8), the *Polyphemus* bioassay (43) and the *Rhodnius* bioassay (44). Until the structure of the hormone was known, many of the bioassay results were presented on an artificial scale of "cecropia units", relating back to the potency of "golden oil". A cecropia unit was later found to be equivalent to 3 ng of actual JH (2). The pitfalls and problems encountered in bioassay have been discussed by Staal (10). He noted that bioassays, if done properly, could be of great use. In the wax test, for example, hormonal agents in concentrations as low as 10^{-6} µg can be measured (10) and therefore these assays seem to be more sensitive than any chemical techniques developed to date.

Elucidation of Structure: Soon after the discovery of JH, the search was underway to find out the chemical nature of this molecule.

Through the work of Gilbert (45) it was determined that JH was a non-saponifiable neutral lipid. Smialek (7,46) isolated several milligrams of the terpenoids farnesol and farnesal from the feces of the yellow mealworm, *Tenebrio molitor*. He found, however, by bioassays that these compounds gave results which could not be reconciled with their being the juvenile hormone, due to their low specific activities. Law and Williams (47) isolated a fraction enriched at least 50,000-fold in JH activity and found that the main component was methyl 9,10-epoxypalmitate. Upon chemical synthesis of this substance they found that the synthetic product was totally inactive as a hormonal agent and theorized that JH was present as a minor component in their final fraction.

Successful purification of JH was achieved by Roller *et al.* (48,49,50) and, in 1967, they obtained 810 μg of pure JH from 875 male cecropia abdomina. Through the use of nuclear magnetic resonance spectroscopy and mass spectrometry of the natural hormone and its catalytic hydrogenation product, the structure was reported (1) to be methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. This structure was soon confirmed by a research group headed by Meyer (4,5,51) who had been seriously working on the problem since 1963. They also discovered a second hormone present in male cecropia that accounted for 13-20% of the biological activity and found that it was the 7-desmethyl homolog of JH, methyl 10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate. Both of these structures have been confirmed as the juvenile hormone of another giant moth, *Hyalophora gloverii* (52), and preliminary evidence has been presented that these are also the juvenile hormones of the silkmoth *Samia cynthia* (53). A third JH molecule, methyl 10,11-epoxy

farnesoate has been reported (54) to be a natural product of the tobacco hornworm, *Manduca sexta*. No experimental details or further reports concerning this latter structure have been published.

The few structural ambiguities of the JH's were solved and the stereochemistry, optical rotation, and chirality of the molecules have been determined (8,55). The cecropia juvenile hormones are: (+)-*cis*-10*R*,11*S*-10,11-epoxy-7-ethyl-3,11-dimethyl-*trans*,*trans*-2,6-tridecadienoate and (+)-*cis*-10*R*,11*S*-10,11-epoxy-3,7,11-trimethyl-*trans*,*trans*-2,6-tridecadienoate.

Chemical Syntheses: The first chemical synthesis of JH was achieved by the Wisconsin group within nine months after publication of the structure (3). This synthesis involved a lengthy procedure with very poor yield of a racemic product. Since then, at least 16 additional syntheses of the JH molecules have been published (56-71), including the elegant syntheses of Corey in which several new reactions involving trisubstituted olefins were originated (72). These chemical syntheses run the gamut from being stereospecific to yielding a rapid method for obtaining a mixed racemic product. A highly active area of research, the chemical syntheses of JH analogs will be discussed in a later section.

Biosynthesis and Metabolism: There seem to be several plausible biosynthetic routes for JH biosynthesis that can be envisioned in view of its structural resemblence to the terpenoid alcohol farnesol in carbon skeleton and positions of unsaturation. Research in this area has revealed that elucidating the biosynthetic pathway of JH has become a difficult problem. Most of the work has been carried out

in vivo on newly emerged adult male cecropia moths; however use of corpora allata organ culture has been reported (73). Metzler *et al.* (11,53) reported that methionine is incorporated exclusively into the methyl ester methyl group and not into the carbon skeleton to any measureable extent. They also found that neither mevalonic acid, farnesol, farnesyl pyrophosphate, nor propionate was utilized in the biosynthesis of the carbon skeleton. Only acetate (and that at a very low level of incorporation) was found to be incorporated into the carbon skeleton of JH. They also found that radiolabeled 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoic acid injected into newly emerged male moths gave rise to radiolabeled JH and proposed that the esterification with methionine was probably the final step in the biosynthesis of JH. Evidence for such enzymatic activity has been shown by Akamatsu and Law (74) who characterized an enzymatic reaction from *Mycobacterium phlei* that promoted esterification of fatty acids using S-adenosylmethionine as the methyl donor and oleic acid as the preferred substrate.

Ajami and Riddiford (73) recently reported that labeled JH could be recovered after incubations *in vivo* with acetate, glucose, methionine, and, to less extent, isoleucine and valine. They also found that bishomofarnesol, farnesol, lysine, 4-methyl-*cis*-3-hexenol, 4-methyl-3-pentenol, propionate, and mevalonic acid did not contribute to the formation of JH. After incubations *in vitro* with corpora allata, they recovered label in the JH fraction only when glucose served as a precursor and concluded that the pathway of carbon incorporation into JH must be significantly different from the pathways leading to fatty

acids and terpenoids commonly found in insects.

In view of the above findings, the idea of a cryptic precursor for JH synthesis could be a reality, i.e., a molecule such as the JH-acid is made during the pupal or late larval stage of growth and stored, possibly as part of a triglyceride, until completion of the molecule by the adult is necessary. Research in this direction is currently underway at the Zoecon Corporation (75).

Although many unanswered questions have been posed in reference to JH biosynthesis, a wealth of information is known about its metabolic fate. It has recently been reported that a specific lipoprotein exists in insect haemolymph that acts as a JH carrier (76) and that the half-life of the molecule varies from several hours to three days in the adult (77,78,79). Catabolism of JH has been studied in at least 20 different species (77-79) and all have been shown to include ester hydrolysis and epoxide hydration. This diol-acid has been found to be totally inactive as a hormonal agent in at least two species tested (79). Excretion of the JH-diol-acid as a glucoside or glucuronide seems to be its final metabolic fate, since conjugated polar metabolites have been found in the feces and incubation of this fraction with glusulase has revealed JH-acid-diol. From the very limited observations of Slade and Zibitt (78), it seems as if JH is metabolized in a similar fashion in mice. It has previously been shown that JH is not toxic to mice after a single dose oral ingestion at a level of 5000 mg/kg body weight (80). Alternative catabolic fates of JH via fatty acid oxidations, as has been shown for the terpene-like phytanic acid (81) in mammals, have not been shown to occur in insects.

Biological Effects: Many functions have been claimed for JH, including egg maturation, accessory sex gland secretion, control of larval and pupal diapause, mating, and general metabolism. The most important property investigated to date, however, has been its morphogenetic activity. Various viewpoints have been taken as to how growth and differentiation are mediated by JH. Williams first suggested that ecdysone functions as the active promotor of growth and metamorphosis and that JH functions as a "brake" on this development (6). Roller and Dahm (8) indicated that JH modified the expression of a molt in such a way as to favor development of larval structures. Wigglesworth (18) maintained that JH possibly has an effect of activating the larval genome. The ability of JH to "reverse" metamorphosis supports the hypothesis for an active role for it. Williams and Kafatos (82,83) have published a new theoretical model for JH function in which master genes (larval, pupal, or adult) are controlled by JH-dependent repressors; successive master genes are derepressed as JH titer decreases throughout the life cycle.

Experimental evidence for direct action of JH at the cellular level has been presented by Lezzi and Gilbert (32,84), who showed that application of JH caused specific chromosome puffs in the Balbiani Ring I of the giant polytene chromosomes of the salivary gland of *C. tentans*. At the same time, several ecdysone specific puffs were decreased and they proposed that an antagonistic relationship existed between these two hormones. Laufer and Holt (34) confirmed the JH results but were unable to find the antagonism proposed by Lezzi and Gilbert. Since these specific puffing patterns can be induced by careful manipulation

of the sodium and potassium levels (84), the possibility exists that these hormones act indirectly on gene activation by altering ion permeability. Ilan *et al.* (85) have postulated that JH exerts control at the level of translation from experiments in *T. molitor*. Williams and Kafatos (82,83), however, argue that JH must act at the level of transcription and have presented evidence for this in their theory. Zalokar (86) demonstrated that stimulation of the coropora allata caused increased incorporation of tritium labeled uridine into RNA in the german cockroach, *Blattela germanica*. Whatever the mode of action of JH is found to be, the effects of the hormone are all encompassing in the insect. Further research in this area will certainly be forthcoming with the availability of tritium labeled JH of very high specific activity (87).

Juvenile hormone has been directly implicated with the reproductive capacity of insects and has been shown to induce vitellogenin synthesis in the monarch butterfly, *Danaus plexippus* (88), induce yolk protein synthesis (89) and the synthesis of female-specific proteins in the cockroach, *Leucophaea maderae* (90), and promote ovarial development in the Douglas-fir beetle, *Dendroctonus pseudotsugae* (91). Juvenile hormone also plays an important role in the programming of the post-embryonic development in the eggs of *H. cecropia* (92,93). The hormone has been shown to induce specific esterases (94) and it seems a well documented fact that extirpation of the corpora allata promotes lipid synthesis in a variety of insects (95). Concomitantly, increasing JH titer corresponds with a decreasing rate of lipid synthesis in *H. cecropia* (96). Juvenile hormone has been found to be a regulator of diapause and incorporation of corpora allata

or application of JH or a JH analog can abruptly terminate diapause (18,97).

Analogs: After the discovery of JH activity in the cecropia moth and the identification of farnesol and farnesal as JH active compounds, the push to find and/or synthesize other JH active molecules was started. At least five hundred compounds have now been synthesized and their possible uses as insecticides investigated. A majority of this work and the structure/activity realtionships found has been reviewed by Slama (9) and the current status of the field has been summarized in a series of articles in *Science* (98). A review of some of the findings with field experiences has also been presented (99).

Several hormonal analogs stand out in their importance due to unusual activity and specificity. One of the most interesting of these is methyl 10,11-epoxy farnesoate, called Bowers' compound since it was synthesized by Bowers *et al.* (100) in 1965. It is amazing that this molecule was synthesized two years before the structure of JH was known and seven years later it has been identified as an actual JH itself. Another highly potent compound was prepared in 1966 by Law, Yuan, and Williams (101) after they bubbled hydrogen chloride gas into an ethanolic solution of farnesoic acid. This mixture is in wide use and has been commercially available for several years. The active principle was identified by Romanuk *et al.* (102) and is an ethyl ester of 3,7,11-trimethyl-7,11-dichloro-2-dodecenoic acid. In 1965, Slama and Williams (103) observed that certain American paper products contained a source of JH activity for the bug *Pyrrochoris apertus*. They determined that the active principle, called the paper factor, was found to have its origin in the wood of certain pulp trees, especially that of the balsam

fir. It is a specific hormonal agent for *P. apertus* and inactive on all other insects studied. Bowers *et al.* (104) identified the active component to be the methyl ester of todomatuic acid, and called it juvabione. An additional compound, specific for the same insect, has since been characterized from balsam fir (105); it is dehydrojuvabione.

Compounds have been synthesized that bear little resemblance to JH and still possess high activity. Aromatic, peptidic, and nonterpenic compounds now exist that show potential for use as insecticidal agents (9) and field tests of many of these are in progress (98). The future of hormonal control of insect populations and Carroll Williams' prediction that JH or JH analogs could become the "third generation" of pesticides may become a reality.

Fatty Acid Methyl and Ethyl Esters

Methyl and ethyl esters of long chain fatty acids have been reported from a wide variety of tissues and organisms, in levels ranging from 0.004% to 18% of the total lipid extracted from guinea pig liver (106), mouse liver (107), human liver (108), human pancreas (109,110), ox pancreas (111), various organs of dog, rat, and monkey (112), corn pollen (113), *Rhizopus arrhizus* (114), and *Tetrahymena pyriformis* (115). The possibility that they are artifacts has been investigated under various conditions of extraction and purification (107) and their presence is usually attributed to solvent or chromatographic catalyzed esterification (116-119). The occurrence of fatty acid methyl and ethyl esters in insects has been reported for grasshopper eggs (120),

bumblebees (121-123), and beetles (124,125). Although no specific physiological function has been determined for these methyl and ethyl esters in insects, Calam (126) pointed out that they could be called pheromones in the broadest sense since they are found in the assembling scent of beetles and have been identified in the territory-marking substances of bees and therefore influence insect behavior and may play a role in insect communication.

Biochemical Fates of Mevalonic Acid

The metabolism of mevalonic acid in plants, insects, and mammals in relation to terpene and cholesterol biosynthesis has been pursued since Tavormina *et al.* (127) established that mevalonic acid was the precursor of the isoprene unit. Since that time, hundreds of research publications have arisen concerning terpene and cholesterol biosynthesis. The results of a majority of this work can be found in a recent Biochemical Society Symposium on substances formed biologically from mevalonic acid (12). Insect terpenoids have been reviewed by Karlson (128) and the capacity for insect synthesis of terpenes but not cholesterol has been under continual investigation (11,13,14,131-133). It is not the aim of this section to present a detailed review of isoprene synthesis and the reader is therefore referred to any of the above references to additional information on this subject.

Reports that mevalonic acid has been incorporated into seemingly non-terpenoid neutral lipid fractions have been presented for the adult housefly (134), *Neurospora crassa* (135), and cardiovascular homogenates (136), however no specific identifications were made. Labeled palmitic, stearic, and oleic acids were tentatively identified

as arising from mevalonate in incubations *in vivo* in the silkmoth, *Bombyx mori* (137), however the techniques used in this study are open to question. Further evidence for mevalonic acid incorporation into non-isoprenoid lipids will be presented in the Discussion.

MATERIALS AND METHODS

MATERIALS

Insects

Hyalophora cecropia, Samia cynthia, and *Callosamia promethea*	General Biological Supply House, Chicago, Ill.
Apis mellifera (without queen)	Wilbanks Apiaries, Claxton, Ga.
Acheta domestica	Fluker's Cricket Farm, Baton Rouge, La.
Olema melanopus	Gift or Dr. S. Wellso
Musca domestica	Cultured as described in Methods. Eggs gift of Dr. R.E. Monroe

Chemicals

The common reagents were all of reagent-grade quality. Special reagents used are listed below.

Solvents

General Solvents	All solvents were routinely redistilled by continuous rotary evaporation before use.
Diethyl Ether	Diethyl ether was redistilled in glass and stored over Type 3A Molecular Sieve (Fisher Scientific Co., Pittsburgh, Pa.)

Chromatographic Supplies

Florisil (acid-washed, 80/100 mesh)	Fisher Scientific Co.
Sephadex LH-20	Pharmacia, Piscataway, N.J.
Silicic Acid (Unisil, 200/325 mesh)	Clarkson Chemical Co., Williamsport, Pa.

Thin-Layer Plates	
Silica Gel G, 250 μm	Quantum Industries, Fairfield, N.J.; Analtech Inc., Newark, Del.
Silica Gel G, 500 μm	Made on a Reeve-Angel Quickfit Spreader, Model 8CR by slurrying 55 g Silica Gel G (Brinkman Industries, Des Plaines, Ill.) with 90 ml glass distilled water.
Silica Gel G, 500 μm, impregnated with 5% Silver Nitrate	Made on the plate spreader by slurrying 55 g Silica Gel G with 90 ml 5% Silver nitrate (w/v)
1% and 3% SE-30 on Supelcoport (100/120 mesh); 15% EGA on Chromosorb WHP (80/100 mesh)	Supelco, Inc., Bellefonte, Pa.
3% XE-60 on Gas-Chrom Q (80/100 mesh); 3% EGSS-X on Gas—Chrom Q (100/120 mesh)	Applied Science Laboratories, State College, Pa.

Lipids

Fatty Acid Methyl Esters	Made by acid-catalyzed methanolysis of fatty acids extracted from Ivory Soap (Procter and Gamble, Cincinatti, Ohio)
Triolein, Fatty Acid Methyl Esters, Fatty Acid Ethyl Esters, Palmitic Acid, Stearic Acid	Applied Science Laboratories
Cholesteryl Palmitate, Cholesteryl Oleate, Cholesteryl Stearate	Supelco, Inc.
Farnesol, Geraniol, Nerolidol, Geranylgeraniol	K and K Laboratories, Jamaica, N.Y.
Squalene, Tripalmitin, Tristearin	Eastman Organics, Rochester, N.Y.
Cholesterol, Oleic Acid	Fisher Scientific Co.
Phospholipid TLC Standards	Gift of Dr. W. Esselman
Total lipid extracted from 100 ml human plasma	Gift of Dr. C. Mapes
Cecropia Juvenile Hormone	Gift of Drs. D.J. Faulkner and M.R. Petersen, Scripps Institute, La Jolla, Calif.

Isotopically Labeled Compounds

[$^2H_3$]Methyl Ester of *Cecropia* Juvenile Hormone; $CH_3C[^2H_2]$-Ethyl Ester of *Cecropia* Juvenile Hormone	Gift of Drs. D.J. Faulkner and M.R. Petersen, Scripps Institute, La Jolla, Calif.
DL- [2-^{14}C]Mevalonic Acid (DBED salt, 14.3 mCi/mmole, free acid, 7.2 mCi/mmole)	New England Nuclear, Boston, Mass.
L- [*Me*-^{14}C]Methionine (10.5 mCi/mmole)	New England Nuclear
DL- [7-*Et*-1,2-$^3H_2$]*Cecropia* Juvenile Hormone (14.1 Ci/mmole)	New England Nuclear
Methyl [1-^{14}C]Palmitate (54.6 mCi/mmole)	Applied Science Laboratories

Scintillation Fluid

DPO Toluene	Prepared by dissolving 4 g PPO and 50 mg POPOP per liter toluene. PPO and POPOP were obtained from Research Products International, Elk Grove Village, Ill.

Quench Gases

Propane, natural grade, 96%	Matheson Gas Products, Joliet, Ill.
P-10 Proportional Counting Gas (90% Argon, 10% Methane)	Matheson Gas Products
Q-10 Quench Gas (98.7% Helium, 1.3% Butane)	Matheson Gas Products

Miscellaneous

M. domestica synthetic diet:

Sodium Oleate	Fisher Scientific Co.
Casein, Yeast Extract, Celluflour, Wesson Salts	Nutritional Biochemicals, Cleveland, Ohio
Bacto-Agar	Difco Labs, Detroit, Mich.
Lipase (Hog Pancreatic), Sodium Taurocholate, Sodium Cacodylate, Osmium Tetroxide	Sigma, St. Louis, Mo.

Lithium Aluminum Hydride	Ventron, Beverly, Mass.
N-Methyl-N'-Nitroso-N-Nitro-guanidine	Aldrich Chemical Co., Milwaukee, Wisc.
10% Palladium on Carbon	Engelhard Industries, Newark, N.J.
Rhodamine 6G	Allied Chemicals, Morristown, N.J.

METHODS

Insect Rearing

Cecropia moth, *Hyalophora cecropia*, cynthia moth, *Samia cynthia*, and promethea moth, *Callosamia promethea*, cocoons were allowed to develop at 32° with a 14 hour photoperiod. After adult emergence, the insects were allowed to mate, and at 6-7 days post-emergence, abdomina from male and female moths were collected, weighed, and either stored in diethyl ether at -20° or homogenized directly. Worker honeybees, *Apis mellifera*, (without queen) were anesthetized with CO_2, counted, weighed, and stored in diethyl ether at -20°. Housecrickets, *Acheta domestica*, obtained as young adults, were sexed, weighed, and stored in diethyl ether at -20°. Cereal leaf beetles, *Olema melanopus*, were homogenized directly after weighing.

Larvae of the common housefly, *Musca domestica*, were cultured axenically on synthetic diets as described (138). One mg cholesterol and 12 ml glass distilled water were added for each gram of dry diet used prior to autoclaving; 30 g of diet were used for a 2.5-l Fernbach flask. Eggs were surface sterilized with 0.2% sodium hypochlorite for 20 minutes and transferred to the sterilized diet. Larvae were reared in total darkness at 35° and were collected as they entered the migrating stage, thus indicating that the majority of the larvae were in the latter

part of the third instar. Approximately 25-35 g of larvae could be obtained from one 2.5-l Fernbach flask. Larvae were homogenized directly after weighing.

Extraction of Lipids

Extraction of Juvenile Hormone: The insect or insect fragment was homogenized in three volumes of diethyl ether in a VirTis Model 23 glass Teflon-capped homogenizer at high speed for five minutes. The residue was subsequently re-homogenized in three volumes of diethyl ether-methanol (9:1) for an additional five minutes. After filtration, this residue was extracted by continuous stirring in ten volumes diethyl ether until no additional lipid could be detected by Land's spot test (187) (about three extractions for 12 hours each). When less than 10 g was extracted, the extraction volumes were increased by five-fold. The combined lipid extracts were pooled, a known amount of the deuterated JH was added (usually 75 µg) and the solution was filtered through a sintered glass funnel of fine porosity containing anhydrous sodium sulfate. The total lipid extract was then taken to dryness under reduced pressure at 35° in a tared round-bottom flask.

Extraction of Fatty Acid Methyl and Ethyl Esters: Housefly larvae were homogenized in a VirTis Model 23 glass Teflon-capped homogenizer at high speed for five minutes in five volumes diethyl ether. To study cuticular lipids as well, the larvae were first slurried gently in ten volumes chloroform (without added alcohol stabilizer) for 30 minutes. The solvent was decanted and the larvae were homogenized as described. In either case, the extract was filtered and the residue was subsequently homogenized in five volumes of diethyl ether-ethyl acetate-acetone (65:30:5). Finally, the residue was refluxed in ten volumes of

diethyl ether-ethyl acetate-acetone (50:30:20) for 90 minutes. After cooling, the extracts were pooled and filtered through anhydrous sodium sulfate in a solvent-washed sintered glass funnel of fine porosity and evaporated to dryness under reduced pressure at 35° in a tared round-bottom flask. After weighing, the total extract was placed in hexane (50 ml/g).

Twenty grams of the synthetic diet were refluxed in the aforementioned solvent mix for 90 minutes and the extract treated as described above. For control experiments, tripalmitin, triolein, tristearin, and oleic acid (100 mg each) were refluxed in 100 ml of the same solvent mix for 90 minutes and treated as described above.

Purification of Lipids

Purification of Juvenile Hormone: The mixtures of protium and deuterium forms of JH were purified according to the methods of Dahm and Roller (52). The total lipid extract was placed in diethyl ether (10 ml/g) in a round-bottom flask and equilibrated to -78° in an ethanol-dry ice bath for fifteen minutes. An equal volume of cold methanol (-78°) was then added and, after one hour, the mixture was filtered under aspirator suction through a sintered glass funnel of medium porosity at -30°. The filtrate was taken to dryness under reduced pressure at 35° and a minimal amount (approximately 2 ml) of benzene-methanol (1:1) was added to the filtrate and it was chromatographed on Sephadex LH-20 which had been swollen and packed in the same solvent mixture. The column, 2.5 x 90 cm, had a void volume of 180 ml, as determined with polyethylene glycol 6000, and a total volume of 450 ml; it could accommodate up to 3 g of lipid. Fractions (2 or 4 ml)

were collected with benzene-methanol (1:1) as the eluting solvent. Fractions containing JH were established by several trial runs with synthetic and radiolabeled JH. At least 95% of the lipid applied to the column was eluted within one column volume. After chromatography, appropriate fractions were pooled and the solvent was evaporated by rotary and micro evaporation under a stream of dry nitrogen. Prior to column chromatography, however, two aliquots (10 µl each from the 2 ml) were taken out and applied directly to a TLC plate; one spot was sprayed with Zinzandee's reagent (molybdenum blue) (139) and the other with α-napthol spray (140) for detection of phospholipids and glycolipids, respectively. The lower limits of detection with these TLC spray reagents are 2 µg for phospholipids and 1 µg for glycolipids, respectively.

Crude JH was further purified by thin-layer chromatography on non-heat-activated Silica Gel G plates (250 µm, Quantum) in paper-lined tanks that had been equilibrated for at least one hour. The first separation was in chloroform-ethyl acetate (2:1) and the second in benzene-ethyl acetate (95:5). When necessary (see below), a third separation was achieved using chloroform-pentane (2:1). A maximum of 10 mg lipid was applied per plate in the first separation and 6 mg lipid in the second or third separations. These will be referred to as TLC-1, TLC-2, and TLC-3 in subsequent sections of the dissertation. The center portion of the plate was covered tightly with Saran Wrap and the edges, including standards, were briefly exposed to iodine vapors to locate the JH band, which was then removed from the plate with a razor blade and the gel eluted with 20-30 ml of diethyl ether-

ethyl acetate (1:1) per plate.

The purification was monitored by gas-liquid chromatography (see instrumental) and was not considered complete until only one major peak was obtained.

Purification of Fatty Acid Methyl and Ethyl Esters: The total extract (larval, diet, or control) was partitioned between hexane and an equal volume of water after which the water phase was extracted three times with an equal volume of hexane. The combined hexane layers were taken to dryness under reduced pressure at 40° in a tared round-bottom flask. The total lipid was subsequently placed in a minimal volume of hexane and chromatographed on Florisil which had been packed as a slurry in hexane. The Florisil had been heated at 120° overnight and then de-activated by the addition of 7 ml water per 100 g and equilibrated with air at 23° for at least two hours before use; one g of Florisil was used for each 50 mg lipid (141). Fatty acid esters were eluted with seven column volumes of 1% diethyl ether in hexane. This fraction was evaporated, weighed, and then chromatographed on pre-coated TLC plates (Silica Gel G, 250 µm, Analtech) in hexane-diethyl ether-acetic acid (80:20:1). A maximum of 10 mg lipid was applied per plate. This solvent system will be referred to as the neutral lipid solvent system in the remainder of the dissertation. After chromatography, the center portion of the plate was covered tightly with Saran Wrap and the edges, including standards, were briefly exposed to iodine vapors. The band migrating with authentic fatty acid esters was marked, the iodine was allowed to evaporate from the plate (about 2 hours at 23°), and this band was scraped with a razor blade into a solvent-

washed sintered glass funnel. The gel was eluted with 20-30 ml of diethyl ether-ethyl acetate (1:1) per plate. For studying cuticular lipids, TLC was used directly in the neutral lipid solvent system using the above techniques. At no time during either the extraction or purification procedures was methanol or ethanol used.

Preparation of Fatty Acid Methyl Esters: Approximately 2 g of Ivory Soap were dissolved in 100 ml distilled water. The solution was acidified to pH 4 with 1 N HCl and the free fatty acids were removed from the top of the solution with a spatula, dried on pieces of filter paper, and then placed in 100 ml of 1 N HCl in methanol. The solution was stirred at 23° for two hours, extracted three times with an equal volume of hexane, and the hexane layer was taken to dryness in a tared round-bottom flask. Working solutions of 10 mg/ml and 1 mg/ml were made up using hexane.

Purification of Standards: All TLC, GLC, GLRC and GC-MS standards were checked for purity by the appropriate technique (see instrumental). Only oleic acid needed further purification; this was carried out by preparative TLC on 500 μm plates in the neutral lipid solvent system using previously described procedures.

In vivo Studies in Housefly Larvae

Radioisotope Administration: In separate experiments, aqueous solutions of DL-[2-^{14}C]mevalonic acid (50 μCi, 10.6 μmole) and L-[*Me*-^{14}C]methionine were mixed directly with 30 g synthetic diet in 2.5-l Fernbach flasks prior to autoclaving. Larval growth under these conditions appeared to be normal. The larvae were collected, extracted,

and the extract purified exactly according to the procedures previously outlined for extraction and purification of JH.

Growth on radiolabeled mevalonic acid was repeated in a separate experiment (50 μCi, 10.6 μmole). The larvae were collected and extracted by procedures outlined for extraction of fatty acid esters; an additional extraction of the final residue by a 90 minute reflux in ten volumes of chloroform-methanol (2:1) yielded a fraction enriched in polar lipids.

DL-[2-^{14}C]Mevalonic acid was supplied as its dibenzylethylenediamine salt. Dibenzylethylenediamine was removed from the mevalonic acid by adding an excess amount of sodium bicarbonate (20 μmole, 1.7 mg in 2 ml water) to free the base. The amine was removed by extraction with 2 ml diethyl ether three times, the dissolved diethyl ether was removed under a stream of dry nitrogen, and an equimolar amount (20 μmole) of HCl was added to neutralize the aqueous solution of the sodium salt. Less than 1% of the radioactivity was lost during this procedure, as determined by liquid scintillation counting of the total ethereal extract (see instrumental).

Radiochemical purity of the mevalonic acid, both before and after autoclaving, was determined by ascending paper chromatography using strips of Whatman No. 3 filter paper in n-propanol-ammonium hydroxide (7:3). The paper strips were scanned on a Packard Radiochromatogram Scanner at 1050 volts using Q-10 quench gas at 300 ml/min. Only one radioactive peak was obtained, but considering the noise level of the stripscanner under the operating conditions employed, the radiochemical purity of the mevalonic acid could only be determined as

at least 97%. The radiochemical purity of the labeled methionine was not checked; it was stated to have been greater than 97.5% by the supplier.

Product Identification from Growth on DL-[2-^{14}C]Mevalonic Acid: The extracted lipid was partitioned by the Folch procedure (142) using ten volumes chloroform-methanol (2:1) and aliquots from the lower phases obtained from the differential methods of extraction (one-tenth of the total extracted from each) were pooled and separated into neutral and polar fractions by silicic acid column chromatography following established procedures (143). Five g of Unisil were packed as a slurry in chloroform and the lipid was applied to the column in chloroform containing 2% methanol. Neutral lipids were eluted with 80 ml of chloroform containing 2% methanol (8 column volumes); polar lipids were eluted with 100 ml of methanol. Thin-layer chromatography of the entire neutral lipid fraction was performed in the neutral lipid solvent system as previously outlined using 6 TLC plates, and the entire polar fraction was chromatographed on 2 TLC plates in a polar lipid solvent system, chloroform-methanol-water (100:42:6). Radioactive bands were identified by means of a Berthold Radioscanner Model 6000 operated at 2100 volts using P-10 quench gas at 40 ml/min. Average efficiency for ^{14}C was 20% and that for ^{3}H was 3% as determined by scanning known amounts of standard radiochemicals on the plate scanner. After brief exposure to iodine vapor to locate mass, the radioactive bands were scraped from the plate with a razor blade and eluted from the gel using 20-30 ml diethyl ether-ethyl acetate (1:1) per plate. After evaporation of the solvent, these fractions were further

subjected to mild alkali-catalyzed methanolysis and acid-catalyzed methanolysis. The fraction migrating with triglyceride as well as the entire neutral lipid fraction were subjected to lipase treatment and total reduction with lithium aluminum hydride.

Approximately 2000 dpm per TLC fraction were used for both methanolysis procedures. The sample was placed in 5 ml of chloroform and then 5 ml of 0.6 N NaOH in methanol and let stand for two hours at 23°. After acidification with 0.26 ml 12 N HCl, 5 ml chloroform and 4 ml water were added, with vigorous mixing by vortex after each addition. After separation, each phase was removed, evaporated to dryness under a stream of dry nitrogen, 1.0 ml toluene was added, and 0.5 ml of this solution was counted in 10 ml DPO toluene in a liquid scintillation counter (see instrumental). Acid-catalyzed methanolysis was accomplished in 2 ml of 1 N HCl in methanol at 80° for four hours. The samples were allowed to cool, an equal volume of hexane was added, and the tubes were shaken vigorously. After separation, the biphasic system was treated identically as described above.

Optimal conditions for enzymatic release of triglyceride fatty acids with hog pancreatic lipase were determined using triolein as substrate. Both the band migrating with triglyceride and the total neutral lipid extract (see silicic acid column chromatography above) were incubated in 0.02 ml 25% sodium taurocholate, 0.2 ml $CaCl_2$, 1.7 ml 1.2 M cacodylate buffer, pH 7.4, and 0.1 ml enzyme (approximately 250 units) for two hours at 37°. The lipid was first evaporated to dryness under nitrogen in a 20 ml screw-capped tube and the reactants

were added in the order listed, with thorough mixing by vortex after each addition. The reaction was stopped by boiling for 2 minutes, the solution was acidified to pH 4 with 1 N sulfuric acid and extracted three times with 4 ml hexane to recover the free fatty acids.

After removal of the solvent, the hexane extract was chromatographed by TLC in the neutral lipid solvent system using a maximum of 10 mg lipid per plate. The band corresponding to free fatty acid was identified by comparison with standards and scraped and eluted from the gel with 20-30 ml diethyl ether-ethyl acetate (1:1) per plate. This fraction was then esterified in 10 ml of diethyl ether at approximately 0° for twenty minutes with diazomethane (generated from about 100 mg N-methyl-N'-nitro-N-nitrosoguanidine). Alternatively, the hexane extract was methylated directly in ethereal diazomethane as described. In either case, the methylated fraction was then rechromatographed in the same solvent system, the plates were radioscanned and the band migrating with authentic fatty acid esters was scraped and eluted from the gel as previously outlined. The fatty acid methyl esters were further subjected to analytical and preparative GLC, GLRC, GC-MS (see instrumental) as well as argentation TLC with subsequent catalytic hydrogenation and osmium tetroxide oxidation of the resultant saturated and unsaturated fractions.

Silver nitrate TLC plates were made immediately before use and heated at 120° for one hour. The plates were stable for no more than 48 hours, even though they were kept in total darkness. A maximum of 4 mg lipid was applied per plate and saturated and unsaturated fatty acid methyl esters were separated in hexane-diethyl ether (8:2).

The resultant bands were visualized under ultraviolet light after lightly spraying the plate with a solution of 0.05% Rhodamine 6G in absolute ethanol. The saturated and unsaturated methyl esters were identified by comparison with authentic standards, the bands were marked, scraped, and the gel eluted with 20-30 ml diethyl ether-ethyl acetate (1:1) per plate. After removal of the solvent, 1.0 ml of methanol was added and the solution was divided equally for hydrogenation and oxidation.

Catalytic hydrogenation was carried out using a five-fold (w/w) excess of 10% palladium on carbon under hydrogen at approximately one pound above atmospheric pressure in the presence of methanol. The reaction was stirred continuously for one hour, the carbon was removed by filtration through a fiberglass filter, and the solvent was removed by evaporation under a stream of dry nitrogen.

Osmium tetroxide oxidation was carried out according to the method of Polito (144). The fatty acid esters were placed in a 20 ml screw-capped vial and 1.6 ml dioxane, 0.2 ml of pyridine, and 0.2 ml of osmium tetroxide in freshly distilled dioxane (50 mg/ml) were added. The mixture was shaken briefly and allowed to stand at 23° for one hour, during which time a precipitate formed. A suspension of sodium sulfite (8.5 ml of 16% Na_2SO_3 in water and 2.5 ml of methanol were mixed immediately before use) was added, after which the mixture was left at 23° for an additional hour. The precipitate was removed by filtration and 20 ml of methanol were added to the filtrate, yielding another precipitate immediately. After filtration, the aqueous methanolic solution was taken to dryness under reduced pressure

and the residue partially dissolved in 5 ml of chloroform-methanol (2:1). The mixture was filtered and the polyhydroxylated product was recovered by removal of the solvent under a stream of dry nitrogen. To this product, 50 µl dry pyridine and 100 µl Regisil (bistrimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane) were added prior to mass spectral analysis.

For total reduction, a known amount of the lipid (about 150 mg) was added to a stirred suspension of a five fold excess (w/w) of finely powdered $LiAlH_4$ in 125 ml diethyl ether. The mixture was refluxed for two hours, cooled in an ice bath, and flushed with nitrogen. An amount of water equal to five times that of the $LiAlH_4$ (w/w, 3.75 ml) was added dropwise with continuous stirring. The flocculent white precipitate was removed by filtration and the filtrate taken to dryness under reduced pressure at 35°. Chromatography of the reduced lipid was carried out on 5 g of Florisil packed as a slurry in hexane. The Florisil had been prepared as previously described; alcohols were eluted with 7 volumes of 50% diethyl ether in hexane (141). One quarter of this fraction was subjected to TLC in the neutral lipid solvent system and the remainder in benzene-ethyl acetate (4:1). After radioscanning, the radioactive bands found were scraped, eluted from the gel, and the sample divided equally in half to be analyzed by GLRC and GC-MS (see instrumental) as either free alcohols or their acetylated derivatives. For derivatization, 0.1 ml dry pyridine, 0.05 ml acetic anhydride, and 0.04 ml triethylamine were added to the dry sample (approximately 10 mg) and let stand overnight at 23°; 0.5 ml water and 2 ml hexane were added,

and after phase separation, the acetates were recovered in the hexane layer.

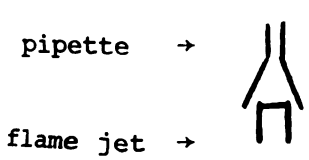
Instrumental

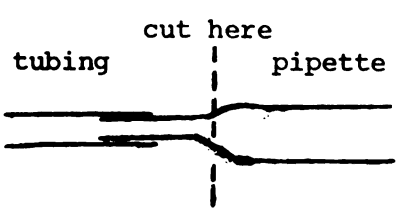
Scintillation Counting: Liquid scintillation counting was performed on a Beckman LS-150 Liquid Scintillation Counter pre-set for 1% counting error. Efficiency was determined from a standard quench curve and was 85-88% for ^{14}C and 55-58% for ^{3}H using 10 ml DPO toluene.

Gas-Liquid Chromatography and Gas-Liquid Radiochromatography: GLC was carried out using an F and M Model 400 Biomedical Gas Chromatograph with hydrogen flame ionization. Helium carrier gas was maintained at a constant flow rate of approximately 30 ml/min. The carrier flow was raised to approximately 60 ml/min for radiochromatography, and the effluent was split 1:10 at the end of the column for mass (flame) and radioactivity (Barber Colman Series 5000 Radioactivity Monitor) determinations, respectively. Radioactive monitoring followed total combustion of the sample at 700° over CuO with subsequent removal of the water formed using magnesium perchlorate and counting of the $^{14}CO_2$ by a proportional ratemeter (145). Propane, natural grade, 96%, was used as quench gas at a flow rate of 8-11% of that of the helium flow entering the ratemeter. Efficiencies were nominally 70% of the effluent entering the monitor for ^{14}C; however this varied considerably, probably due to incomplete combustion of the sample and/or variances in the flow rate of either the carrier or quench gases. The ratemeter was calibrated daily with a standard ^{226}Ra source to obtain a Geiger-Meuller plot (voltage vs. cpm) and the high voltage

was then set to be mid-range on the plateau portion of this curve (usually 1800-2100 volts). The lag time observed between mass and radioactive responses was probably caused by the relatively long path (approximately 2 m) that the effluent had to traverse and the inherent slowness of response of the proportional ratemeter as compared to that of the flame ionization detector. The geometry of the combustion tube (5" x 3/8") also created the possibility of a mixing chamber for the effluent gas and thus a cause for zone spreading. The practical lower limit of detection under the normal operating conditions was usually 500-600 dpm per radioactive component, if there were more than one in a mixture. A major porblem encountered when using the lowest sensitivity scale (300 cpm) was the electronic noise present in the laboratory. When the preparative ultracentrifuge was running (it is on the same power bank), ratemeter response was illicited whenever this machine either started or ended a run.

Preparative GLC was carried out in the following manner. After several trial runs had established the retention time of the compound(s) of interest an injection was made, timing was commenced, and the hydrogen and air were shut off after the solvent front emerged. At pre-selected times, a 9" disposable pipette (Pasteur type) which had been cut off at one end to snugly fit around the flame jet was placed over the jet and collection was carried out for the appropriate time. A two foot length of Teflon tubing had previously been placed over the other end of the pipette. A diagram of the pipette and tubing is shown below:





The pipette and tubing were then rinsed with approximately 20 ml of hexane into a test tube, the solvent removed under a stream of dry nitrogen, and the fractions of interest analyzed by GC-MS or by additional GLC trapping by the above techniques to determine radioactive peaks when the radioactivity present was too low to use the monitor.

Combined Gas Chromatography-Mass Spectrometry: Combined GC-MS including single scanning, computer-controlled repetitive scanning, and manual and computer-controlled single and multiple ion detection were carried out on an LKB Model 9000, interfaced to a dedicated PDP-8/I minicomputer (8K core, two 32K discs) for data acquisition and interactive data reduction and display. The GLC inlet consisted of a silanized coiled glass column with helium carrier gas maintained at a constant flow rate of approximately 35 ml/min. In several cases, the direct probe inlet of the LKB was also used.

The following basic operating conditions of the mass spectrometer were kept as constant as possible over the several years encompassed by the above projects: ion source temperature of 290°; molecular separator temperature of 240°; trap current of 60 μA; full accelerating voltage of 3.5 kV; entrance and exit slits of 0.8 and 1.2 mm, respectively; and a nominal resolution of approximately 700-900. Spectra were recorded at both 22.5 and 70 eV ionizing potentials.

The computer system originally described by Sweeley *et al.* (146) has been expanded to include a Tektronix Model 4002A storage scope with keyboard terminal and a Tektronix Model 4601 hard copy unit. The system has also been modified to include computer-controlled repetitive

scanning with subsequent output of selected ions (mass chromatography) (147).

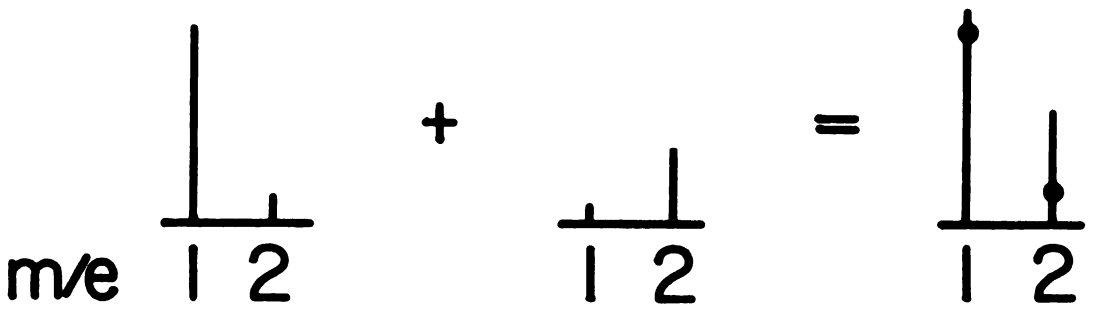
The standard accelerating voltage alternator (AVA) accessory of the LKB was used for single and multiple ion detection, manually for the determination of JH, and under computer-control for the quantitative determination of the levels of fatty acid methyl and ethyl esters. The levels of these fatty acid esters were determined by single ion monitoring of a selected ion on all three channels of the AVA with the aid of computer-control of fine focus, data acquisition, reduction, and display (148). The proportion of protium form of JH in preparations was determined with continuous recording of the intensities of two selected ion pairs. The lower mass of the pair was first focused by manual changing of the magnetic field strength; the upper mass was then focused by lowering the accelerating voltage using coarse and fine voltage dividers which allow up to a 10% lowering of the full accelerating voltage. The areas of each ion envelope were measured by triangulation and the ratio of protium to deuterium forms was determined. A blank ratio of these ions for the pure reference deuterium form was obtained both before and after a series of analyses and this was subtracted from the observed isotopic ratios of the samples. To validate this mathematical approach, a general model for isotope dilution calculations and therefore reverse isotope dilution calculations was devised.

Mathematical Model for Isotope Dilution Calculations*: The basic situation in its simplist form where an isotopically labeled compound is added to its non-labeled form producing a mixture is illustrated in Figure 1. A specific ion is selected as a linear measure for each of the isotopic forms of the compound and these two *m/e* values are then measured in the labeled form, the non-labeled form, and in the final mixture. In the mixture, both ions will contain a contribution from each of the two ions. Due to the many variables of mass spectrometric analysis, absolute intensities are not generally accurate enough for reliable quantitative measurements. To minimize the effects of long term variables and greatly enhance the reliability of the method, ratios of the intensities of the two ions are used for the quantitative calculations. Figure 2 illustrates the composition of the mixture. I_{L_1} and I_{N_1} represent the contributions at one mass from the labeled and non-labeled forms, and I_{L_2} and I_{N_2} indicate the contributions at the other mass. Since the ratios for the two ions in each isotope will remain constant, mathematical solutions for the intensity of each component in the mixture can be obtained, since there are two equations with two unknowns. In terms defined from Figures 1 and 2, $I_{L_2} = R_L \times I_{L_1}$ and $I_{N_2} = R_N \times I_{N_1}$, therefore:

$$I_{L_1} = I_{M_1} \times \frac{R_M - R_N}{R_L - R_N}$$

* This approach was devised by Dr. J.F. Holland and R.E. Teets and is included in the dissertation with their permission. It has been presented as a preliminary communication to the American Society for Mass Spectrometry (21th Annual Conference, San Francisco, May, 1973) and extended by the author to include reverse isotope dilution calculations.

FIGURE 1. The Isotope Dilution Method



nonlabeled labeled mixture

$$R_N = \frac{I_2}{I_1} \qquad R_L = \frac{I_2}{I_1} \qquad R_M = \frac{I_2}{I_1}$$

FIGURE 2. Composition of the Mixture

and,

$$I_{N_1} = I_{M_1} \times \frac{R_L - R_M}{R_L - R_N} .$$

It has usually been assumed that the measured ion intensities are directly and linearily related to amounts of the molecular isotopic forms from which they arise. This, of course, is fundamental to the analytical capability of the technique of stable isotope dil ion. In fact, each of the ions is usually selected to attain this linearity if possible. The quantitative identification in terms from Figure 2 may be defined as:

I_{L_2} = the measure of the labeled molecule, and

I_{N_1} = the measure of the non-labeled molecule.

Ideally,

$$I_{L_2} = k_L C_L \quad \text{and} \quad I_{N_1} = k_N C_L,$$

and both k_N and k_L are constants.

The ratio of the amounts of the two forms contributing to the mixture may be represented as I_{L_2} / I_{N_1}. Solving for this ratio in terms of measurable quantities,

$$I_{L_2} / I_{N_1} = \frac{R_M - R_N}{1 - R_M/R_L}$$

and substituting for the intensities,

$$\frac{C_L}{C_N} = \frac{k_L}{k_N} \times \frac{R_M - R_N}{1 - R_M/R_L} .$$

When $k_L = k_N$ and $R_M << R_L$,

$$C_L / C_N = R_M - R_N .$$

It is clearly recognized that k_L and k_N are complicated constants, if constants at all and that they may be further expanded into several factors, some of which will be definable and measureable, and include such factors as isotope fragmentation effects or concentration dependent responses.

The mathematical approach outlined above is for a case where a labeled species is added to a non-labeled one. In reverse isotope dilution experiments, the reverse is occurring since a non-labeled species is being diluted with a large excess amount of a labeled species. Again referring to Figures 1 and 2, the roles of the labeled and non-labeled species are interchanged and the mathematical method may be directly transposed. In this case, such as has been done in the work presented in this dissertation, a new general equation can be defined as:

$$I_{N_1} / I_{L_2} = \frac{R_M - R_L}{1 - R_M / R_N} .$$

Again, when $k_L = k_N$ and $R_M << R_N$, the equation reduces to:

$$C_N / C_L = R_M - R_L .$$

The application of this latter set of equations will be demonstrated in Results.

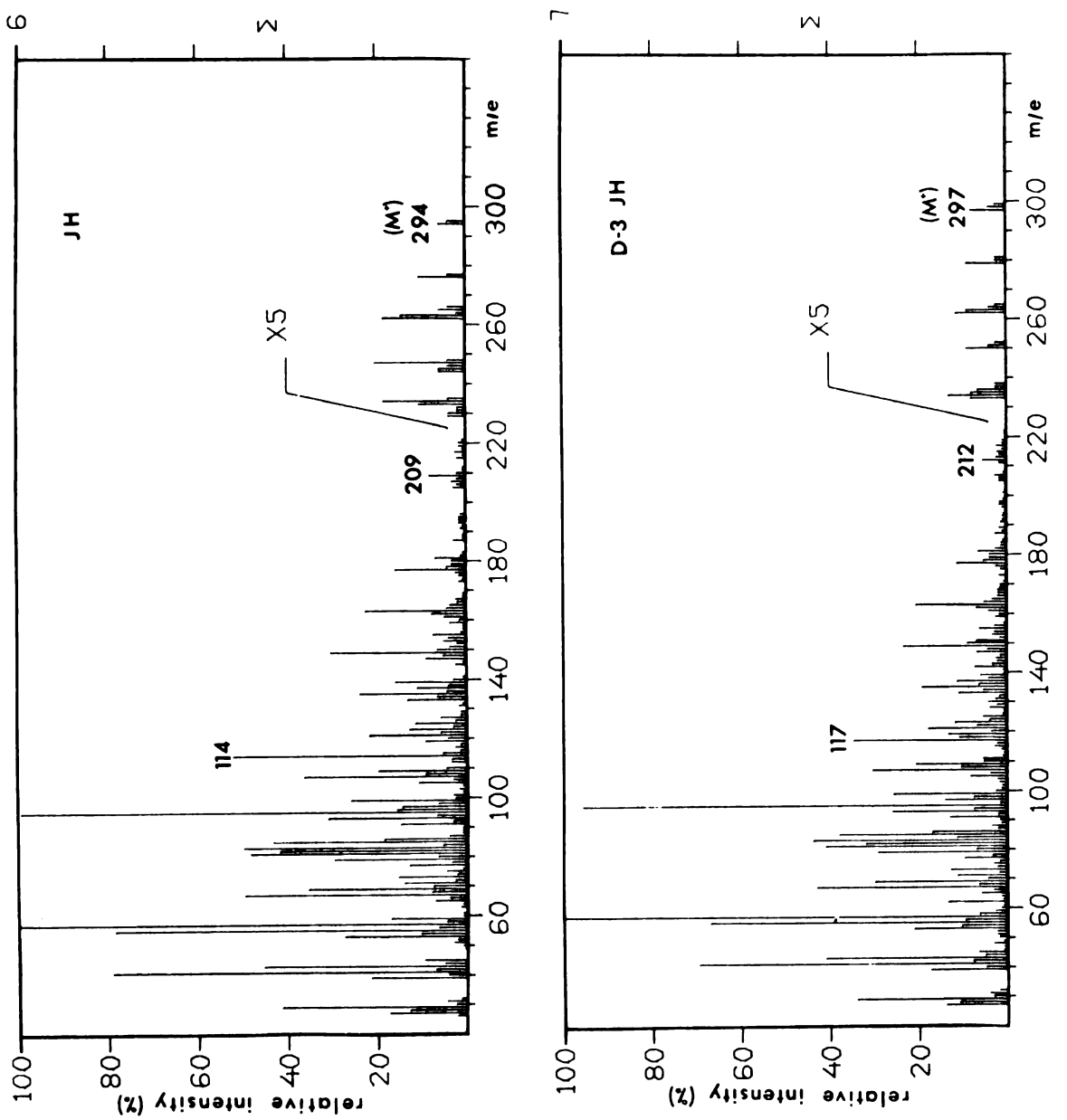
RESULTS

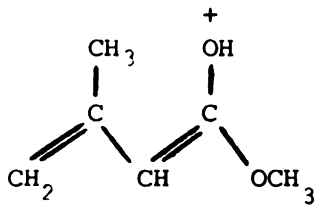
Quantitative Determination of Juvenile Hormone

Mass Spectra of Juvenile Hormone: Mass spectra of the protium and deuterium forms of synthetic juvenile hormone (JH) are presented in Figure 3. The fragmentation pathways of both cecropia C_{17}- and C_{18}-juvenile hormones have been presented elsewhere (36,55,149) and will not be discussed in detail. The masses of five ions containing the methyl group were shifted by 3 amu in the deuterated form and were therefore appropriate possibilities for measurement of protium and deuterium forms in mixtures. The molecular ions at *m/e* 294 and at *m/e* 297, as well as those for loss of water (*m/e* 276 and *m/e* 279) and for loss of water plus an ethyl group (*m/e* 247 and *m/e* 250) were too low in intensity to be used for analyses of very small amounts of sample. The rearrangement ions at *m/e* 114 and at *m/e* 117 and also those at *m/e* 209 and at *m/e* 212 were considerably more intense and were therefore suitable for measurement of isotopic ratios. A detailed study of the electron impact induced behavior of terpenoid esters of the juvenile hormone class has been carried out by Liedtke and Djerassi (149). In their study of methyl 10,11-epoxy farnesoate and several of its deuterated derivatives they found that the ions at *m/e* 114 and at *m/e* 195 (the ion analogous to *m/e* 209 in JH) are monomolecular species having the elemental compositions $C_6H_{10}O_2$ and $C_{12}H_{19}O_2$, respectively. The probable structure of these ions in JH are:

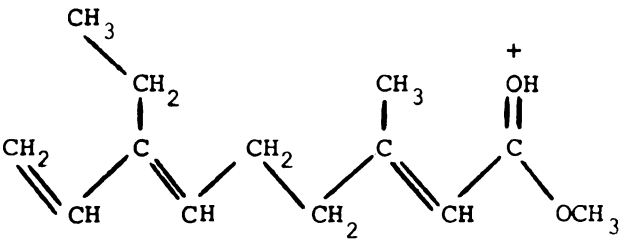
FIGURE 3. Mass Spectra of Protium and Deuterium Forms of Synthetic *Cecropia* C_{18}-Juvenile Hormone

Mass spectra were recorded at an ionizing potential of 70 eV on an LKB 9000 Gas Chromatograph-Mass Spectrometer. The GLC inlet consisted of a coiled glass column (1.4 m x 3 mm) packed with 1% SE-30 on Supelcoport (100/120 mesh) operated isothermally at 170°. Helium carrier gas was maintained at a constant flow rate of 35 ml/min.


JH
relative intensity (%)
m/e
114
209
(M+)
294
X5
D-3 JH
relative intensity (%)
m/e
117
212
(M+)
297
X5



A (*m/e* 114)



B (*m/e* 209)

The origin of the ion at *m/e* 114 was studied in detail by these investigators but no conclusion was reached as to how it is formed since three mechanistic pathways could easily account for it. All three proposed pathways involve McLafferty type rearrangements with a 6-, 8-, or 10-membered transition state. It is not surprising, therefore, that a fragmentation isotope effect was observed in the formation of the A and B ions from the deuterated form. This isotope effect was determined by measurement of the intensities of these ions in relationship to those of *m/e* 57 and *m/e* 95 (fragments not containing the methyl ester function) in several spectra and was found to decrease the abundance of *m/e* 117 in the deuterated form to 80% of that observed for *m/e* 114 in the protium form. The B ions exhibited an isotope effect which decreased the abundance of *m/e* 212 to 89% of that observed for *m/e* 209. These factors were taken into consideration in a correction of raw isotopic abundance for mass spectral data (see next section).

A mass spectrum of the synthetic deuterated ethyl ester is not presented. Those ions containing the methyl ester group noted above were shifted by 16 amu to *m/e*'s 310, 292, 263, 225, and 130 in the deuterated ethyl ester. The rest of the mass spectrum was, for all intensive purposes, identical to those presented in Figure 3. The protium form of the ethyl ester was never received; therefore,

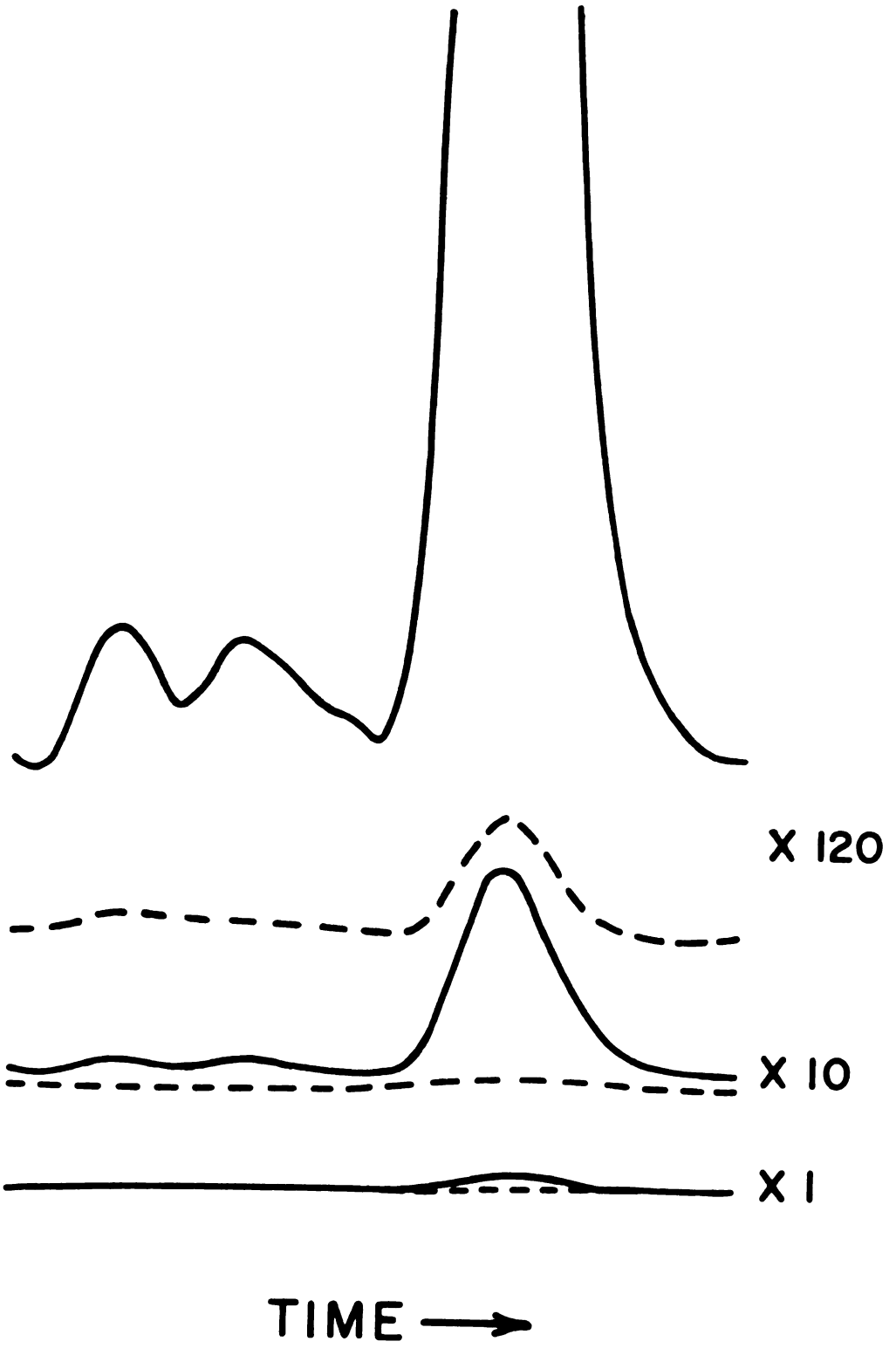
detailed studies of the ions suitable for measurement of isotopic ratios (*m/e* 130 and *m/e* 225) could not be undertaken.

AVA Technique for Quantitative Determination of Juvenile Hormone: A graphic representation of the galvanometer tracing of a synthetic mixture of the protium and deuterium forms in a ratio of 1:1000 is presented in Figure 4. The solid line represents *m/e* 117 and the broken line, *m/e* 114. The areas were measured by triangulation and the ratio obtained after normalization with the appropriate scale factor. The intensity of *m/e* 114 in the pure reference deuterated compound was low and somewhat variable. The intensity of *m/e* 209 was much higher and more variable. These may arise from another molecular species, including a small impurity of protium form and a contribution from gas chromatographic "bleed". In the latter case, *m/e* 207 is a well characterized ion arising from column bleed or "leaking" of silicone-based liquid phases and has an elemental composition of $C_5H_{15}O_3Si_3$ (150); *m/e* 209 is therefore an isotope peak of *m/e* 207. The blank ratio at ion pair *m/e* 209/212 in the pure reference deuterated form was therefore highly dependent upon the conditioning of the SE-30 column at the time of analysis. In order to separate the individual ions at nominal mass *m/e* 209, a higher resolution would have been needed (at least 2000) since the exact mass of ion B in JH is 209.1536 and that for the nearest isotopic species arising from column bleed is 209.0371 ($C_5H_{15}{}^{16}O_2{}^{18}O_1Si_3$).

The blank value for the ratio at ion pair *m/e* 114/117 in the deuterated form varied between 23 and 70 parts per thousand (ppt) and that at *m/e* 209/212 ranged from 108 to 205 ppt. Average values for

FIGURE 4. Graphic Representation of a Galvanometer Tracing of Selected Ions from a Synthetic Mixture of Protium and Deuterium Forms of *Cecropia* C_{18}-Juvenile Hormone

Continuous recording of the intensities of ions *m/e* 114 (broken line) and *m/e* 117 (solid line) in a synthetic mixture of protium and deuterium forms of juvenile hormone in a ratio of 1:1000. See Figure 3 for operating conditions.


X 120
X 10
X 1
TIME

duplicate determinations of the blank ratio were therefore obtained before and after a series of analyses and were subtracted from the observed isotopic ratio of the samples. This difference represents $R_M - R_L$ (see mathematical section of Methods), and is proportional to the amount of protium form present in the sample. However, to be an exact measure of the concentration of protium form, C_N, the ratio of the factors k_N/k_L must be known. In these cases, k_N/k_L equaled 0.80 at ion pair *m/e* 114/117 and 0.89 at ion pair *m/e* 209/212 as determined from the fragmentation isotope effects described. The raw isotopic abundance was therefore multiplied by the appropriate factor to obtain a corrected isotopic ratio. Since C_L (deuterated form added) is known, and $R_M << R_N$:

$$C_N = 0.80\ (R_M - R_L)\ C_L \qquad \text{at } m/e \text{ pair } 114/117, \text{ and}$$

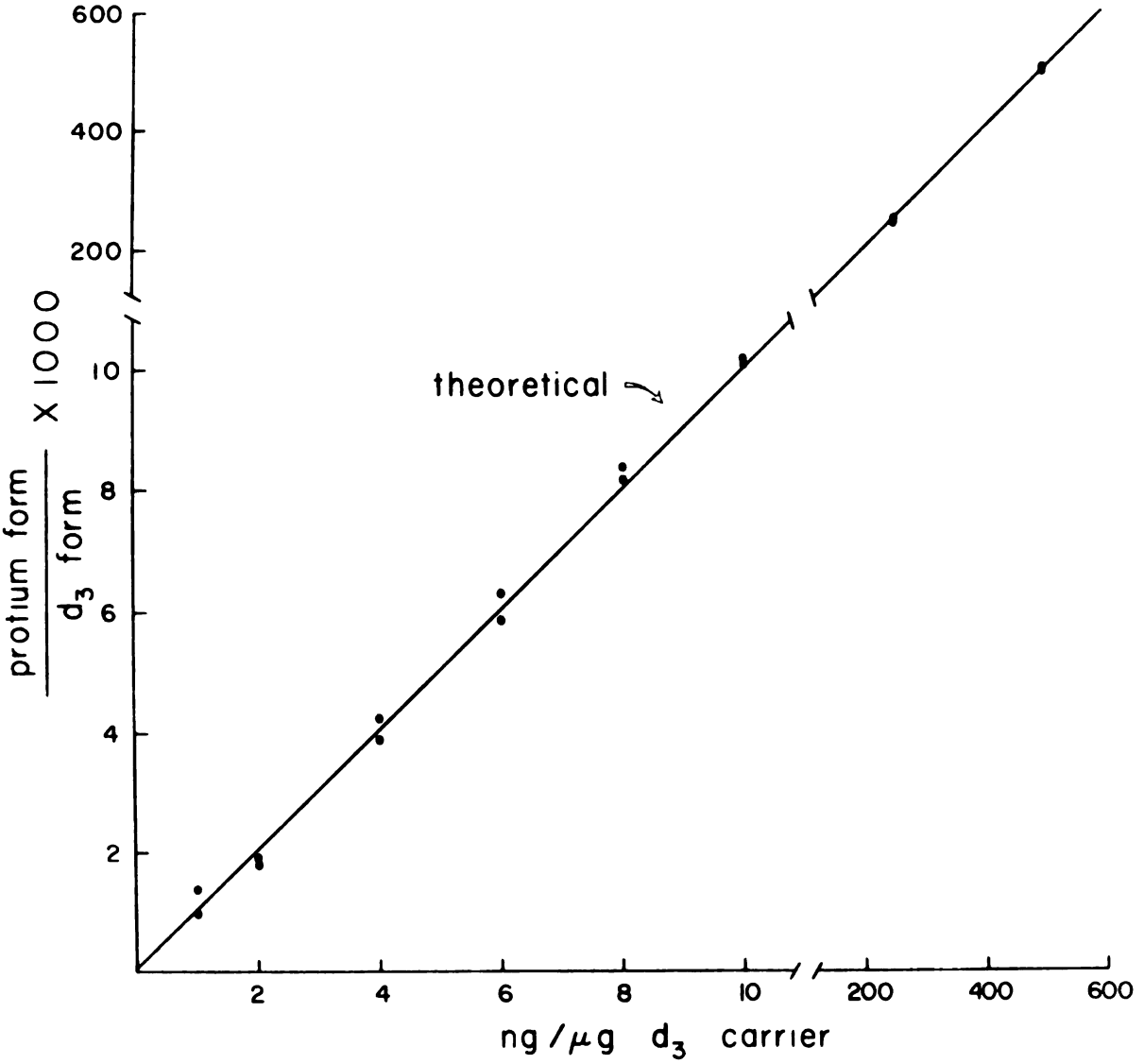
$$C_N = 0.89\ (R_M - R_L)\ C_L \qquad \text{at } m/e \text{ pair } 209/212.$$

The value C_N represents the total amount of protium form in the sample; therefore, this was appropriately normalized to present the results on the basis of number of insects studied or weight of lipid extracted.

A linear relationship was observed for the variation of the corrected isotopic ratios with the amount of protium form of synthetic JH added in known mixtures, as shown in Figure 5. These data were obtained from ratios at *m/e* pair 114/117. Least-squares analysis of the data gave a regression line with a slope coefficient of 1.01 and an intercept of -0.25. Thus, the corrected isotope ratio provided an accurate quantitative measure of the natural JH isolated from crude lipid extracts along with added deuterium carrier form.

FIGURE 5. Ratios of Peak Areas of the Protium and Deuterium Forms versus the Composition of the Injected Material

Corrected isotopic ratios (see Results) for ion pair *m/e* 114/117 observed for known mixtures containing 1 to 500 ng protium form in 1000 ng deuterium form juvenile hormone. See Figure 3 for operating conditions.


600
400
200
10
8
6
4
2
protium form / d_3 form X 1000
theoretical
2
4
6
8
10
200
400
600
ng / μg d_3 carrier

Purification of Juvenile Hormone: Yields of lipids at the various stages of JH purification (see Methods) are listed in Table 1. Recoveries of synthetic and radiolabeled JH placed through the purification procedures averaged 93% (two determinations). Quantitative recovery of the added deuterated hormone, however, was not essential, provided sufficient sample was recovered in relatively pure form for mass spectral analysis (approximately 30-40 μg). The underlying assumption made throughout the work was that the added deuterated form acted not only as an internal standard but as a carrier molecule for the much smaller amounts of natural protium form present and that there would be no differential loss of either form during any of the purification procedures. This assumption was never specifically tested, however it seemed to be valid and is currently being used in the quantitative determination of prostaglandin levels in biological fluids and tissues (151). Losses of hormone probably occurred at the low-temperature precipitation step since the residue obtained was no re-extracted; losses definitely occurred at the stage of Sephadex LH-20 column chromatography since fractions were chosen carefully to contain only those that were highly enriched in JH as evidenced by GLC. Additional losses resulted with each TLC step since the portion of the plate exposed to iodine vapor (both side edges) was not scraped and therefore approximately 5% of the JH per plate was lost. The total recovery of JH in these purifications was probably 50-70% as evidenced by the relative detector responses obtained upon GLC after each of the various stages, seen in Figure 6 which shows the GLC monitoring of part of the purification procedure of JH from a

TABLE 1. Purification of Juvenile Hormone from Various Insects

Insect	Sex	N[a]	Wet Weight (g)	Yield (mg)					
				Total Lipid	Low-temp. filtrate	LH-20 fraction	TLC-1[b]	TLC-2[c]	TLC-3[d]
H. cecropia	M	4.9[e]	2.31	721	650	35	9.2	2.3	
H. cecropia	M	1	0.287	173	158	8	5.9	3.2	
H. cecropia	M	1	0.489	292	262	18	2.7	0.4	
H. cecropia	F	32	18.37	3480	3148	133	35.0	2.7	
S. cynthia	M	7	1.99	710	247	16	5.8	0.8	
S. cynthia	F	12	2.68	534	232	9	N.D.	1.1	
C. promethea	M	13	1.69	703	456	25	3.2	0.9	
C. promethea	F	15	4.45	478	333	19	4.0	1.0	
M. domestica (larvae)	M,F	∿7300	98.52	2790	2118	38	4.9	1.0	0.2
A. domestica	M	149	56.6	3138	653	40	14.5	2.1	1.1
A. domestica	F	182	91.2	6471	1892	68	16.1	0.9	

A. mellifera (without queen)	F	1035	112.98	4343	1541	115	16.1	2.2	0.5
O. melanopus	M,F	∿3500	113.17	1189	493	45	6.4	0.5	

[a] N represents the number of organisms used.

[b] R_f in chloroform-ethyl acetate (2:1) was 0.68.

[c] R_f in benzene-ethyl acetate (95:5) was 0.45.

[d] R_f in chloroform-pentane (2:1) was 0.36.

[e] An aliquot of the total lipid extracted from 10 male abdomina was used.

single male cecropia abdomen.

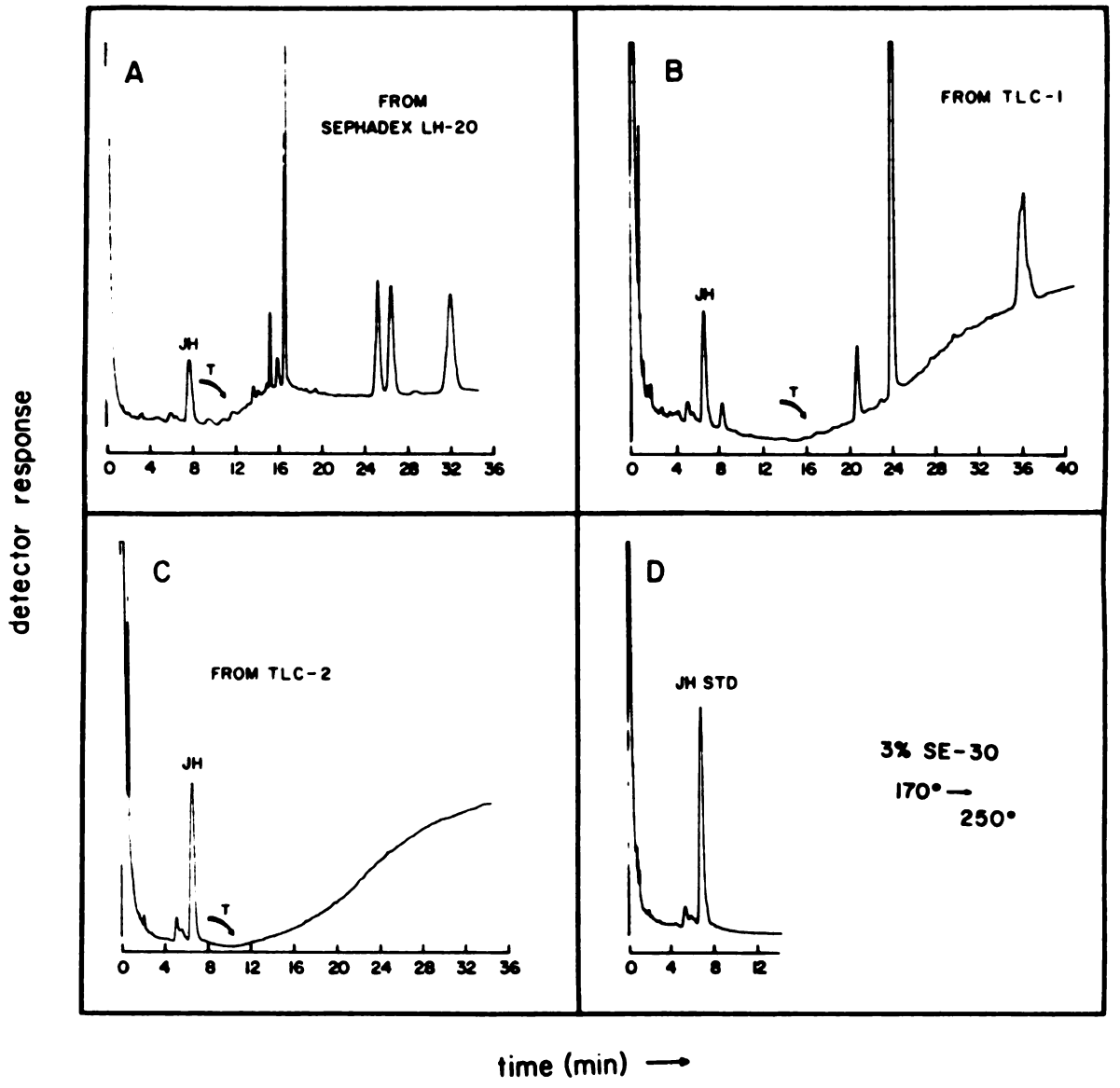
Gas-liquid chromatography was performed isothermally at 170° on 3% SE-30 until the JH peak was identified by its retention time and then, at the arrow (T) in each part of Figure 6,the temperature of the GLC oven was turned up to 250° to elute other volatile components present in the fraction. In these particular runs, 1% of the total sample weight was injected onto the column each time and it is evident that the JH peak increased in size with each injection, thus giving an indication of the purification achieved. The two small partially resolved peaks eluting directly before JH are impurities of synthesis (152) and were not identified; they were helpful as markers in monitoring the purification procedure since they co-purified with JH and indicated that the peak observed at the correct retention time for JH was probably the actual hormone and not another compound with an identical retention time on the SE-30 column.

It is also evident from Figure 6D that the standard deuterated JH was not pure. It was determined by GLC that JH represented 75.0% (three determinations) of the pure synthetic standard received from Drs. Faulkner and Petersen. It was also determined that there was little (0.8%) difference in the differential detector responses of JH and methyl palmitate, methyl ricinoleate, and methyl stearate; this implied that the peak observed for JH was a direct measure of the total amount of hormone present (both protium and deuterium forms) in any given sample.

Biological activity of the protium or deuterium forms of the synthetic JH was not tested by this investigator. They both, however,

FIGURE 6. Gas-Liquid Chromatography of Juvenile Hormone

A, B, and C are gas chromatograms of an aliquot (1 µl out of 100 µl) of the volatile components obtained from fractions after Sephadex LH-20 column chromatography, TLC-1, and TLC-2, respectively. D is a gas chromatogram of the pure reference protium form of synthetic juvenile hormone. GLC was carried out on an F and M Model 400 with flame ionization detector using 3% SE-30 on Supelcoport (100/120 mesh). Carrier gas (helium) was maintained at a constant flow rate of 30 ml/min. At the arrow, T, the temperature was turned up from 170° to 250°.


A
FROM
SEPHADEX LH-20
JH
T
0 4 8 12 16 20 24 28 32 36
B
FROM TLC-1
JH
T
0 4 8 12 16 20 24 28 32 36 40
C
FROM TLC-2
JH
T
0 4 8 12 16 20 24 28 32 36
D
JH STD
0 4 8 12
3% SE-30
170° →
250°
detector response
time (min) →

were tested by other investigators and gave highly active responses in both the *Galleria* Wax Test (42,153) and the *Oncopeltus* Bioassay (154,155).

The low-temperature precipitation step afforded at least an 8% to almost 80% purification of the extract since this procedure eliminated most of the polar lipids present in the total extract. No phospholipid or glycolipid could be detected using the TLC spray reagents molybdenum blue or α-napthol for detection of phospholipids and glycolipids, respectively. Under these conditions, amounts of either of these two classes of lipids present could not have been greater than 2 mg each in the low-temperature filtrate. Therefore, even in the case where the least amount of total lipid was purified (173 mg from one male cecropia abdomen), no more than 2.5% of the filtrate could have been phospholipid or glycolipid. In most cases, it was probably no more than 0.5% of the filtrate. The degree of purification at this stage was therefore dependent upon the polar lipid content of the insect. From the limited data that Fast has collected over a period of years from a variety of sources (156,157), the degree of purification achieved at this step did parallel the amounts of polar and neutral lipids found in the various insects studied.

Column chromatography on Sephadex LH-20 in benzene-methanol (1:1) yielded approximately a 20-fold purification of the filtrate. Fractions were chosen with care and an aliquot of each fraction obtained in the area of JH elution was monitored by GLC. Figure 6A shows a GLC tracing of the volatile components of the fractions eluted between 244 and 256 ml in the purification of JH from a single male cecropia moth abdomen.

The reproducibility of the LH-20 column was quite good; with a new column the JH fractions were found in an elution volume of between 260-280 ml. After the column had packed more tightly, with use several times, the JH fractions were usually found within an elution volume of 240-250 ml.

Figure 7 shows an elution profile of lipid extracted from cereal leaf beetles after the low-temperature precipitation step; 4 ml fractions were collected. The area of JH elution is marked and at least four peaks were observed. The fraction at the apex of each of the four peaks was tentatively identified by TLC in the neutral lipid solvent system. Peaks labeled I, II, III, and IV were thought to be composed of triglyceride, sterol ester, free sterol, and free fatty acid, respectively. For positive identification of these neutral lipid fractions, 50-60 µg of each fraction was subjected to mass spectrometry using the direct probe inlet of the LKB 9000. Through mass spectrometric analysis, an additional component not observed on TLC was identified in peak II. These spectra are presented in Figures 8 and 9 and will be discussed only in terms of the characteristic ions that allowed the identifications.

The ions designated A and A' in Spectrum I (Figure 8) are indicative of the acyl moieties of triglycerides (RCO+). The palmitoyl ion is at A (*m/e* 239) while A' has been designated for the ions at *m/e*'s 261, 263, and 265, representing linolenoyl, linoleoyl, and oleoyl groups, respectively. Increasing unsaturation in the acyl moieties causes abundant formation of $[RCO-1]^+$ (158); these ions are seen at *m/e*'s 260, 262, and 264. The steroyl ion at *m/e* 267 is small, indicating

FIGURE 7. Elution Profile of Cereal Leaf Beetle Lipid on Sephadex LH-20

Gel filtration on Sephadex LH-20 was carried out with benzene-methanol (1:1) as the eluting solvent. 4 ml fractions were collected. The area of juvenile hormone elution is marked.

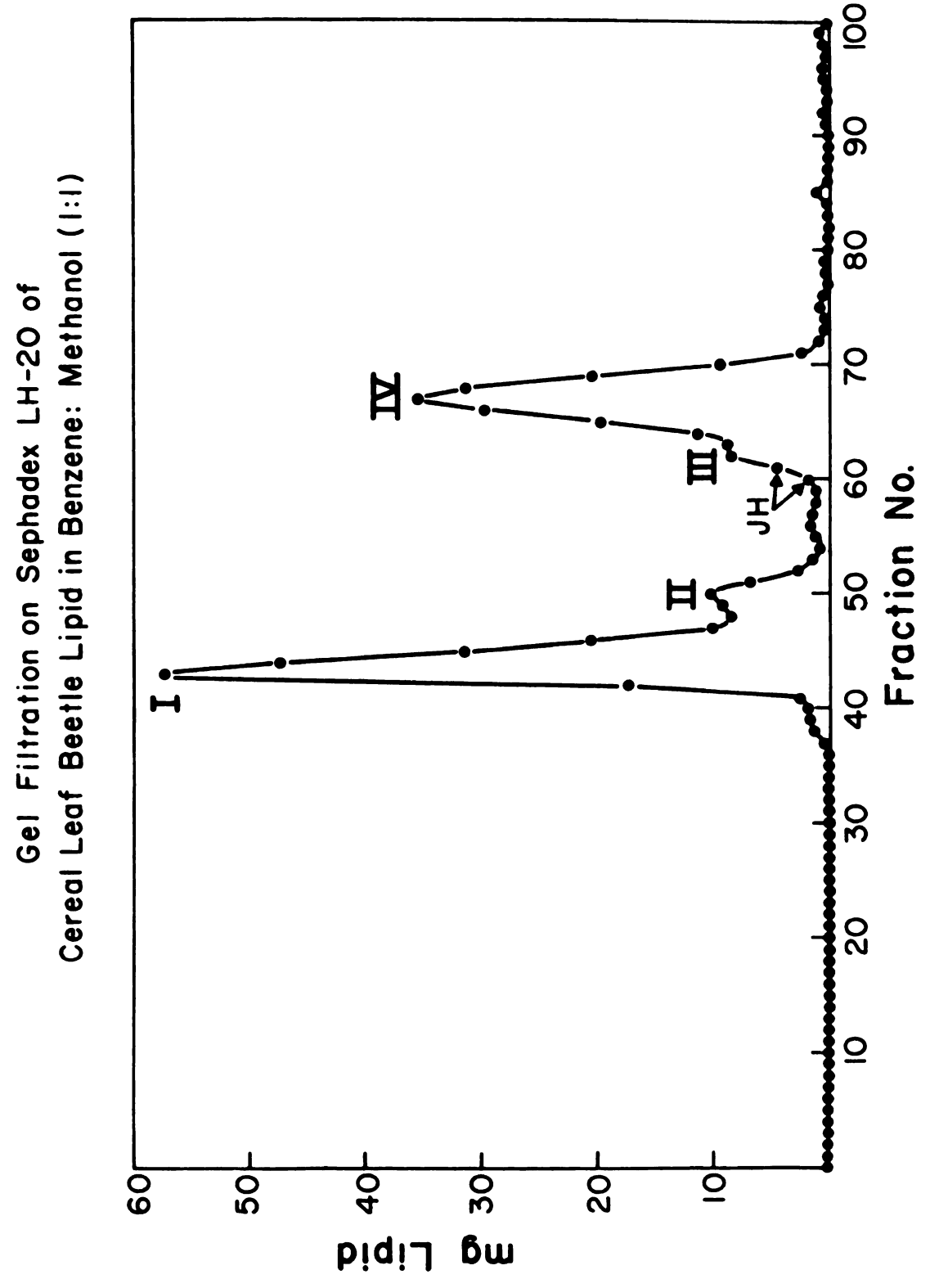
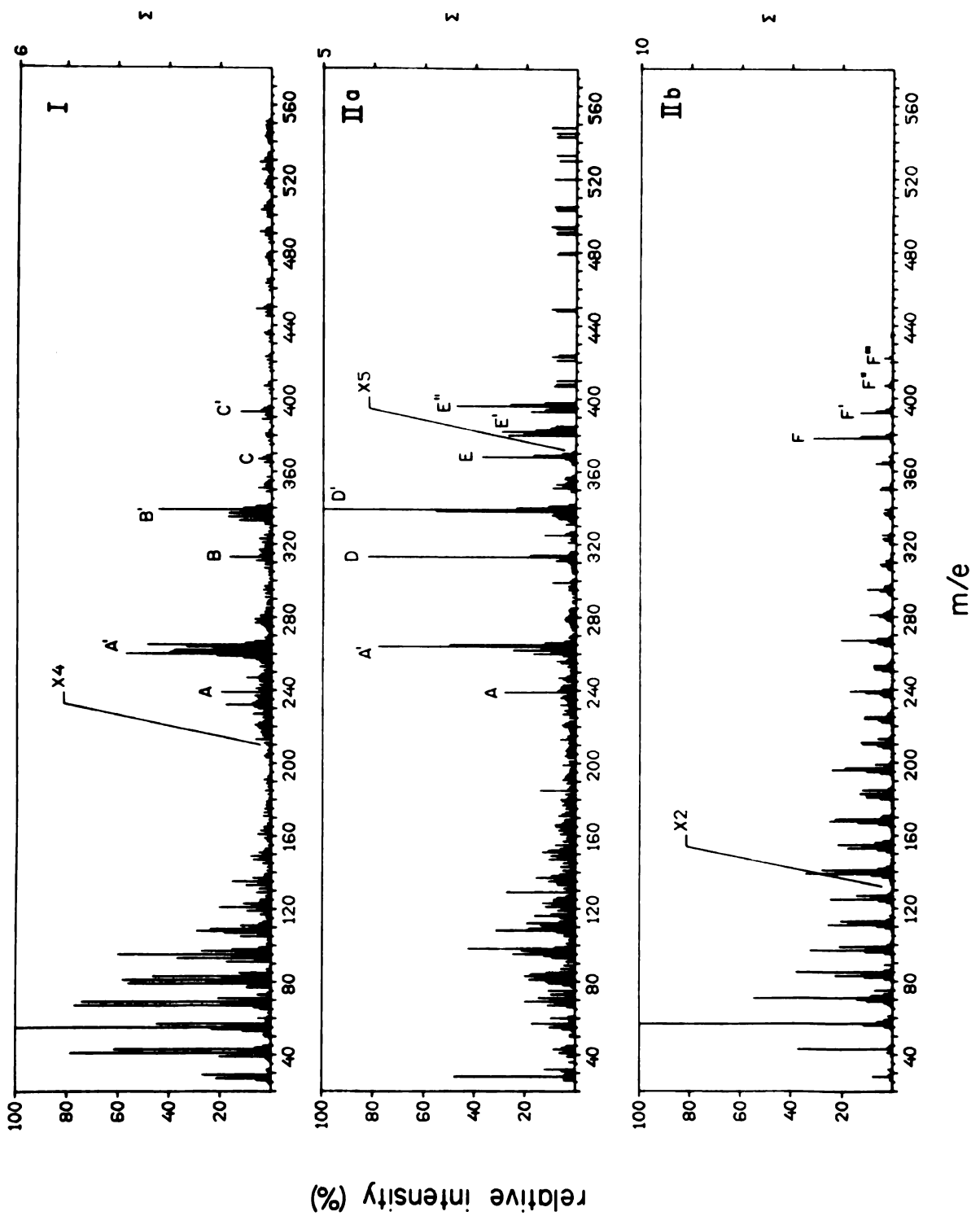

Gel Filtration on Sephadex LH-20 of
Cereal Leaf Beetle Lipid in Benzene: Methanol (1:1)
mg Lipid
60
50
40
30
20
10
I
II
JH
III
IV
10
20
30
40
50
60
70
80
90
100
Fraction No.

FIGURE 8. Mass Spectra of Peaks I and II Obtained from LH-20 Column Chromatography

Mass spectra were obtained via the direct probe inlet of an LKB 9000 gas chromatograph-mass spectrometer. Spectra I and IIb were recorded at an ionizing potential of 70 eV. Spectrum IIa was recorded at an ionizing potential of 22.5 eV. See Figure 7 for location of the corresponding peaks from the LH-20 column.


I
IIa
IIb
A
A'
B
B'
C
C'
D
D'
E
E'
E"
F
F'
F"
F"'
X4
X5
X2
relative intensity (%)
m/e

that there was very little stearic acid in the triglycerides of cereal leaf beetles. Other ions characteristically observed in the mass spectra of triglycerides occur at $[RCO+74]^+$ and $[RCO+128]^+$. The B ion, located at *m/e* 313, B' ions, grouped at *m/e*'s 335, 337, and 339, the C ion at *m/e* 367 and the C' ions at *m/e*'s 389, 391, and 393 are $[RCO+74]^+$ and $[RCO+128]^+$, respectively. Another characteristic feature in the mass spectra of triglycerides is the occurrence of a series of ions at $[RCO+128+14n]^+$. Although these ions have not been specifically noted in Spectrum I, they are present. Due to the limitations of the computer system at the time this spectrum was obtained (the computer was not calibrated to determine masses above *m/e* 550), molecular ions as well as those for $[M-RCOO]^+$ and $[M-RCOOCH_2]^+$ were not recorded. From the above data and that obtained from TLC, it was concluded that peak I (Figure 7) is a mixture of triglycerides primarily composed of palmitic, oleic, linoleic, and linolenic acids.

The fraction analyzed from peak II (Figure 7) yielded two distinct volatile compounds as the temperature of the direct probe tube was increased. Spectrum IIa (Figure 8) was recorded as the direct probe tube was heated to a temperature of approximately 90° and it was concluded to be consistent with that expected for a mixture of sterol esters for the follow reasons. The A and A' ions indicate esterified long chain acyl moieties (RCO+) and are present in high abundance; the intensities of the palmitoyl and oleoyl ions (*m/e* 239 and *m/e* 265) indicate that they are the major fatty acids present. The ions designated E, *m/e* 368, E', *m/e*'s 380 and 382, and E'', *m/e* 396, are $[M-RCOO]^+$ and represent the steroid nucleus. Although it is not

specifically noted, the ion at *m/e* 129 is formed from sterols with a Δ^5-3-β-ol configuration (159) and it was therefore concluded that the esterified sterols might be cholesterol, 24-methylene cholesterol, campesterol, and β-sitosterol, respectively. These are the only naturally occurring sterols prevalent in the plant kingdom consistent with the above findings (159). The intensity of the ion at *m/e* 368 in relationship to those at *m/e*'s 380, 382, and 396 indicates that cholesterol is the major esterified sterol. The ions designated D and D', at *m/e* 313 and at *m/e* 339, are $[RCO+74]^+$ and arise from cleavage occurring in the A ring of the steroid; they are 26 amu apart and contain the acyl aide chain. Molecular ions were not recorded due to the limitations of the computer system already noted. The reduced abundance of the ions in the lower mass range in this spectrum was due to the fact that it was recorded at an ionizing potential of 22.5 eV.

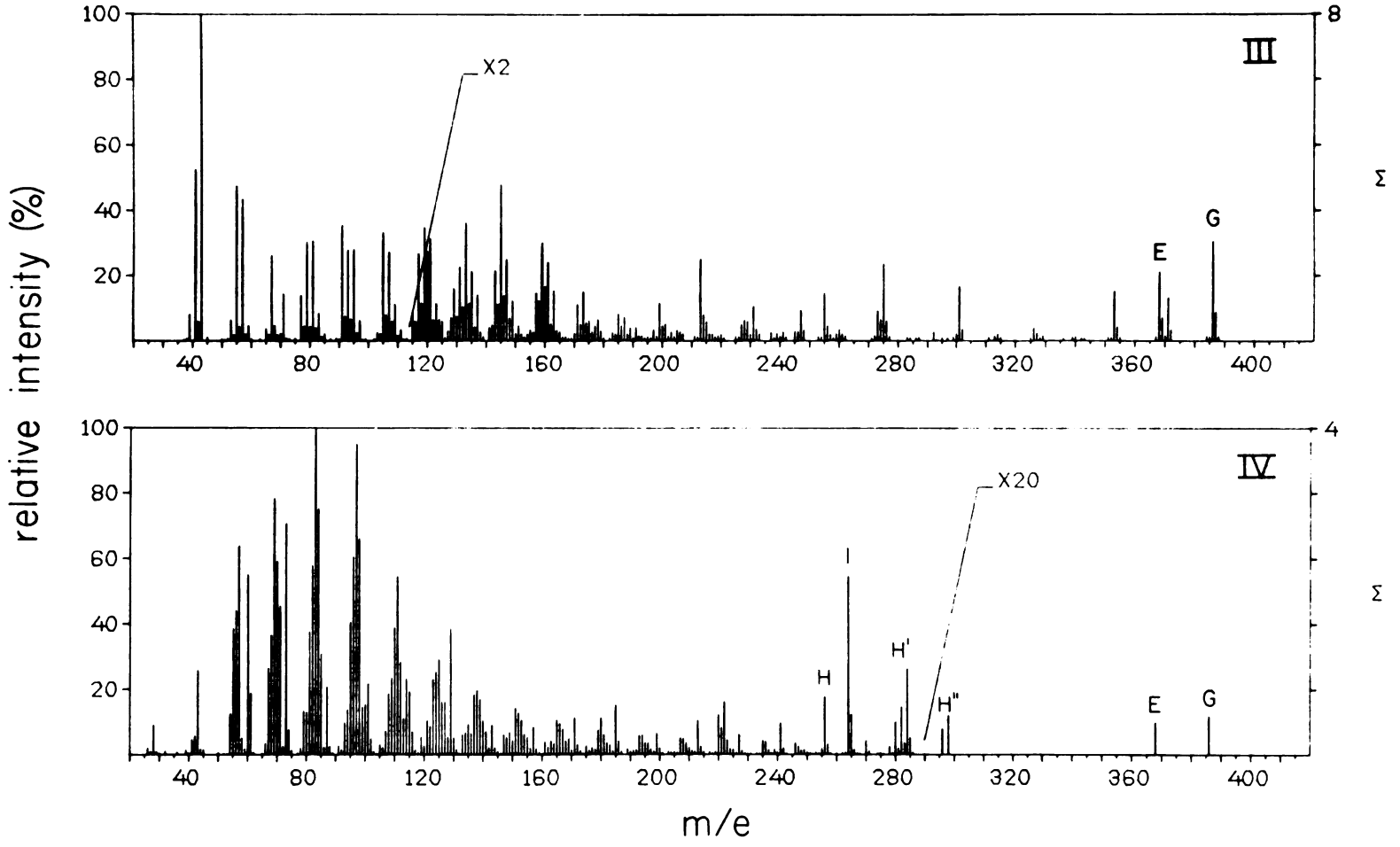
Spectrum IIb was obtained as an additional compound was volatilized from the direct probe tube as it was heated to 120°. This spectrum (Figure 8) is consistent with that predicted for a mixture of C_{27} to C_{30} monounsaturated and saturated hydrocarbons, having molecular weights of 378, 392, 408, and 422 (ions F, F', F'', and F''', respectively). The regular pattern of ions, 14 amu apart throughout the entire spectrum with a base peak at *m/e* 57 (C_4H_9), is characteristic of the mass spectra of long chain hydrocarbons. The presence of hydrocarbons in peak II (Figure 7) was not indicated by TLC. This was probably due to the fact that hydrocarbons and sterol esters have similar R_f values in the nuetral lipid solvent system used (see Table 4).

Spectrum III (Figure 9) is very similar to that of free cholesterol (160). The molecular ion (G) at *m/e* 386 and that for loss of water (*m/e* 368, ion E as in Spectrum IIa), as well as ions located at *m/e* 371 ($[M-15]^+$) and *m/e* 353 ($[M-15-18]^+$), indicate a C_{27} Δ^5-3-β-ol sterol (159). Molecular ions for other sterols are not present in this spectrum, although they were observed in another spectrum, taken at an ionizing potential of 22.5 eV, and were assumed to be due to mono- and diunsaturated C_{28} and monounsaturated C_{29} sterols. It was concluded from the data obtained from TLC and the above findings that peak III (Figure 7) is a mixture of free sterols, tentatively assumed to be cholesterol, 24-methylene cholesterol, campesterol, and β-sitosterol. Cholesterol is the major free sterol present.

Spectrum IV in Figure 9 has all the characteristic ions expected for a mixture of free fatty acids. Molecular ions at *m/e*'s 256, 270, 278, 280, 282, 284, 296, and 298 are derived from palmitic, hepatadecanoic, linolenic, linoleic, oleic, stearic, nonadecenoic, and nonadecanoic acids, respectively. For clarity, these ions have been grouped and labeled H, H', and H'' in this spectrum. The ion labeled I at *m/e* 264 represents loss of water from oleic acid. This is a prominent feature in the mass spectra of monounsaturated fatty acids (160). Ions at *m/e*'s 60, 73, and 129 are analogous to those shifted by 14 amu and found at *m/e*'s 74, 87, and 143 in the spectra of fatty acid methyl esters (see later sections). The reduced intensity of these ions is due to the fact that this spectrum was recorded at an ionizing potential of 22.5 eV. The small ions at *m/e*'s 368 and 386 (E and G) are probably due to the presence of a small amount of cholesterol in

FIGURE 9. Mass Spectra of Peaks III and IV Obtained from LH-20 Column Chromatography

Mass spectra were obtained via the direct probe inlet of an LKB 9000 gas chromatograph-mass spectrometer. Spectrum III was recorded at an ionizing potential of 70 eV. Spectrum IV was recorded at an ionizing potential of 22.5 eV. See Figure 7 for location of the corresponding peaks from the LH-20 column.


relative intensity (%)
III
IV
X2
X20
E
G
H
I
H'
H"
Σ
m/e
72

this fraction. It was concluded that peak IV (Figure 7) is a mixture of free fatty acids consisting primarily of palmitic, linoleic, oleic, and stearic acids.

Mass spectra of authentic reference standards of each of the above classes of neutral lipids, except hydrocarbons, were obtained with the direct probe inlet system of the LKB 9000 and agreed well with the expected fragmentation pathways discussed above.

The separation achieved using Sephadex LH-20 in benzene-methanol (1:1) was therefore chiefly that of molecular sieving effects. Most of the lipid applied to the column (at least 95%) was eluted within one column volume; elution by subtle differences in polarity of solvent vs. compound (commonly encountered using LH-20) (188) were probably not of great importance. After this work had been completed, Roller's group published a report using benzene-acetone (1:1) as the eluting solvent for the LH-20 step in JH purification (11). Although this solvent system may afford a better purification of JH it was never attempted since the system in use had been well characterized and seemed to work well in the purification procedure.

The fraction obtained after two stages of thin-layer chromatography was substantially more pure than that obtained after the LH-20 column step and was highly enriched in JH. A gas chromatogram of the volatile components obtained after TLC-1 is presented in Figure 6B and it is evident that the sample still contained contaminating lipid. Usually one major peak was obtained upon GLC after TLC-2 (Figure 6C) and the mass spectrum of this compound was identical to that of the synthetic deuterated JH with minor exceptions for contributions from

any protium form in the sample. A third TLC separation was included when gas chromatography indicated that there were major impurities still present in the fraction obtained after TLC-2. In these cases the gas chromatogram of an aliquot of the volatile components obtained after TLC-3 resembled that shown in Figure 6C, thus indicating that purification was complete enough for mass spectral analysis. When this additional TLC step was included, recoveries of JH were lower due to the obligatory losses previously noted on the portions of the plate exposed to iodine vapor.

Levels of Juvenile Hormone: The results of assays for JH in seven insects are presented in Table 2. Those for the three species of moths were carried out on adult abdomina and the others were done on young adults, except for the housefly larvae. The number of insects analyzed was dependent for the most part upon the number available. In cases where there was an abundance of insects (houseflies, crickets, and honeybees) enough insects were extracted so that the total lipid did not exceed 6 g since the LH-20 column could not accomodate more than 3 g lipid.

For clarity, the results of observed and corrected isotopic ratios have been presented in parts per thousand (ppt). The error in measurement averaged 13% and was independent of which ion pair was used for the assay. In the earlier stages of these analyses, relatively larger amounts of deuterium carrier form was added (experiments 1-4, Table 2), however this was decreased to a minimal level (75 μg) as expertise with handling insect lipid extracts was obtained. At this latter level of added deuterated carrier form, the lower limit

TABLE 2. Assay of *Cecropia* C_{18}-Juvenile Hormone

Exp.	Insect	Sex	N[a]	*m/e* pair	Protium/deuterium ratio (ppt) Sample	Background
1	*H. cecropia*	M	4.9[c]	114/117	87.5 ± 0.7[d]	70.4 ± 1.0
				209/212	193.4 ± 1.8	175.4 ± 0.4
2	*H. cecropia*	M	1	114/117	36.1 ± 0.3	27.1 ± 0.2
3	*H. cecropia*	M	1	114/117	51.1 ± 0.1	41.7 ± 0.2
4	*H. cecropia*	F	32	114/117	55.8 ± 1.7	27.1 ± 0.2
				209/212	204.0 ± 4.0	172.5 ± 0.5
5	*S. cynthia*	M	7	114/117	34.4 ± 0.2	23.4 ± 0.2
				209/212	121.2 ± 0.3	108.4 ± 0.3
6	*S. cynthia*	F	12	114/117	32.1 ± 0.4	23.4 ± 0.2
				209/212	118.7 ± 0.6	108.4 ± 0.3
7	*C. promethea*	M	13	114/117	53.9 ± 0.5	40.1 ± 0.6
				209/212	221.5 ± 0.8	205.0 ± 1.0
8	*C. promethea*	F	15	114/117	47.2 ± 0.6	40.1 ± 0.6
				209/212	210.0 ± 0.7	198.5 ± 0.5
9	*M. domestica* (larvae)	M,F	∿7300	114/117	44.3 ± 0.2	39.5 ± 0.2
				209/212	162.3 ± 0.8	157.0 ± 0.6
10	*A. mellifera* (without queen)	F	1035	114/117	92.2 ± 1.1	40.6 ± 0.2
				209/212	205.2 ± 2.1	159.2 ± 0.9
11	*A. domestica*	M	149	114/117	63.2 ± 0.4	43.9 ± 0.1
				209/212	175.2 ± 0.8	153.6 ± 0.4
12	*A. domestica*	F	182	114/117	250.6 ± 1.5	40.1 ± 0.6
				209/212	326.7 ± 9.3	132.0 ± 2.0
13	*O. melanopus*	M,F	∿3500	114/117	106.5 ± 0.5	23.4 ± 0.2
				209/212	200.8 ± 1.3	108.4 ± 0.3

[a] N represents the number of organisms used.

[b] Corrected for isotope effect as described in Results.

TABLE 2. Assay of *Cecropia* C_{18}-Juvenile Hormone (CON'T)

Exp.	Protium/deuterium ratio (ppt)		μg carrier form added	μg JH / g Lipid	ng JH / Organism
	Difference	Corrected ratio[b]			
1	17.1 ± 1.0	13.7 ± 1.0	607	11.53 ± 0.84	1697 ± 124
	18.0 ± 1.8	16.0 ± 1.8	607	13.47 ± 1.52	1982 ± 223
2	9.0 ± 0.3	7.2 ± 0.3	247	10.28 ± 0.43	1778 ± 74
3	9.4 ± 0.2	7.5 ± 0.2	247	6.35 ± 0.16	1853 ± 49
4	27.8 ± 1.7	22.4 ± 1.7	750	4.83 ± 0.37	525 ± 40
	31.5 ± 4.0	28.0 ± 4.0	750	6.03 ± 0.86	656 ± 94
5	10.9 ± 0.2	8.7 ± 0.2	75	0.92 ± 0.02	93 ± 2
	12.8 ± 0.3	11.4 ± 0.3	75	1.20 ± 0.03	122 ± 3
6	8.7 ± 0.2	7.0 ± 0.4	75	0.98 ± 0.07	44 ± 3
	10.3 ± 0.6	9.2 ± 0.6	75	1.29 ± 0.09	58 ± 4
7	13.8 ± 0.5	11.0 ± 0.5	75	1.17 ± 0.06	63 ± 3
	16.5 ± 1.0	14.7 ± 1.0	75	1.57 ± 0.11	85 ± 6
8	7.1 ± 0.6	5.7 ± 0.6	75	0.89 ± 0.09	29 ± 3
	11.5 ± 0.7	10.2 ± 0.7	75	1.60 ± 0.13	51 ± 4
9	4.8 ± 0.2	3.8 ± 0.2	75	0.10 ± 0.01	0.039 ± 0.002
	5.3 ± 0.8	4.7 ± 0.8	75	0.13 ± 0.02	0.048 ± 0.008
10	51.6 ± 1.1	41.3 ± 1.1	75	0.71 ± 0.02	3 ± 0.08
	46.0 ± 2.1	40.9 ± 2.1	75	0.71 ± 0.04	3 ± 0.15
11	19.3 ± 0.4	15.4 ± 0.4	75	0.37 ± 0.01	8 ± 0.2
	21.6 ± 0.8	19.2 ± 0.8	75	0.46 ± 0.02	10 ± 0.4
12	210.5 ± 1.5	168.4 ± 1.5	75	1.95 ± 0.03	69 ± 1
	194.7 ± 9.3	173.4 ± 9.3	75	2.01 ± 0.11	71 ± 4
13	83.1 ± 0.5	66.5 ± 0.5	75	4.19 ± 0.03	1.4 ± 0.01
	92.4 ± 1.3	82.2 ± 1.3	75	5.19 ± 0.08	1.8 ± 0.03

[c] An aliquot of the total lipid extracted from 10 male abdomina was used.

[d] Each value represents 2-4 determinations ± standard error of the mean.

of sensitivity can be determined as being approximately 150 ng protium form in the total extract assuming a ratio of 1:500 as the lowest ratio acceptable for a reliable measurement. The absolute limit of sensitivity is dependent upon the precision of the blank value. For example, the variation in the blank ratio at ion pair *m/e* 114/117 in experiment 1 (Table 2) was 1.0 ppt; therefore a ratio of 1:1000 would be the absolute limit of detection. At *m/e* pair 209/212 the variation was 0.4 ppt; therefore a ratio of 1:2500 could presumably be analyzed. These absolute limits of detection were never reached in any of the experiments done.

The results obtained for the levels of JH in these organisms have been simplified in Table 3 for ease of comprehension. When expressed on a per organism basis, the results point out sexual dimorphism; female moths contain less JH than males. Interestingly, however, female crickets contain an 8-fold higher level of JH than their male counterparts. When the results are expressed on the basis of weight of lipid, which is a more standard and satisfactory method of data presentation due to variability of the water content of insects, only the housefly larvae and the cereal leaf beetles stand out due to their low and high levels, respectively. The cecropia moth (both male and female) contains a higher amount of JH than others in their order.

When the deuterated form of JH was carried through the entire purification procedure in the absence of crude insect lipid there was no increase in the ratio at *m/e* pair 114/117 or at *m/e* pair 209/212 above the blank ratio. When the deuterated form of JH was carried

TABLE 3. Levels of *Cecropia* C_{18}-Juvenile Hormone[a]

Insect	Sex	µg/g Lipid	ng/Organism
H. cecropia	M	10.41	1827
	F	5.43	591
S. cynthia	M	1.06	108
	F	1.14	51
C. promethea	M	1.37	74
	F	1.25	40
M. domestica (larvae)	M,F	0.12	0.044
A. mellifera (without queen)	F	0.71	3
A. domestica	M	0.42	9
	F	1.98	70
O. melanopus	M,F	4.69	1.6

[a] A standard deviation of ± 13% can be attributed to each of the above figures as a measure of relative precision.

through the entire purification procedure in the presence of the total lipid extracted from 100 ml of human plasma, there was no increase in the observed ratios above the blank at either of the two ion pairs.

Only one analysis was carried out using the deuterated ethyl ester form of JH. One male cecropia moth abdomen was extracted, 50 µg of the deuterated ethyl ester carrier was added and purification proceeded exactly as with the methyl ester. The blank value at ion pair *m/e* 128/130 was 30.7 ± 0.5 ppt (three determinations) and that for the sample was 30.4 ± 0.4 ppt (three determinations). Since no difference was obtained, it was concluded that male cecropia moth did not contain this form of JH at a level of 100 ng/organism, again assuming 1:500 as the lowest ratio acceptable for accurate isotopic ratio measurements. This does not preclude the possibility that the ethyl ester is not present at lower levels. Since the protium form was not available, further detailed study with larger numbers of insects was discontinued.

Occurrence and Levels of Fatty Acid Methyl and Ethyl Esters in Housefly Larvae

Purification of Fatty Acid Esters: A summary of the recoveries of lipid extraction and purification from housefly larvae is presented in Table 5. The amount of extracted lipid varied between 1.6% and 3.5% of wet weight, which is significantly lower than the previously reported values of 4.9% and 7.1% lipid in *M. domestica* larvae (156). The low yield can be attributed to the fact that alcohol was not used in any of the procedures; re-extraction of the final residue with

TABLE 4. Analysis of Lipids by Thin-Layer Chromatography[a]

Compound	R_f	
	Hexane-diethyl ether-acetic acid (80:20:1)	Benzene-ethyl acetate (4:1)
Cholesterol	0.09	0.33
Geraniol	0.13	0.31
Farnesol-Band 1	0.13	0.38
Farnesol-Band 2	0.14	0.43
Fatty Alcohols	0.16	0.38
Nerolidol	0.24	0.53
Free Fatty Acids	0.25	N.D.
Geranylgeraniol	0.28	0.66
Triglycerides	0.48	0.82
Juvenile Hormone	0.50	N.D.
Fatty Acid Esters	0.56	N.D.
Cholesterol Esters	0.74	0.71
Squalene	0.75	0.80

[a] Silica Gel G, 250 μm, Analtech, Inc.

TABLE 5. Purification of Fatty Acid Methyl and Ethyl Esters from *Musca domestica* Larvae and Synthetic Diet

Experiment	Wet Weight (g)		Total Extract (g)	Hexane Soluble Fraction (g)	Florisil Column (mg)	Thin-Layer Chromatography (mg)
	larvae	diet				
1	114.99		N.D.	2.52	33.6	1.6
2	35.00		1.05	0.73	10.2	0.4
3	64.24		1.65	1.00	19.2	1.2
4[a]	30.74		1.42	1.09	70.8	4.8
5[a]	25.60		1.34	0.96	50.0	3.5
6		20.00	N.D.	0.56	43.5	0.1

[a] Insects in these experiments were first slurried gently in chloroform for 30 minutes for removal of cuticular lipids prior to extraction.

chloroform-methanol (2:1) yielded additional lipoidal material. However this material was not analyzed since alcohol had been used in the workup. Recovery studies with pure methyl and ethyl palmitate, as measured by gravimetric, radioisotopic, and gas chromatographic techniques, indicated a total recovery in the initial extraction of 95.9% (mean value of three determinations).

A water wash of the total extract was necessary since significant amounts of water soluble material (mainly pigments and gut contents) in the larvae were extracted with acetone (a moderately hydrophilic solvent). The color of the total extract was usually brown, that of the discarded water phase was usually bright orange, and that of the hexane soluble lipid became a light yellow. The Florisil column afforded at least a 15-fold purification of the extract and in some cases up to a 75-fold purification was acheived (Table 5). The column was not further eluted and the Florisil was discarded. Thin-layer chromatography in the neutral lipid solvent system provided a fraction which was highly enriched in fatty acid esters and suitable for analysis by GC-MS. The separation of neutral lipids obtained in this solvent system can be seen in the picture of the TLC plate presented in Figure 17 as well as in the R_f values presented in Table 4. The separation of fatty acid methyl and ethyl esters from triglycerides and sterol esters was complete.

Identification of Fatty Acid Esters: The fatty acid esters were identified by means of continuous repetitive scanning over a mass range of *m/e* 6-400 at intervals of 8 seconds (mass chromatography) (147,161), with subsequent output of selected masses or normalized

bargraphs. Figure 10, which is the total ion intensity of each scan for a typical run (experiment 1, Table 5), shows that the separation of the methyl and ethyl esters was quite satisfactory on the SE-30 column used in this study. Mass spectra recorded at the apex of each peak were in good agreement with those reported for long-chain saturated and monounsaturated fatty acid methyl and ethyl esters, respectively (162,163), as well as authentic standards run under similar conditions.

Figure 11 shows the normalized bar graphs for the spectra taken at scans 44 and 59. These spectra show a base peak at *m/e* 74 and at *m/e* 88, representing the characteristic McLafferty rearrangement ions of long chain esters. They also show major ions at *m/e* 87 and at *m/e* 101 which represent cleavage between the β and γ carbon atoms for the methyl and ethyl esters, respectively. The ions at *m/e* 143 and at *m/e* 199 in the spectra of methyl esters represent fragmentation at C-7 and C-11 and probably include charge localization on the carbonyl carbon; they are 14 amu higher in the spectra of ethyl esters, at *m/e* 157 and at *m/e* 213. The molecular ions at *m/e* 270 and at *m/e* 284, the ions at *m/e* 227 ($[M-43]^+$) and at *m/e* 241 ($[M-29]^+$) which are shifted by 14 amu to *m/e* 241 and *m/e* 255, and the ion at *m/e* 239 which represents loss of methoxy and ethoxy radicals from the molecular ion and is present in each spectrum, are consistent with the unequivocal identification of these compounds as methyl palmitate and ethyl palmitate, respectively. Mass spectra of the other methyl and ethyl esters have not been presented, but agreed in all respects with the predicted fragmentation pathways and molecular ions for the other esters identified.

Mass chromatograms of *m/e* 74 and *m/e* 88, the Type H rearrangement

FIGURE 10. Total Ion Intensity of Fatty Acid Methyl and Ethyl Esters

Mass spectra were continuously recorded at 70 eV over a mass range of *m/e* 6-400 at intervals of 8 seconds on an LKB 9000 gas chromatograph-mass spectrometer using the computer system described in Methods. The subscripts denote chain length and degree of unsaturation and the superscripts, the type of ester found. GLC was performed isothermally at 160° on 1% SE-30 (100/120 mesh on Supelcoport) using a coiled glass column (1.6 m x 3 mm). Helium carrier gas was maintained at a constant flow rate of 35 ml/min.

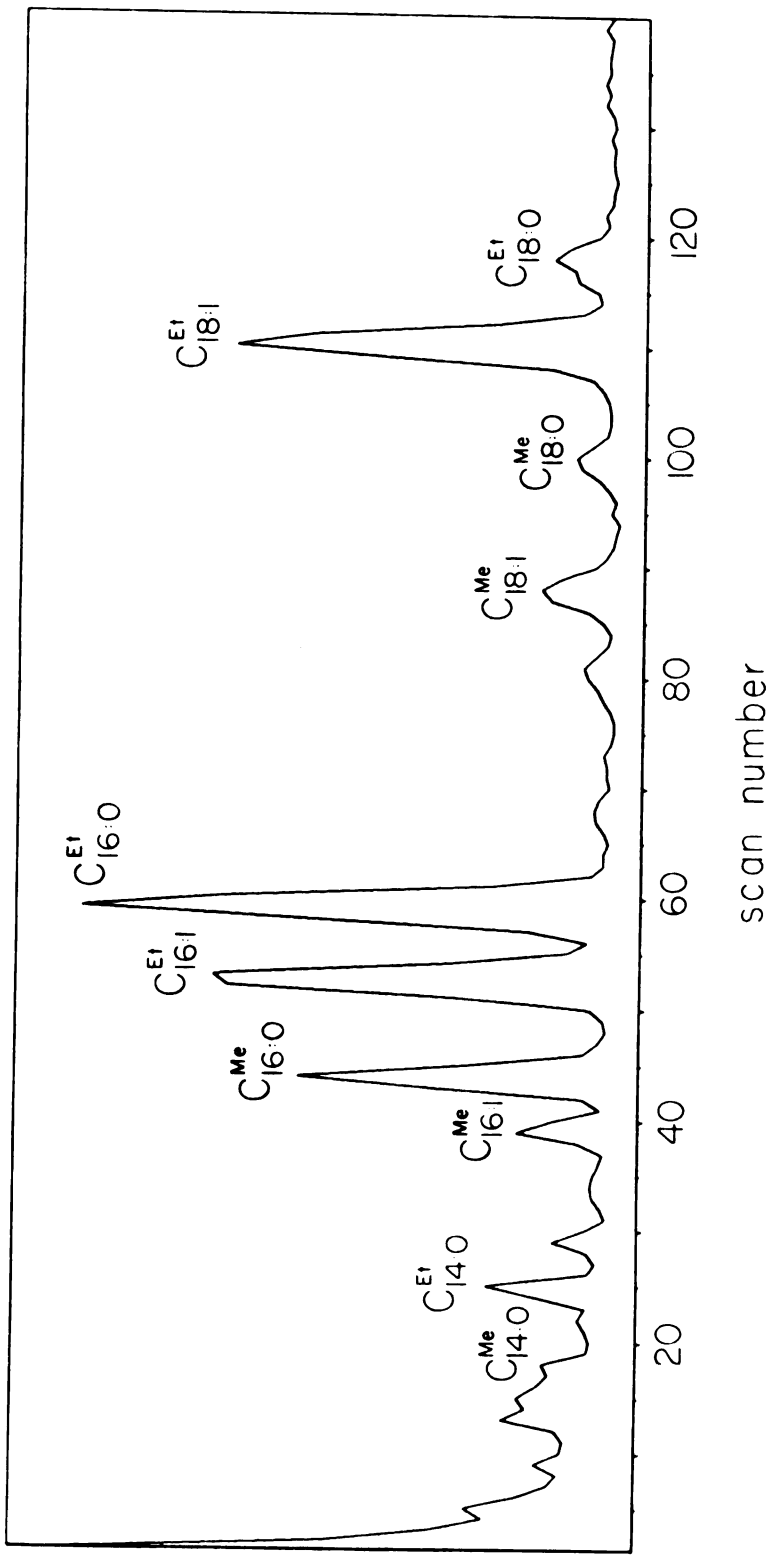
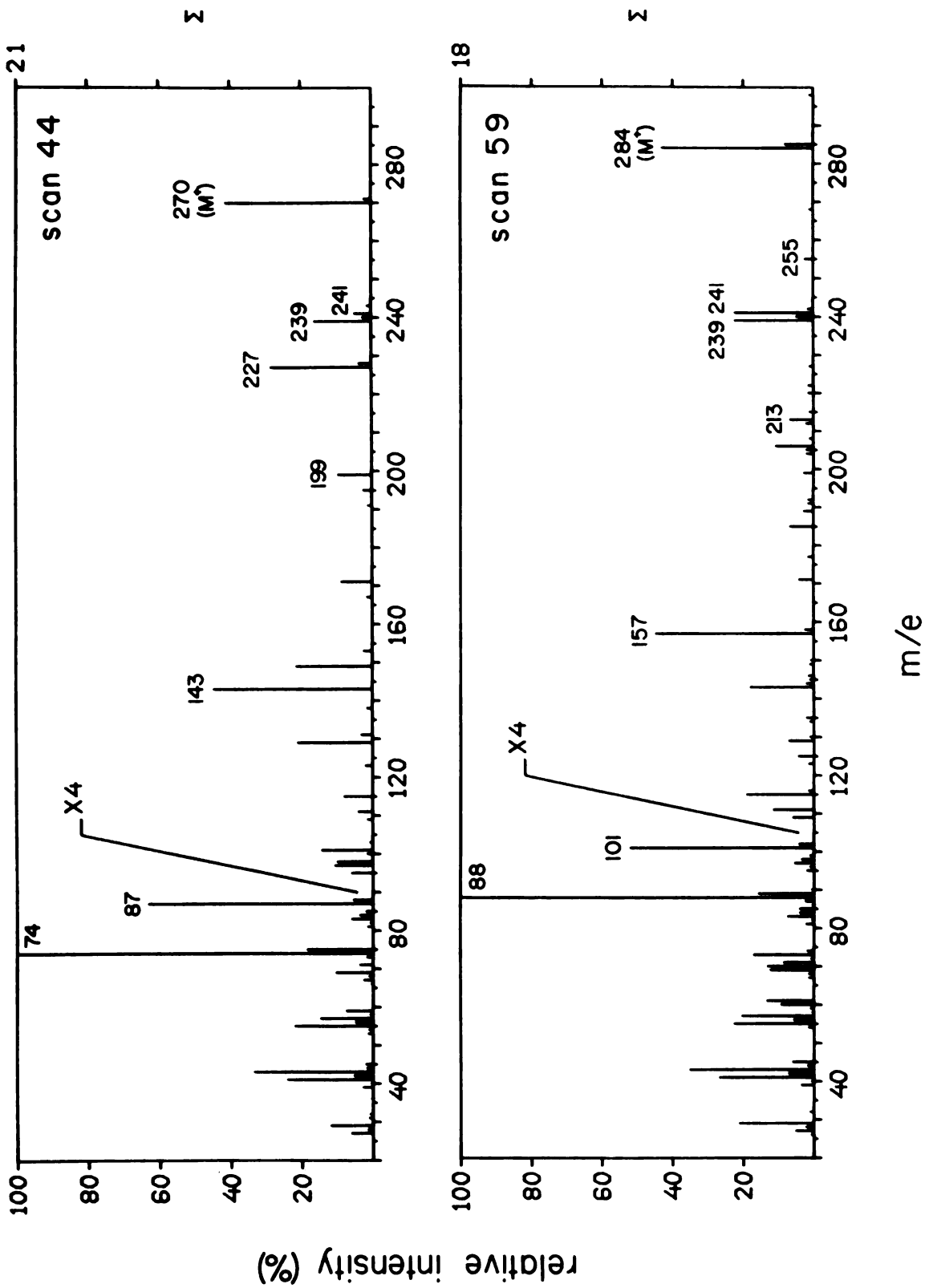

relative intensity
scan number
20
40
60
80
100
120
$C^{Et}_{14:0}$
$C^{Me}_{14:0}$
$C^{Me}_{16:1}$
$C^{Me}_{16:0}$
$C^{Et}_{16:1}$
$C^{Et}_{16:0}$
$C^{Me}_{18:1}$
$C^{Me}_{18:0}$
$C^{Et}_{18:1}$
$C^{Et}_{18:0}$

FIGURE 11. Mass Spectra of Methyl and Ethyl Palmitate

Normalized bargraphs of spectra taken at scans 44 and 59 (see Figure 10). Characteristic ions have been labeled and are discussed in Results.


scan 44
21
Σ
270 (M+)
241
239
227
199
143
X4
87
74
scan 59
18
Σ
284 (M+)
255
239 241
213
157
X4
101
88
100
80
60
40
20
40
80
120
160
200
240
280
relative intensity (%)
m/e

ions, are shown along with the total ion intensity chromatogram in Figure 12. The intensities of these ions lined up exactly with those of each of the esters identified. Mass chromatograms of each molecular ion were located in the predicted place in relation to the total ion intensity chromatogram. Upon temperature programming, no additional peaks for fatty acid esters were found.

Assay of Fatty Acid Esters: Differential analyses were made with the aforementioned McLafferty rearrangement ions at *m/e* 74 and at *m/e* 88, respectively, representing the long-chain methyl and ethyl esters. The ions, base peaks for the saturated straight chain esters, are assumed to be directly proportional to concentration in the range of concentrations used in this investigation. They are significantly depressed in monounsaturated fatty acid esters, however, in which *m/e* 55 becomes the base peak. Mass spectral data of reference mixtures of saturated and monounsaturated esters were recorded under similar conditions; multiplication by a factor of 4.0 was necessary to correlate the responses of *m/e* 74 and of *m/e* 88 for the monounsaturates to that of the saturates. Although a standard curve such as that shown in Figure 13 was not run with each experiment, selected points were repeated with every determination.

Figure 14 shows the computer output of a typical run (experiment 3, Table 5) of both ions, *m/e* 74 and *m/e* 88 (mass fragmentograms (148,164)) for one of the three AVA channels. Although the mass fragmentogram for *m/e* 74 shows only those peaks arising from fatty acid methyl esters, that for *m/e* 88 shows extra peaks other than those representing ethyl esters. These additional peaks at *m/e* 88 line up exactly with those

FIGURE 12. Mass Chromatograms of *m/e* 74 and *m/e* 88 and Total Ion Intensity Chromatogram of Fatty Acid Methyl and Ethyl Esters

Operating conditions are the same as those described in the legend to Figure 10.

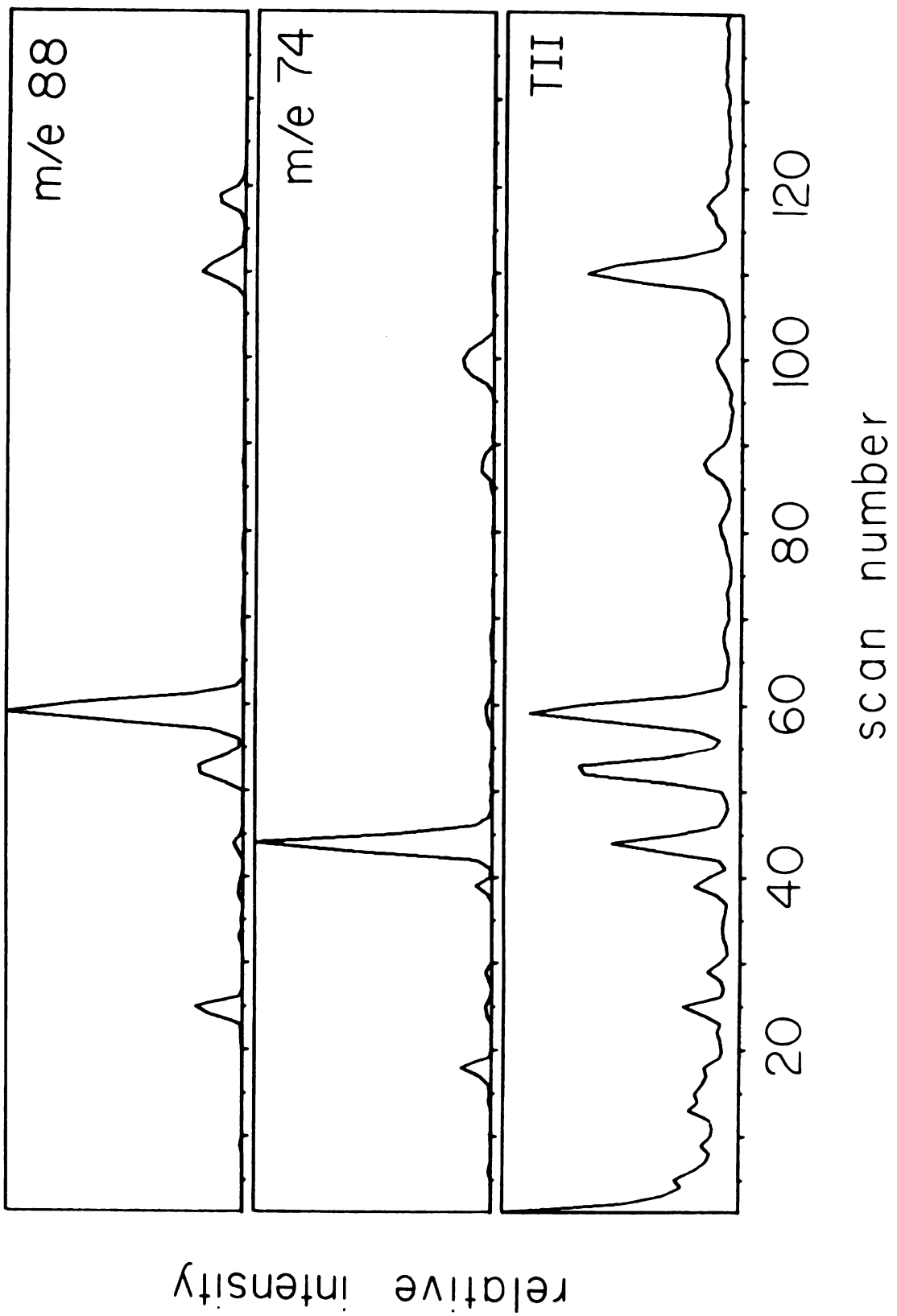

m/e 88
m/e 74
TII
20
40
60
80
100
120
scan number
relative intensity

FIGURE 13. Intensity of *m/e* 74 versus Sample Size of Methyl Palmitate Injected

Areas were calculated by computer integration; each point represents the mean value of six determinations. Methyl palmitate was chromatographed isothermally at 160° on 1% SE-30.

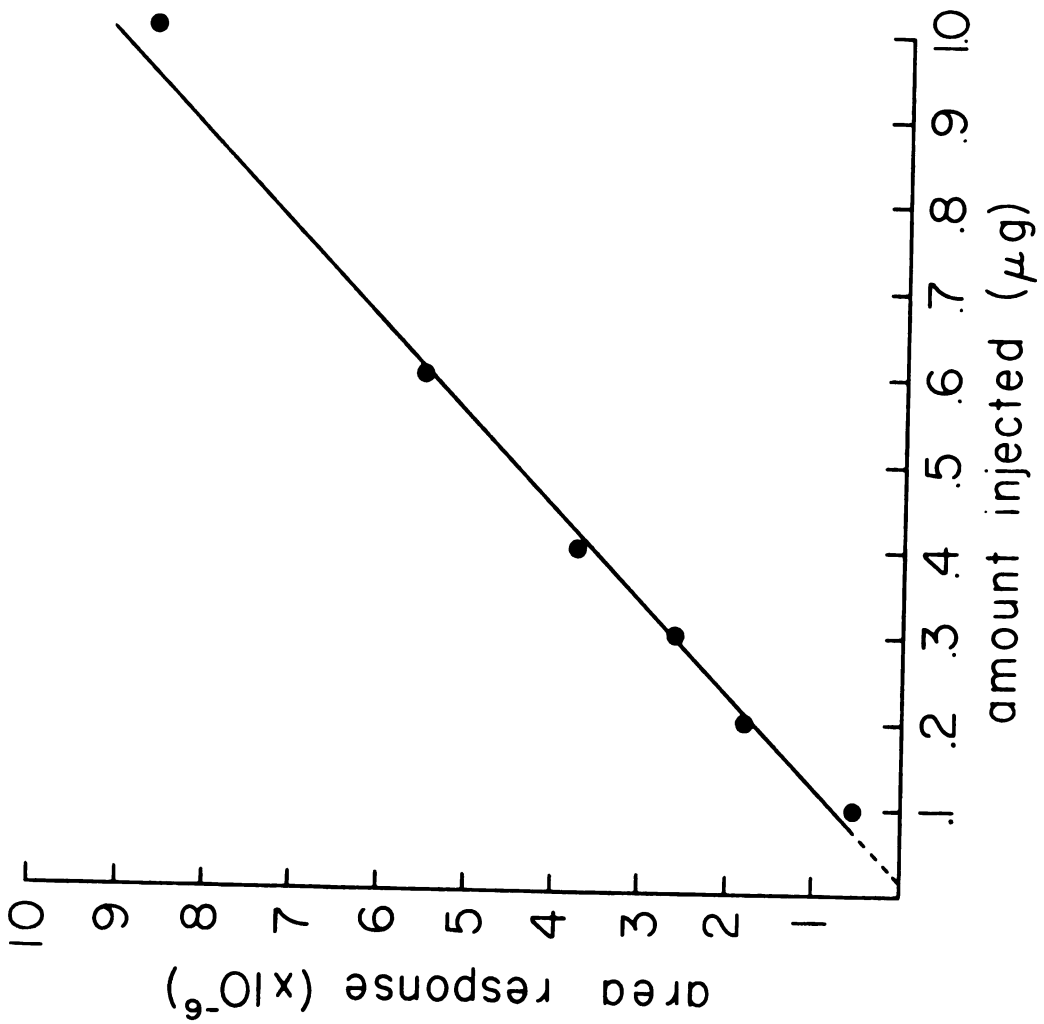
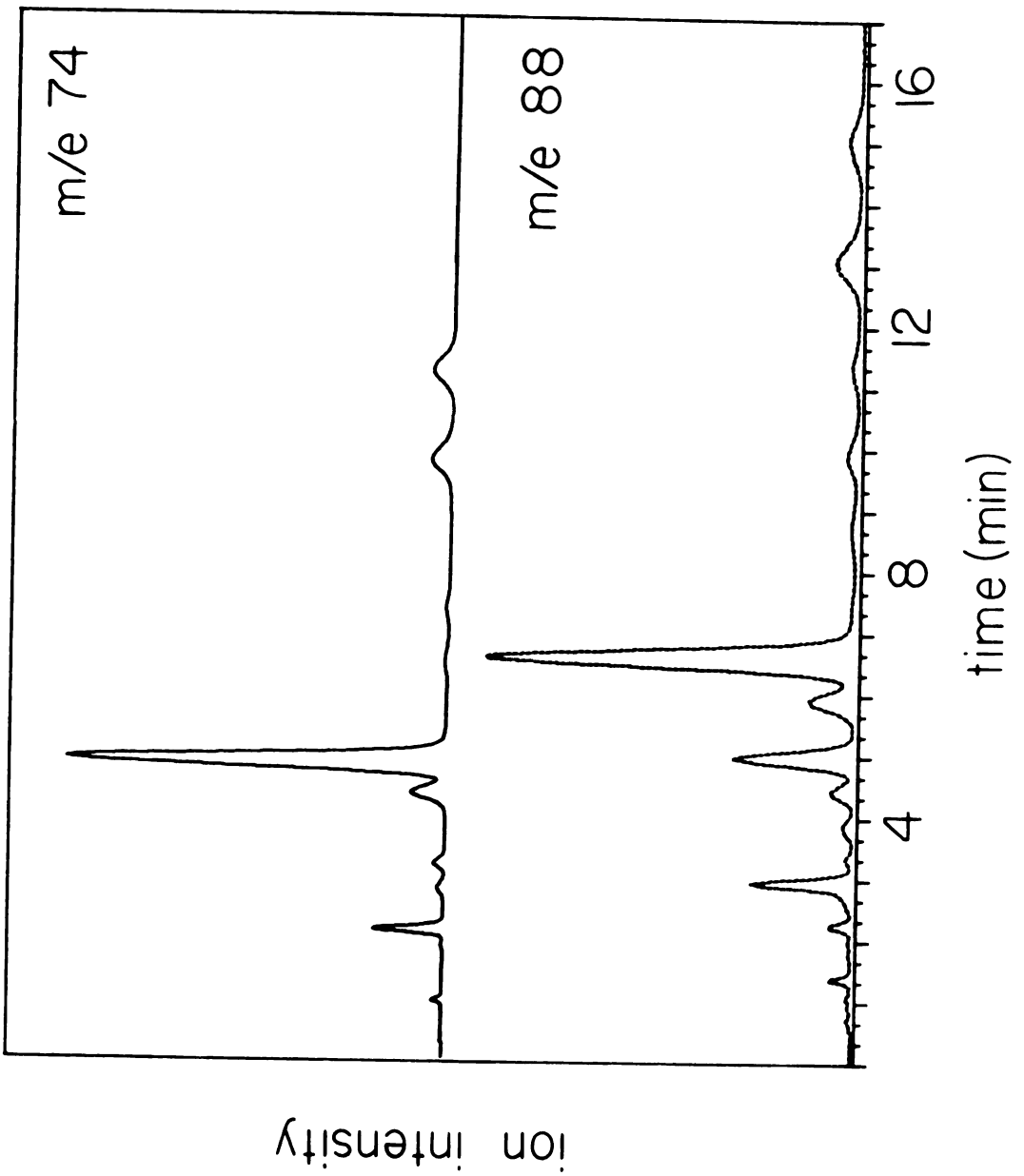

area response ($\times 10^{-6}$)
1
2
3
4
5
6
7
8
9
10
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
amount injected (μg)

FIGURE 14. Specific Ion Intensity Chromatograms of *m/e* 74 and *m/e* 88

Specific ion intensity chromatograms (mass fragmentograms) were obtained at 70 eV with the aid of the computer system described in Methods. GLC was performed isothermally at 160° on 1% SE-30. This is the analysis of fatty acid esters performed in experiment 3 (Table 5).


m/e 74
m/e 88
4
8
12
16
time (min)
ion intensity

at *m/e* 74 and represent a ^{13}C species stable isotope ion of *m/e* 87 (β-γ cleavage of the methyl esters). To resolve the two ions at nominal mass *m/e* 88, a much higher resolution (at least 20,000) would be needed since the ^{13}C isotope ion of *m/e* 87 ($^{12}C_3{}^{13}C_1H_7O_2$) has an exact mass of 88.0478 and *m/e* 88 from the ethyl esters has an exact mass of 88.0522 ($C_4H_8O_2$).

The linearity of response and sensitivity of the mass spectrometer-computer system were tested with methyl palmitate. As shown in Figure 13, the linearity over the range of 0.1 to 0.8 μg ester injected was acceptable; loss of linearity at lower amounts injected was probably due to adsorption on the gas chromatographic column. Least-squares analysis of the data points gave a regression line with an X-intercept of 22 ng as the absolute limit of detection under the operating conditions employed.

Levels of Fatty Acid Esters: The total amount of methyl and ethyl esters, 5.20 and 13.14 nmole/g wet weight (Table 6), represents 0.0071% and 0.0188%, respectively, of the extracted lipid. Ethyl esters were about two and a half times as abundant as methyl esters and in both cases, methyl and ethyl palmitoleate, palmitate, and oleate were the predominent species, with palmitate being the most abundant. Five independent determinations were carried out, and, for each sample, a precision of 4% between runs was maintained. The relative distributions of naturally occurring fatty acid esters does not vaty greatly from that observed for the total fatty acid distribution in the housefly (157), with the exception of linoleate. In these experiments, linoleate was not added to the diet and the insects therefore contained no

TABLE 6. Levels of Fatty Acid Methyl and Ethyl Esters in *Musca domestica* Larvae and Synthetic Diet[a]

Fatty Acid Ester		*Musca domestica*		Diet	
Methyl	Ethyl	(nmole/g)	(% of total)	(nmole/g)	(% of total)
14:0		0.30 ± 0.11	(1.6)	0.06	(1.6)
16:1		1.46 ± 0.61	(8.0)	0.07	(1.8)
16:0		1.89 ± 0.54	(10.3)	1.66	(43.8)
18:1		1.29 ± 0.47	(7.0)	1.01	(26.6)
18:0		0.26 ± 0.10	(1.4)	0.77	(20.3)
TOTAL		5.20 ± 0.58		3.57	
	14:0	0.72 ± 0.24	(3.9)		
	16:1	4.03 ± 1.44	(22.0)		
	16:0	4.53 ± 1.60	(24.7)	0.22	(5.8)
	18:1	3.53 ± 0.59	(19.2)		
	18:0	0.33 ± 0.14	(1.8)		
	TOTAL	13.14 ± 2.28			

[a] Each value represents the mean of five experiments ± S.D. For each individual experiment, 6-9 determinations were made with an overall relative precision of 4%. Only one experiment was performed on the diet. Values were not corrected for losses during purification.

polyunsaturated fatty acids. Esters below C_{14} or above C_{18} were not detected under the conditions employed, but the existence of other esters in the housefly larvae at very low levels is not precluded.

To test whether these esters were of cuticular origin, cuticular lipids were removed from the larvae by a special extraction procedure. Approximately 2% of the total lipid was obtained; further purification revealed the existence of both methyl and ethyl esters, at levels which represented only 2.3% and 4.7% of the total methyl and ethyl esters, respectively, extracted from intact larvae. Furthermore, the distribution of these esters was markedly different from that of the whole insect in that the saturated esters were far more prominent than the unsaturated ones. The cuticular fatty acid methyl esters found in *Tenebrio molitor* showed a similar pattern (125). This pattern may be variable with temperature as has been shown in the temperature-dependent changes in phospholipid fatty acids in *M. domestica* larvae (165). At any rate, the esters found in this study were not localized to any special extent in the cuticle.

The synthetic diet was analyzed once and contained about the same level of methyl esters as that found in the larvae (Table 6). Ethyl palmitate was the only ethyl ester detected, however, and the total level was much lower. The methyl esters, therefore, could have arisen from the diet, but this explanation is unsuitable for the quantity and variety of ethyl esters found.

Since it is a well recognized fact that methyl and ethyl esters can be made spontaneously from alcoholic solutions of triglycerides (107,117,118,119) the use of methyl or ethyl alcohol in the

extraction or purification procedures was deliberately avoided. Neither methyl or ethyl oleate was detected when purified oleic acid was placed through the purification procedures. Control experiments in which tripalmitin, triolein, and tristearin (100 mg each) were carried through the purification procedures, failed to reveal significant amounts of methyl or ethyl palmitate, oleate, or stearate. When these triglycerides were placed in chloroform-methanol (2:1) overnight at neutral pH at 23° approximately 0.001% of the lipid was detected as methyl ester. Although the solvents were not purified extensively before use and theoretically as little as 50 nl of alcohol per 100 ml solvent would have been sufficient for synthesis of esters in the levels detected, the control experiments ruled out the possibility that the esters were artifacts. A recent report indicated the ability of Florisil to catalyze the formation of fatty acid esters, but apparently this happens only when lipids interact with the chromatographic support for a period of at least two hours (119). In all of the purification carried out, the esters were eluted within 15 minutes after application to the column.

In vivo Studies in Housefly Larvae

Search for Radiolabeled Juvenile Hormone: The incorporation of ^{14}C substrates into the lipids of housefly larvae is reported in Table 7. Approximately 35% of the total incorporated methionine and 85% of the incorporated mevalonate was recovered in the neutral lipid fractions. The neutral lipid fractions from experiments 1 and 2 were chromatographed on Sephadex LH-20 after low-temperature precipitation as

TABLE 7. Incorporation of ^{14}C Substrates into Housefly Lipids during Larval Growth

Exp.	Substrate	Incorporation					
		Total Lipid		Neutral Lipid		Polar Lipid	
		dpm	%	dpm	%	dpm	%
1	L-[*Me*-^{14}C]methionine[a] (50 µCi)	4,113,924	3.74	1,466,260[b]	1.33	2,647,664	2.41
2	DL-[2-^{14}C]mevalonic acid[c] (50 µCi)	7,031,645	12.78	6,241,000[b]	11.35	790,645	1.44
3	DL-[2-^{14}C]mevalonic acid[c] (50 µCi)	8,017,384	14.58	6,560,625[d]	11.93	1,456,758	2.65

[a] % incorporation based on total amount of radioactivity administered.

[b] Determined as low-temperature filtrate (see Methods).

[c] % incorporation based on 50% of radioactivity administered.

[d] Determined by silicic acid column chromatography (see Methods).

described (see Methods). The elution profiles for both mass and radioactivity for each experiment are presented in Figures 15 and 16, for growth on radiolabeled mevalonic acid and methionine, respectively. Note the difference in radioactivity scales; the region of JH elution is shown. Mass spectra of the lipid obtained from the various fractions were not taken; however, the major peak at fractions 43-45 in each profile were tentatively identified as being triglyceride by co-chromatography with authentic standards. In the same way, the minor peaks at fractions 50-51, 61-62 and 67-69 in each profile were identified as sterol ester, free sterol, and free fatty acid, respectively. The profiles are similar to that obtained for mass from the lipid purified from cereal leaf beetles (Figure 7). Since fly larvae contain much smaller quantities of free sterol and free fatty acids, these peaks are much smaller than those found for the cereal leaf beetles (156). In both cases, radioactivity incorporation into the neutral lipids followed the mass quite closely.

The fractions containing deuterated JH were purified by TLC and the volatile components obtained after TLC-2 were subjected to gas-liquid radiochromatography (GLRC) as described (see Methods). No radioactive JH was found; mass response for the mixture of protium and deuterium forms was quite similar to that shown in Figure 6C; however, radioactive response for the injected material gave nothing above background. Assuming total incorporation of each radiolabeled substrate, without dilution, into the JH molecule, the theoretical amount of radioactive hormone present would have been around 2100 dpm for growth on labeled mevalonic acid and 3200 dpm for growth on labeled methionine. The failure to find

FIGURE 15. Elution Profile of Mass and Radioactivity on Sephadex LH-20 After Growth of Housefly Larvae on DL-[2-^{14}C]Mevalonic Acid

Gel filtration on Sephadex LH-20 was carried out with benzene-methanol (1:1) as the eluting solvent after low-temperature precipitation. 4 ml fractions were collected. The area of JH elution is marked.

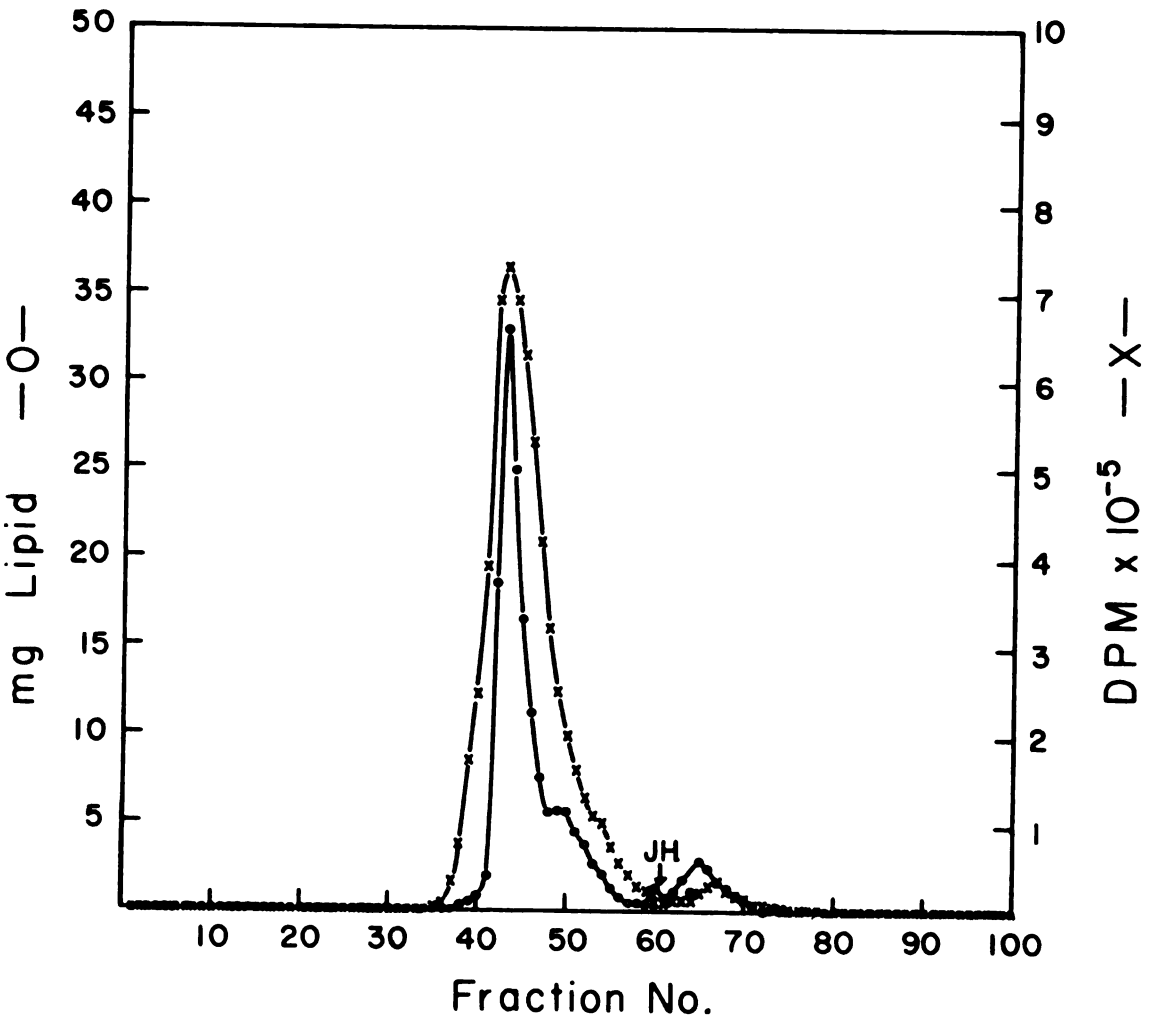
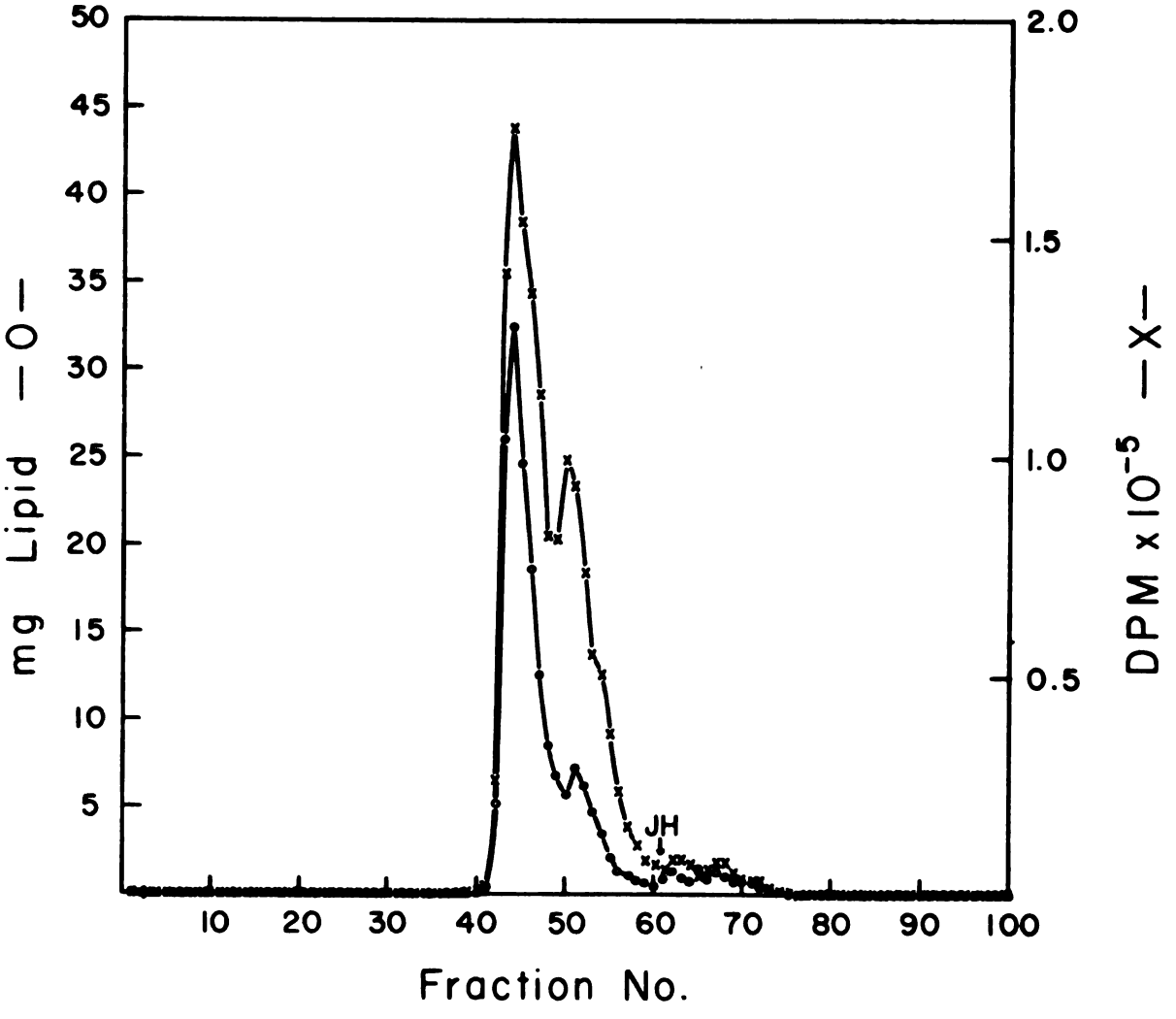

mg Lipid —O—
DPM x 10^{-5} —X—
JH
Fraction No.
50
45
40
35
30
25
20
15
10
5
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100

FIGURE 16. Elution Profile of Mass and Radioactivity on Sephadex LH-20 After Growth of Housefly Larvae on L-[*Me*-^{14}C]Methionine

Conditions were the same as those for Figure 15. Note the difference in radioactivity scales from that in Figure 15.


mg Lipid —O—
DPM x 10⁻⁵ —X—
Fraction No.
JH
50
45
40
35
30
25
20
15
10
5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
80
90
100

labeled JH and problems with sensitivities and limits of detection will be discussed later in the dissertation.

Prior to JH purification an aliquot (one-twentieth) of each total extract was subjected to TLC in both the neutral and polar lipid solvent systems to assess where the radioactivity had been incorporated into total lipid. A radioscan of the total lipid from larvae grown on labeled methionine in the polar lipid solvent system is presented in Figure 17. Phospholipids were identified by spraying with Zinzandee's reagent and co-chromatography with authentic standards. Radioactive bands were chromatographically identical to phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and assumed neutral lipid at the top of the plate. The radioscan closely resembles that obtained for the lipid extracted from housefly larvae grown on labeled methionine in a similar experiment by Moulton *et al.* (166). Radioactive incorporation into these two phospholipids is expected since flies cannot synthesize N-methyl groups (i.e., choline) and incorporation into these phospholipids appears due to "C-1" metabolism through folic acid mediated reactions. The neutral lipid was not specifically identified but a radioscan of this total lipid extract in the neutral lipid solvent system (not presented) showed that the majority of the radioactivity was found at the origin (approximately 60%) and in a diffuse band migrating with triglyceride (approximately 35%). A very minor radioactive band was seen in the area of sterol esters. This distribution correlates well with the predicted distribution of radioactive neutral lipids as evidenced from chromatography on LH-20 (see Figure 16).

The radioscan of the total extract from growth on mevalonic

FIGURE 17. Thin-Layer Radiochromatography of the Total Lipid Extract Obtained After Growth of Housefly Larvae on L-[*Me*-^{14}C]Methionine

The total lipid extract was chromatographed on silica gel G (250 μm, Quantum) in chloroform-methanol-water (100:42:6) and scanned on a Berthold Model 6000 Radioscanner. A picture of the TLC plate showing mass was aligned with the scan by the marker (dye containing [^{14}C]scurose) spotted on each end of the plate. PS=phosphatidyl serine, PE=phosphatidyl ethanolamine, NL= neutral lipid. Bands were identified by co-chromatography with authentic standards.

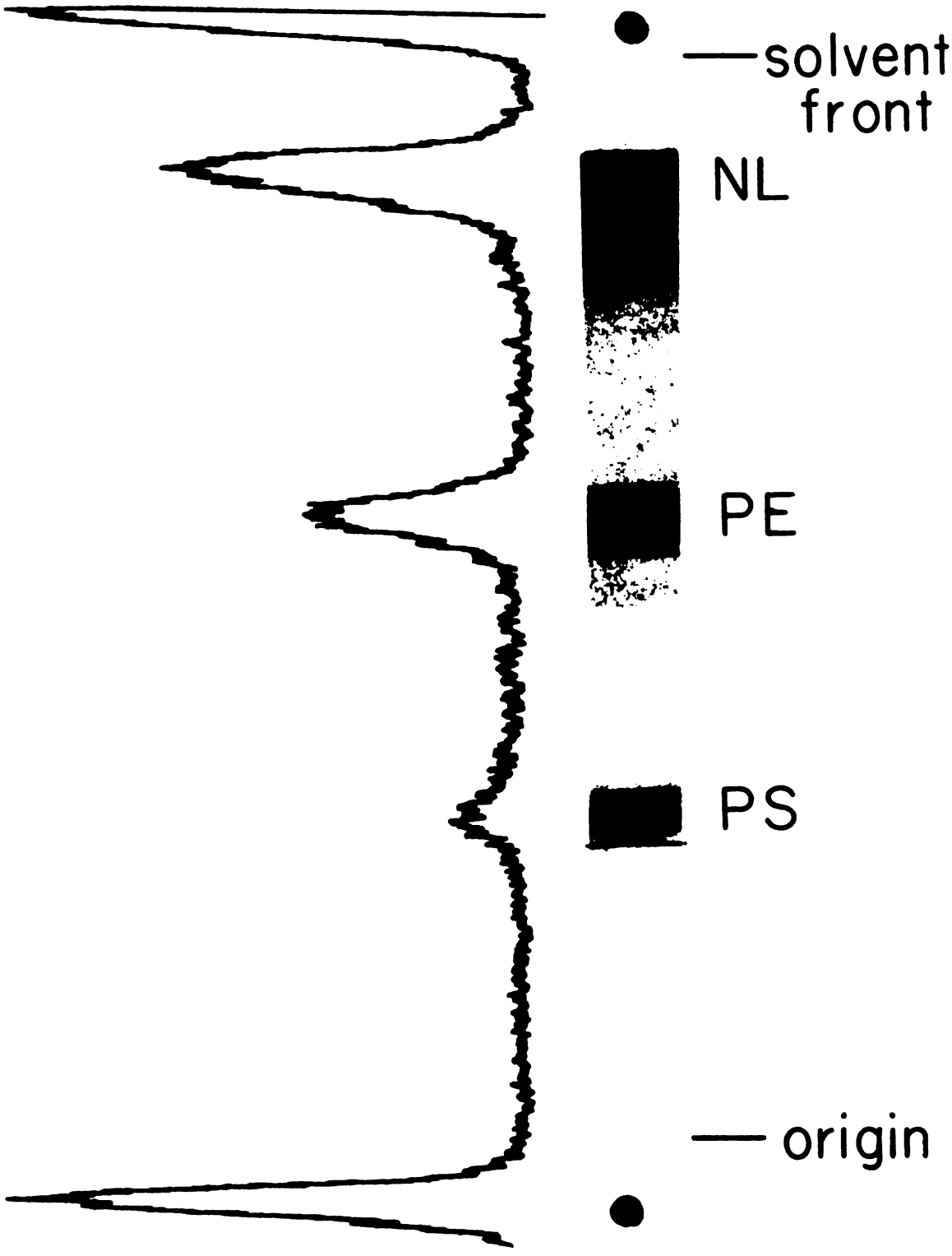
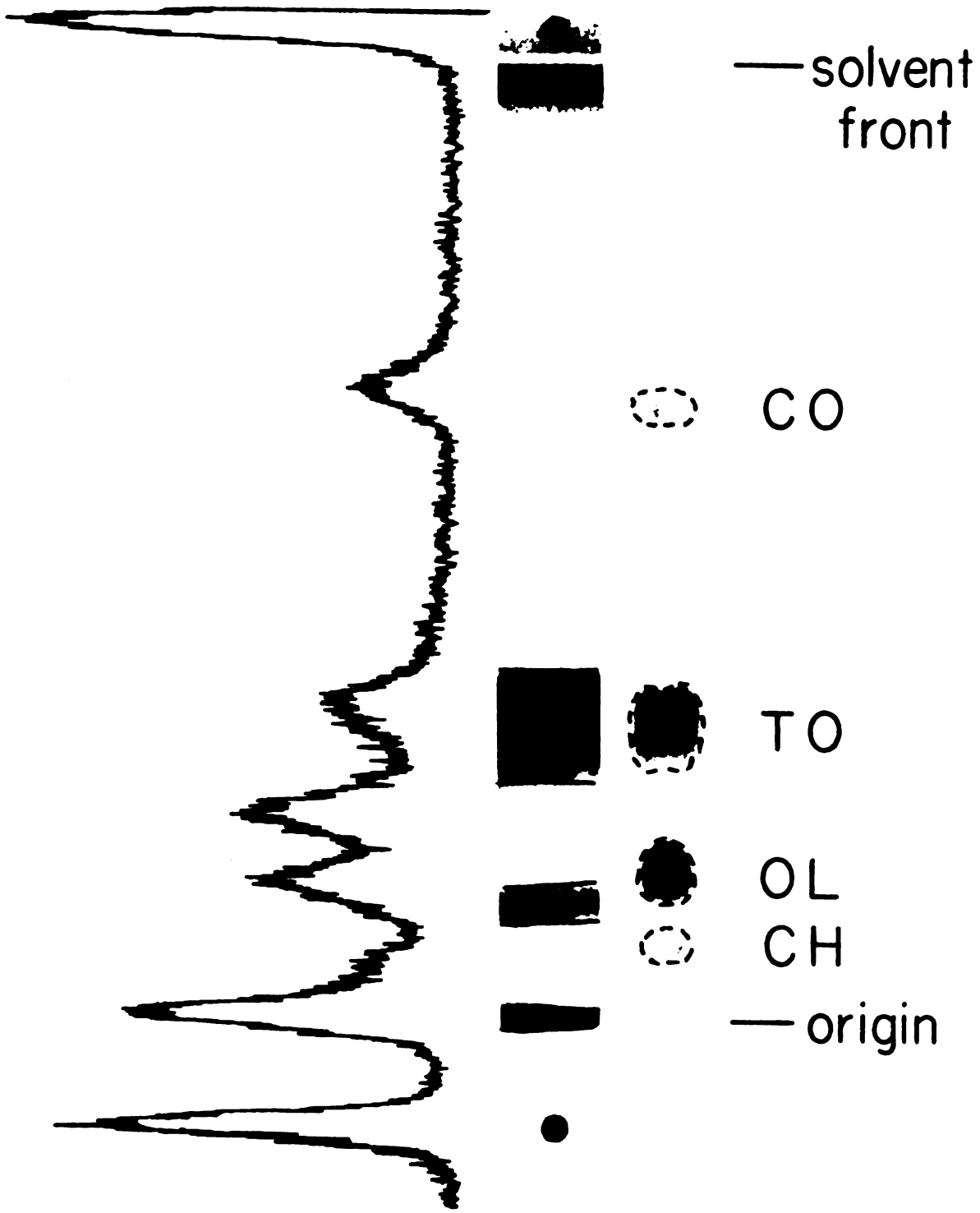

solvent front
NL
PE
PS
origin

FIGURE 18. Thin-Layer Radiochromatography of the Total Lipid Extract Obtained After Growth of Housefly Larvae on DL-[2-^{14}C]Mevalonic Acid

The total lipid extract was chromatographed on silica gel G (250 μm, Quantum) in hexane-diethyl ether-acetic acid (80:20:1) and scanned on a Berthold Model 6000 Radioscanner. A picture of the TLC plate was aligned with the scan by the marker dye spotted on each end of the plate and the standards. CH=cholesterol, OL=oleic acid, TO=triolein, CO=cholesteryl oleate.


solvent front
CO
TO
OL
CH
origin

acid, along with standards, is presented in Figure 18. Thin-layer chromatography was performed in the neutral lipid solvent system. A radioscan of this lipid after TLC in the polar lipid solvent system is not presented since greater than 90% of the radioactivity (determined by triangulation and the limit of sensitivity compared to noise levels) was found to be in a fraction that migrated near the solvent front and was thus assumed to be neutral lipid. Since no radiolabeled JH was found in either extract and the distribution of radioactivity in the neutral lipid solvent system obtained from growth on mevalonic acid seemed quite interesting in that radioactivity was detected as migrating with each class of neutral lipids, this was pursued in an additional experiment (experiment 3, Table 7) to determine where mevalonate had been incorporated into these lipids.

Product Identification from Growth on DL-[2-^{14}C]Mevalonic Acid: The level of incorporation of mevalonic acid into the lipids of housefly larvae in a second experiment (experiment 3, Table 7) was slightly higher than that obtained in the first experiment with growth on labeled mevalonic acid although the total amount of neutral lipid as determined by two independent methods was surprisingly similar. The increased incorporation into the polar lipid fraction in the second experiment is not clear. Radioscans of a portion of the total lipid extracted after TLC in both the nuetral and polar lipid solvent systems gave results that were for all intensive purposes identical to those previously obtained. At least five radioactive bands can be discerned (Figure 18) and since three of these migrated with authentic standards (free fatty acid, triglyceride, and sterol

ester) it was of interest to determine what would happen when these fractions were separately treated by mild alkali-catalyzed methanolysis and acid-catalyzed methanolysis.

The results of these procedures as well as the distribution of radioactivity on the TLC plate are shown in Table 8. Although most of the methanolyses did not give clear cut results, and some of the radioactivity was lost during these procedures for unknown reasons, it was evident that the fraction migrating with authentic triglyceride (R_f 0.32-0.62) had the same characteristics as authentic triglyceride under these conditions: the majority of it was resistent to mild alkali-catalyzed methanolysis and labile to acid-catalyzed methanolysis. This would be consistent with a hypothesis that the mevalonic acid had been incorporated into some type of fatty acid that was esterified as part of a triglyceride and therefore the fatty acid would be present in several neutral lipid fractions. Careful analysis of the triglyceride fatty acids was therefore undertaken using mild conditions so that the structure of any molecules would not be chemically altered.

Analyses were made of total lipids and triglycerides from TLC using hog pancreatic lipase as an agent for release of fatty acids from triglycerides. After incubation, the liberated fatty acids were esterified with diazomethane to make methyl esters for analysis by GLRC. Figure 19 shows two radioscans of the resultant fatty acid methyl ester fractions along with known standards. In lane A, methyl esters had been made from free fatty acids released by lipase after the acids had been separated from the rest of the extract by TLC.

TABLE 8. Distribution of Radioactivity after Thin-Layer Chromatography and Mild Alkali-Catalyzed and Acid-Catalyzed Methanolyses

Band No.	0	1	2	3	4
R_f[a]	0.00-0.12	0.12-0.18	0.18-0.32	0.32-0.62	0.62-1.00
% distribution after TLC[a,b]	32.6	22.8	19.3	16.8	8.5
Aklali-catalyzed[c]					
Upper phase (%) (water soluble)	31.6	18.2	20.4	16.4	45.8
Lower phase (%) (organic soluble)	57.8	67.2	64.0	83.0	45.8
Acid-catalyzed[c]					
Upper phase (%) (hexane soluble)	42.7	51.3	48.2	77.3	24.4
Lower phase (%) (methanol soluble)	41.3	30.5	33.3	18.7	18.4

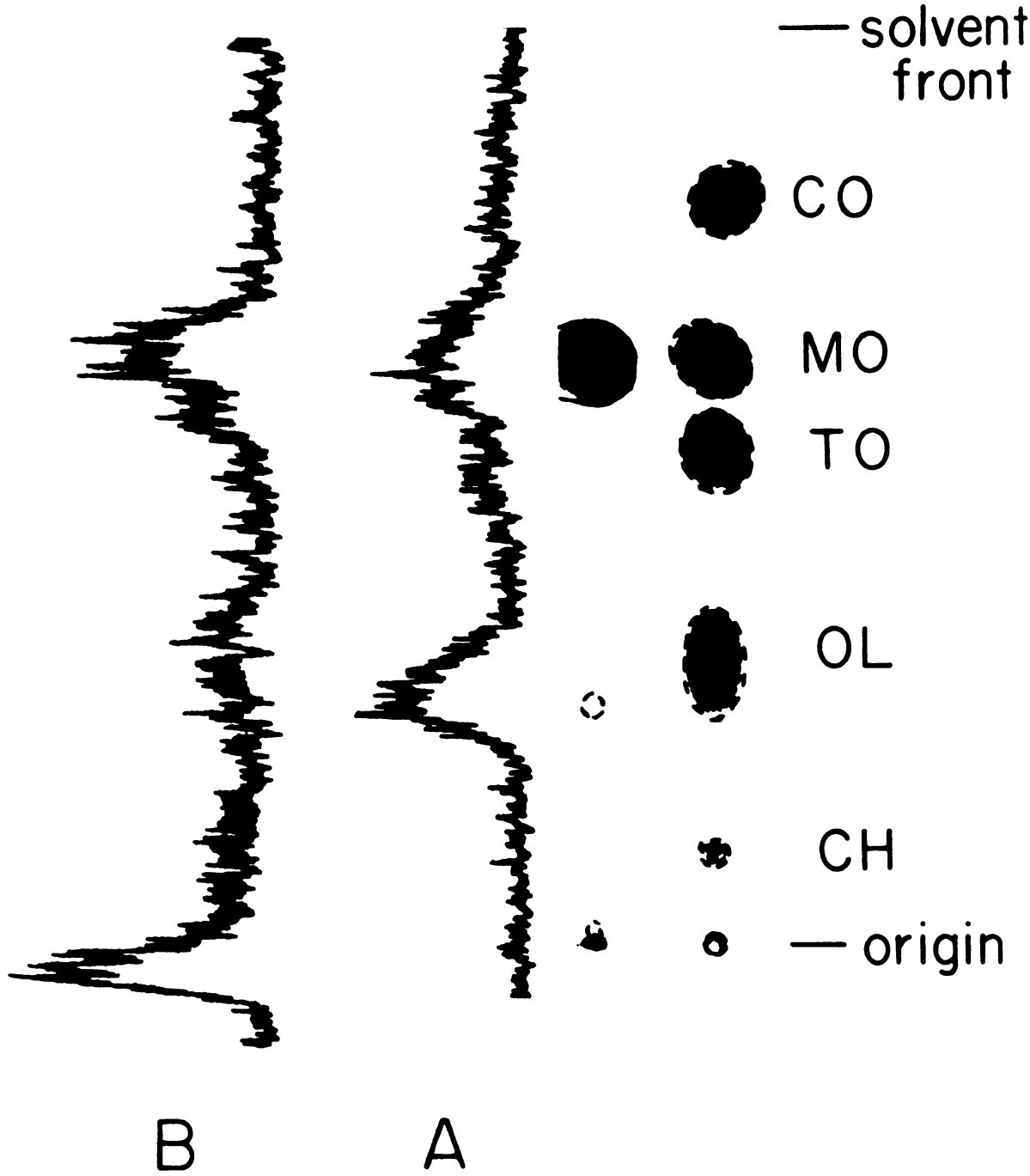
[a] Silica Gel G, 250 μm, Analtech Inc., hexane-diethyl ether-acetic acid, (80:20:1).

[b] Radioactive regions were identified as described in Methods. See also Figures 18 and 19.

[c] Carried out according to procedures outlined in Methods.

FIGURE 19. Thin-Layer Radiochromatograms of Fatty Acid Methyl Esters

Free fatty acids released by hog pancreatic lipase were esterified with diazomethane (A) after separation of the free fatty acid fraction by TLC and (B) on the total extract obtained after lipase treatment. TLC was carried out on pre-coated plates of silica gel G (250 μm, Analtech) in hexane-diethyl ether-acetic acid (80:20:1). A picture of the TLC plate was aligned with the standards. CH=cholesterol, OL=oleic acid, TO=triolein, MO=methyl oleate, CO=cholesteryl oleate.


— solvent front
CO
MO
TO
OL
CH
— origin
B
A

There are two defined regions and at least one undefined radioactive region on this plate (lane A). One corresponds to fatty acid methyl ester (MO, methyl oleate), the other to free fatty acid (OL, oleic acid), and the third possibly to triglyceride (TO, triolein). Incomplete esterification was probably not due to diazomethane resistent fatty acid but to an insufficient amount of diazomethane since lane B shows the resultant methyl esters when diazomethane treatment involved a large excess (approximately 250 mg generator used). This latter fraction (lane B) was obtained without previous TLC to separate out the free fatty acids prior to esterification. A significant amount of radioactivity was therefore still associated with the origin (presumed polar lipids) and other places on the plate. The lack of finding a radioactive area in the region of free fatty acids on the plate supports the aforementioned hypothesis since any free fatty acid in the total extract would be esterified. The additional radioactivity was probably due to either lipase resistent triglyceride or end-product inhibition during the reaction. In the original experiments, done to delineate optimal conditions for lipase treatment, the best enzymatic hydrolyses still showed about 10-20% of the starting triolein after the incubation. Radioactive mono- and diglycerides could have been made and this could also account for the occurrence of the other radioactive regions seen in lane B. When compared with a picture of the TLC plate presented in Figure 18, it can be seen that the separation of neutral lipids was far better on the TLC plate shown in Figure 19 although the identical solvent system was used. This can be explained by the fact that the chromatography presented in

Figure 18 was carried out on a pre-coated TLC plate supplied by Quantum Industries and that shown in Figure 19, a pre-coated TLC plate from Analtech, Inc. Differences in lipid separation between these two brands of pre-coated TLC plates has previously been noted by other workers in this laboratory (167) and no explanation can be given for this observation.

The fatty acid methyl ester fraction obtained from TLC was chromatographed isothermally at 160° on 3% SE-30 for analysis by GLRC (see Methods). Both mass and radioactivity tracings are presented in Figure 20. As can be seen, radioactive response was detected in a region between C_{16} and C_{18} fatty acid methyl esters (Figure 21). No attempt was made to keep the mass response on scale suring the GLRC process since a definite overload (approximately 2 mg) was injected onto the column in order to have the required number of dpm's (approximately 1000) for a definitive radioactive response. The background averaged 60 cpm and the radioactive response that becomes apparent near the end of the C_{16} region can be attributed to the zone spreading previously noted. For positive identification of these methyl esters, combined GC-MS was used with continuous repetitive scanning over a mass range of *m/e* 6-500 at intervals of 8 seconds. The total ion intensity chromatogram of this run is presented as Figure 21. The identification of the methyl esters was determined according to the criteria outlined for fatty acid esters described in a previous section of the dissertation and their gas chromatographic and mass spectral resemblence with authentic standards run under similar conditions. The shape of the peaks was due to the obligatory overload

FIGURE 20. Gas-Liquid Radiochromatography of Fatty Acid Methyl Esters

Chromatography of the fatty acid methyl esters separated by TLC (see Figure 19) was carried out on 3% SE-30 isothermally at 160°. Analysis by GLRC was carried out as described in Methods. The peaks are identified in Figure 21.

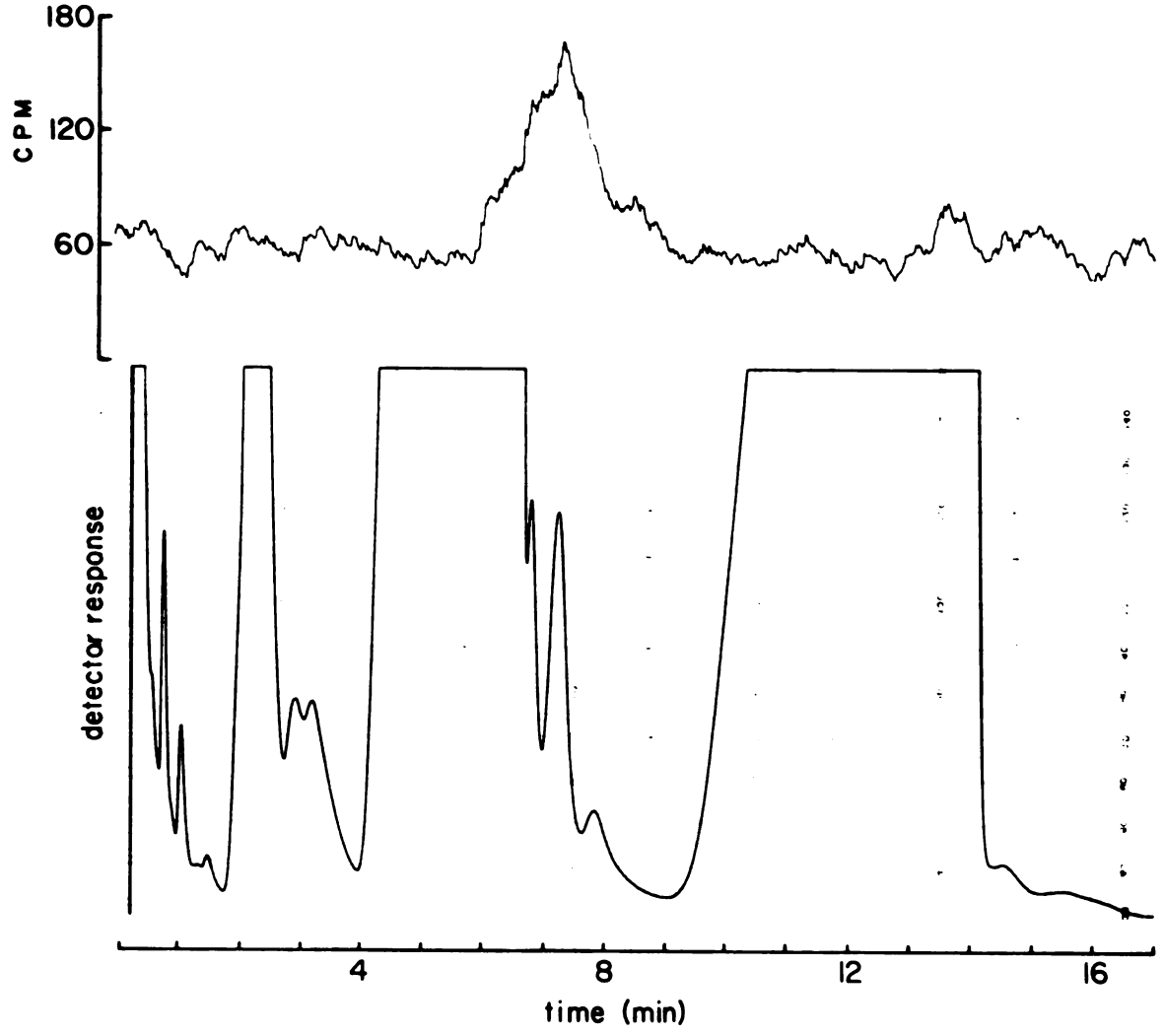
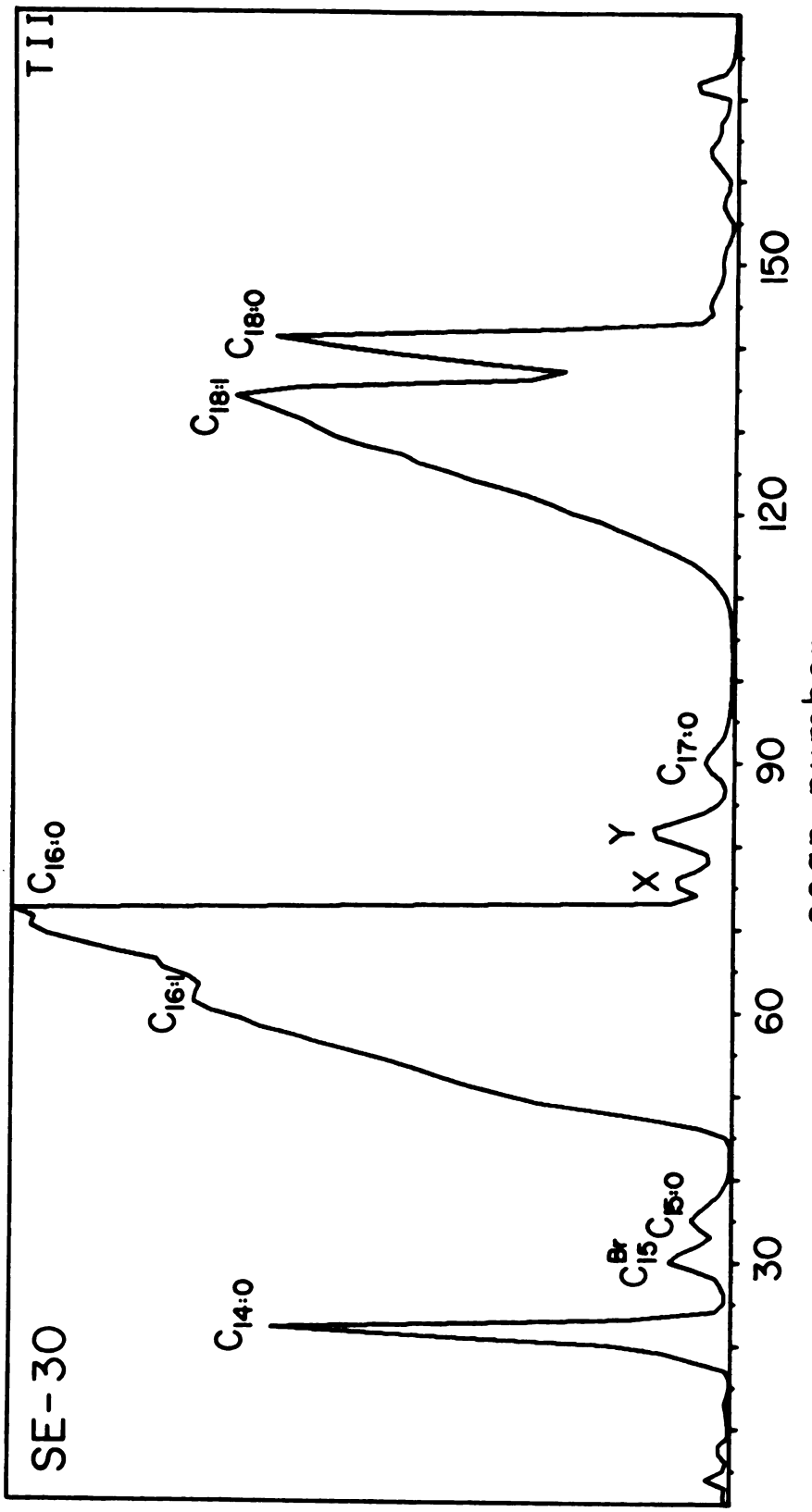

180
120
60
CPM
detector response
4
8
12
16
time (min)

FIGURE 21. Total Ion Intensity Chromatogram of Fatty Acid Methyl Esters

The fatty acid methyl esters obtained by TLC (see Figure 19) were subjected to analysis by GC-MS. Mass spectra were recorded continuously over a range of *m/e* 6-400 at intervals of 8 seconds. The subscript denotes chain length and degree of unsaturation. The GLC inlet consisted of a coiled glass column (1.4 m x 3 mm) packed with 1% SE-30. Esters were separated isothermally at 165°.


SE-30
III
$C_{14:0}$
C_{15}^{Br}
$C_{15:0}$
$C_{16:1}$
$C_{16:0}$
X
Y
$C_{17:0}$
$C_{18:1}$
$C_{18:0}$
30
60
90
120
150
scan number
relative intensity

injected necessary to obtain a mass spectrum of the radioactive unknown(s). Due to the zone spreading either component X, or more likely Y, was the radioactive substance.

The mass spectra of X and Y were difficult to interpret since relatively large amounts of methyl palmitate were still tailing on the SE-30 column and even when scans on either side of the peak(s) of interest were subtracted as background, there was not enough mass spectral information present for elucidation of structure. Preparative gas-liquid chromatography was therefore carried out; the results of this can be seen in Figure 22 (SE-30). The resultant mass spectra showed that the preparative GLC had highly enriched the sample since only small amounts of methyl palmitoleate and methyl palmitate were present. It seemed, however, that compound Y was a mixture of two components since the mass spectrum indicated two compounds with molecular weights of 284 and 282 which could represent a saturated and a mono-unsaturated C_{17} methyl ester, respectively. Accordingly, GLC was performed on a polar column (EGSS-X) and an additional peak was separated (Z, Figure 22, EGSS-X). The first major peak, X, was found to be the only radioactive component, evidenced by trapping of the GLC effluent and subsequent scintillation counting of the trapped effluent (there was not enough radioactivity at this point to use GLRC analyses).

Mass spectra of peaks X, Y, and Z are presented in Figure 23. Compounds X and Y have molecular weights of 284, consistent with that of saturated C_{17} methyl esters. However, these spectra are not in excellent agreement with that of authentic methyl heptadecanoate.

FIGURE 22. Total Ion Intensity Chromatograms of Fatty Acid Methyl Esters after Preparative Gas-Liquid Chromatography

Mass spectra were recorded as decribed in the legend to Figure 21. Esters were separated isothermally at 165° on 1% SE-30 and at 145° on 3% EGSS-X. The subscript denotes chain length and degree of unsaturation. Unknowns are labeled X, Y, and Z.

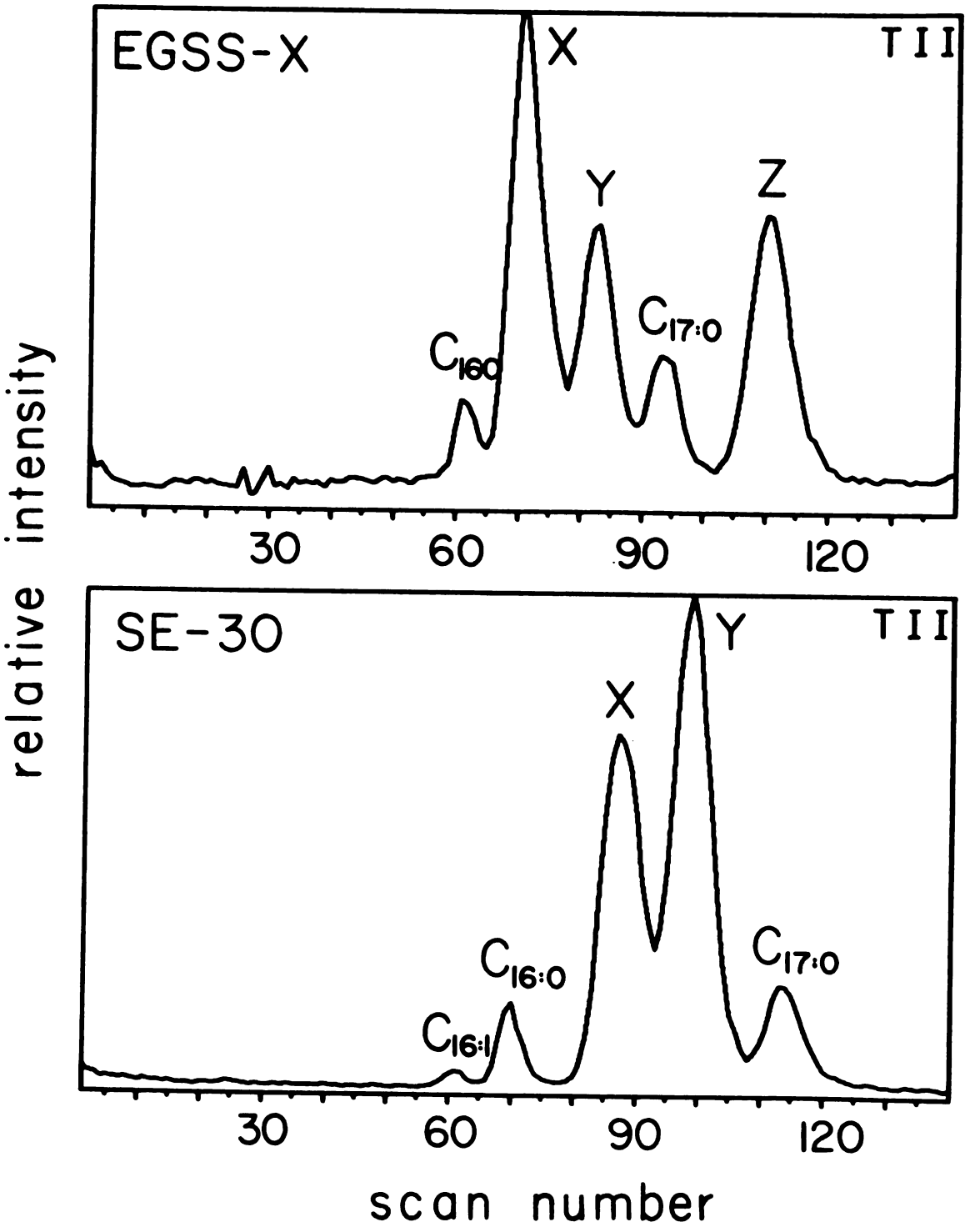
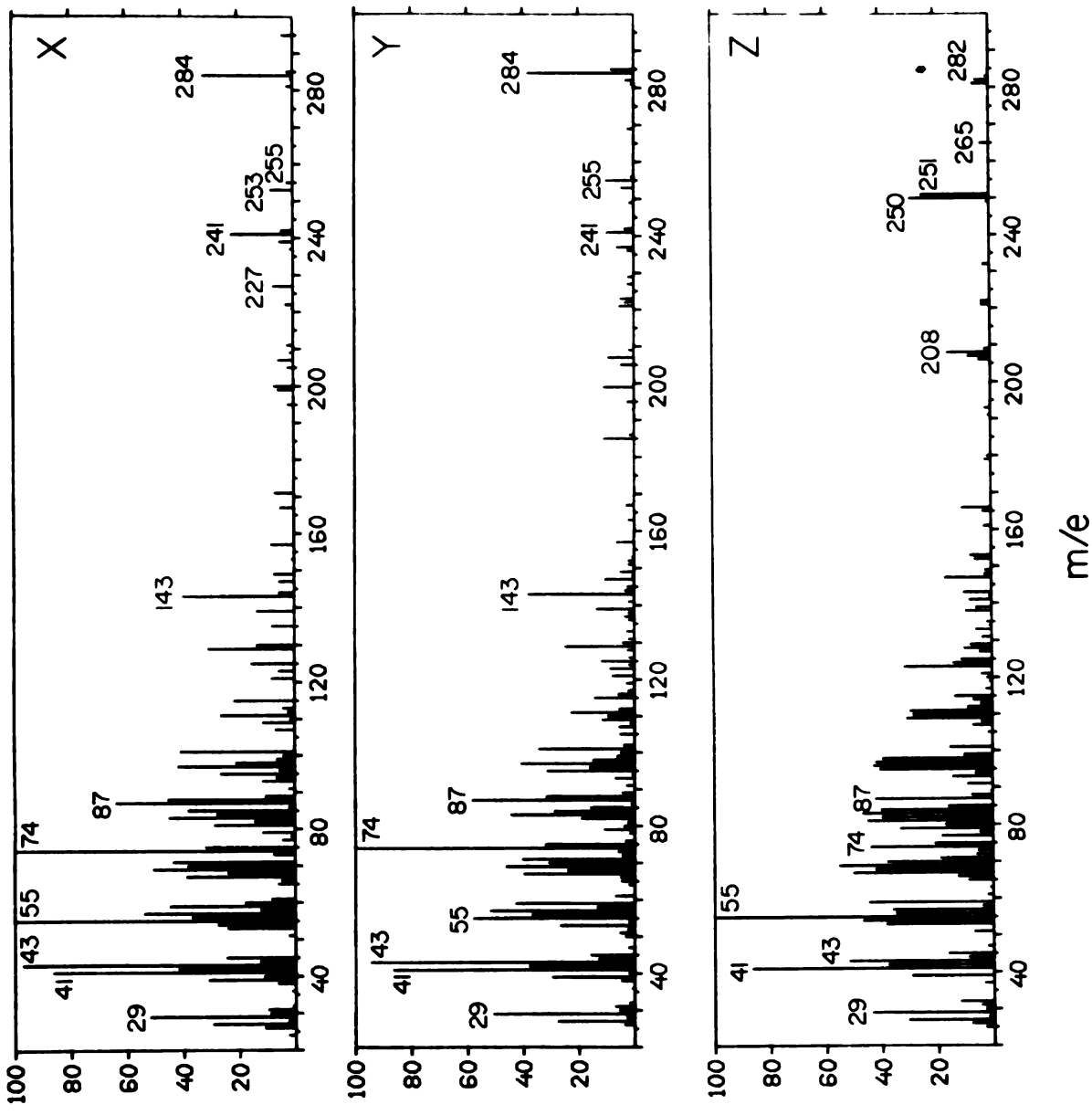

EGSS-X
X
Y
Z
TII
C160
C17:0
30
60
90
120
SE-30
Y
X
TII
C16:0
C16:1
C17:0
30
60
90
120
relative intensity
scan number

FIGURE 23. Mass Spectra of Compounds X, Y, and Z

Spectra were recorded at an ionizing potential of 70 eV. Ions of interest are noted and discussed in the text.


X
Y
Z
relative intensity (%)
m/e
100
80
60
40
20
29
41
43
55
74
87
143
208
227
241
250
251
253
255
265
282
284
40
80
120
160
200
240
280

The compounds were identified and the spectra will be discussed in greater detail in the following paragraphs.

The spectrum of compound Z is consistent with that of a monounsaturated C_{17} fatty acid methyl ester since ions at *m/e*'s 250 and 251 representing $[M-31]^+$ and $[M-32]^+$ for loss of methoxy and methanol radicals, respectively, are greatly enhanced in monounsaturated fatty acid methyl esters (160). The ion at *m/e* 208, representing $[M-74]^+$, is also characteristically seen in these spectra. The ion at *m/e* 281 in this spectrum and also in the spectra of X and Y was presumed to be from incomplete background subtraction by the computer system since this ion is a well characterized one, arising from gas chromatographic "bleed" and having the molecular composition of $C_7H_{21}O_4Si_4$ (150); it is probably not an $[M-1]^+$ or an $[M-3]^+$ ion from the parent compounds at *m/e*'s 282 or 284 in any of these spectra. The ions at *m/e* 74 and *m/e* 87 in spectrum Z indicate a methyl ester function unsubstituted on the α or β carbon atoms; they are depressed due to the unsaturation in the molecule. Other possible structures consistent with a molecular weight of 282 could be methyl esters of a cyclopropane fatty acid or a C_{16} hydroxy fatty acid. According to the rules of Campbell and Naworal (168), the M/M-32 ratio for a cyclopropane fatty acid methyl ester should be around 5-11/100 and that for a monounsaturated fatty acid methyl ester, 15-22/100. Spectrum Z gave a M/M-32 ratio of 18/100 which was consistent with that of an unsaturated fatty acid methyl ester. Although there is an ion at *m/e* 265 which could represent $[M-17]^+$ for loss of hydroxyl radical thus indicating a hydroxy fatty acid, further experiments involving

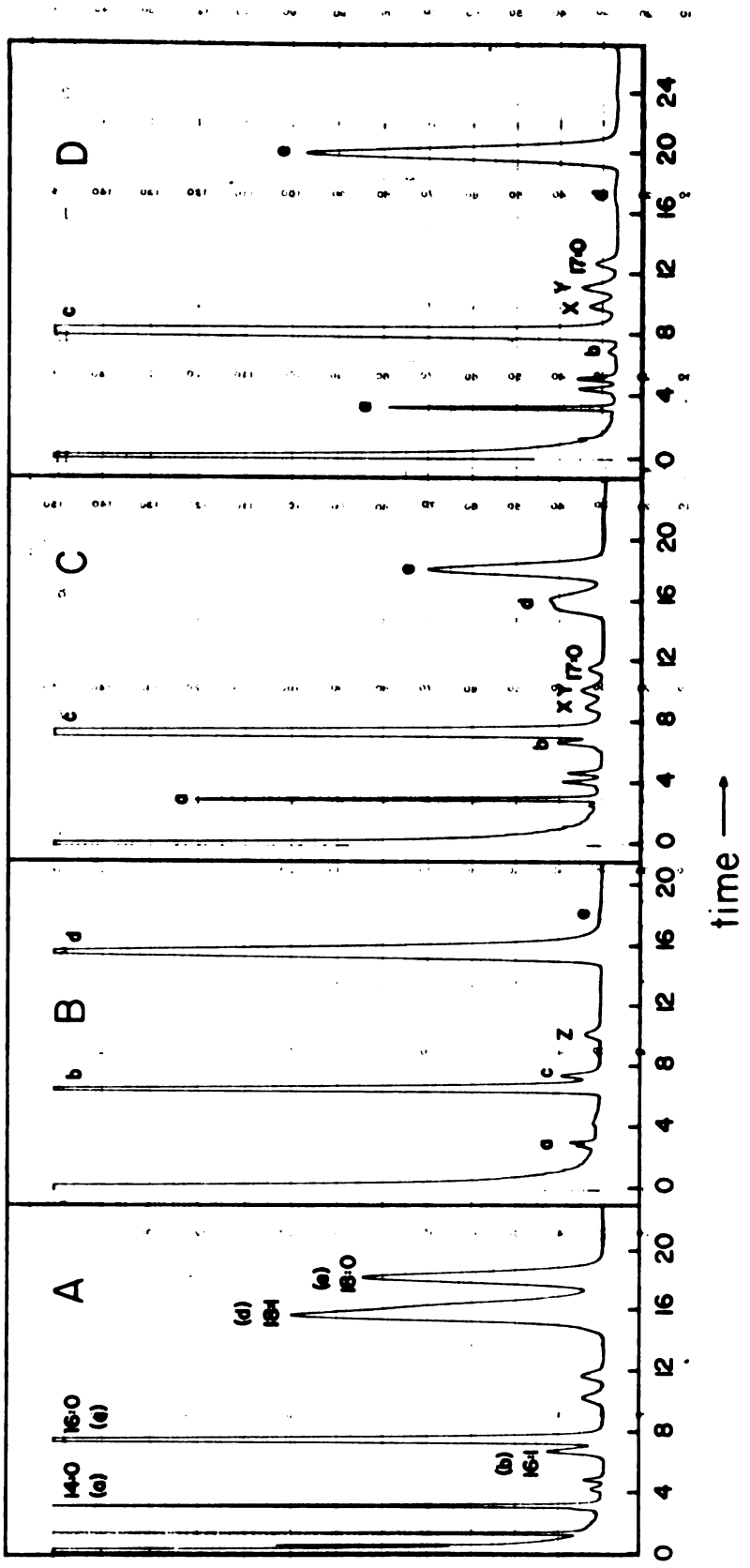
osmium tetroxide oxidation and catalytic hydrogenation ruled out this possibility.

Gas chromatographic analysis of the catalytic hydrogenation product of the sample obtained from preparative GLC showed only two peaks. Mass spectral analysis of these peaks indicated the presence of a small amount of methyl palmitate and an unknown compound which gave a very confusing mass spectrum. The latter spectrum was uninterpretable and it therefore seemed necessary to separate the fatty acids according to their degree of unsaturation prior to catalytic hydrogenation to identify compound Z, since hydrogenation of the total sample obtained from preparative GLC gave compounds that seemed to be chromatographically similar.

Argentation TLC was used to separate the fatty acid methyl esters and Figure 24 shows the resultant GLC tracing of an aliquot of each sample obtained after the bands had been scraped and eluted from the gel. Figure 24A is a tracing of a standard mixture of fatty acid methyl esters prepared from acid-catalyzed methanolysis of the fatty acids extracted from Ivory Soap; the peaks are identified by carbon number and degree of unsaturation and have been given letter designations for clarity in peak identification in the other gas chromatograms in Figures 24 and 27. Figure 24B, which is a gas chromatogram of the monoene fraction, showed that small amounts of methyl palmitate and methyl stearate (c and e) still remained but the majority of the sample consisted of methyl palmitoleate and methyl oleate (b and d). The reverse was true for the saturated fraction as evidenced by the gas chromatogram shown in Figure 24C. Successful separation of the fatty acid methyl esters according to their degree of unsaturation was therefore acheived.

FIGURE 24. Gas Chromatograms of Fatty Acid Methyl Esters after Argentation Thin-Layer Chromatography and Catalytic Hydrogenation

Gas-liquid chromatography was carried out isothermally at 160° on 3% SE-30. A is a chromatogram of the standard methyl esters of Ivory Soap (see Methods). These have been identified by carbon number and degree of unsaturation and given letter designations for clarity in presentation of the other chromatograms. B and C are chromatograms of the monoene and saturated fractions obtained after argentation TLC, respectively and D is the chromatogram of the catalytic hydrogenation product of the saturated fraction.


A
B
C
D
14:0 (a)
16:0 (a)
(b) 16:1
(c) 18:1
(d) 18:0
a
b
c
d
Z
X Y 17:0
time
detector response

It was also obvious that components Y and Z had been separated by this technique, as was predicted from the resutls obtained on the polar and non-polar GLC columns used. The mass spectra of compounds X, Y, and Z obtained from argentation TLC were in good agreement with those shown in Figure 23.

Catalytic hydrogenation of the monoene fraction obtained from the argentation TLC step should have then yielded insight into the identity of compound Z. Due to haste a mistake was made and the saturated fraction was subjected to catalytic hydrogenation instead of the monoene fraction. This, however, gave greater insight into the identity of compounds X and Y. A gas chromatogram of the catalytic hydrogenation product of the saturated fraction is shown in Figure 24D. There was almost no unsaturated fatty acid methyl ester present. Mass spectra of this sample were taken continuously over a range of *m/e* 6-400 at intervals of 8 seconds and the results enabled tentative identification of compounds X and Y. The total ion intensity chromatogram of this run is shown in Figure 25 and the shape of the methyl palmitate peak is distorted due to one bad data point on the computer system for some unknown reason.

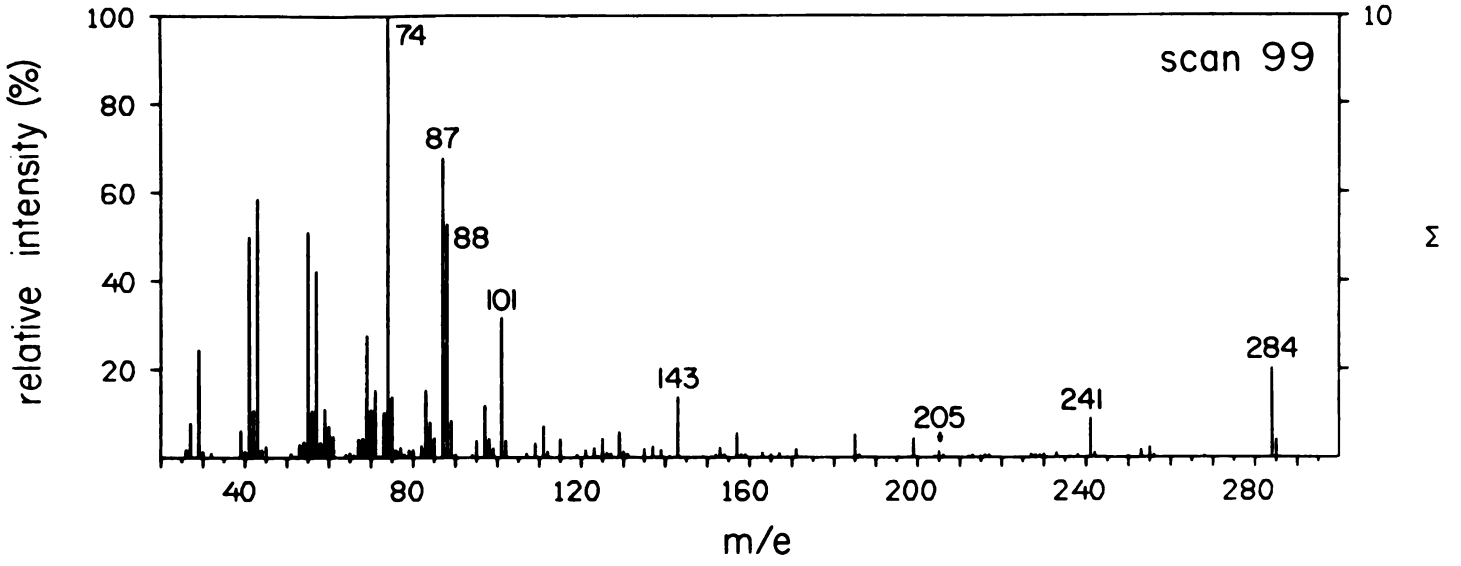
The spectrum of X agreed well with that shown in Figure 23 and was identified as a C_{17} saturated iso fatty acid methyl ester. The ratio of M-29/M-31/M-43 agreed well with that found by Campbell and Naworal (168) for iso fatty fatty acid methyl esters and the ion at *m/e* 219 ($[M-65]^+$) which is indicative of iso fatty acid methyl esters (169) was also present. Although this ion was not present in the spectrum of X in Figure 23, it was present in each of the spectra taken over

scans 85-90 in the total ion intensity chromatogram shown in Figure 25. The ions at *m/e* 227 ($[M-57]^+$) and at *m/e* 143 (cleavage at C-7) are consistent with this identification.

Compound Y gave a mass spectrum (Figure 25, scan 99) that appeared to be a mixture of two components: methyl esters of a saturated C_{17} anteiso fatty acid and an α-methyl fatty acid or a mixture of α- and β-methyl fatty acids. The ratio of M-29/M-31/M-43 calculated from this spectrum was consistent with that of an anteiso fatty acid methyl ester according to the rules of Campbell and Naworal (168). The ion at *m/e* 255 is larger than that at *m/e* 253 and both are significantly smaller than the ion at *m/e* 241. The ion at *m/e* 205 ($[M-79]^+$) is diagnostic in the mass spectra of anteiso fatty acid methyl esters (169). The ions at *m/e* 88 and at *m/e* 101, however, are much larger than expected and it therefore implicated the presence of an additional compound in this mass spectrum. All the ions in a mass spectrum should be made in equal amounts at the same time if a single compound is present and being eluted into the analyzer from the GLC column. When the spectra taken over scans 95-100 were analyzed, it became obvious that compound Y included an α-methyl or mixture of α- and β-methyl methyl esters since the ratio of *m/e* 88 to *m/e* 87 (Figure 26) was constantly changing during the elution of the peak from the GLC column. The ions at *m/e* 88 and at *m/e* 101 arise from the McLafferty rearrangement and β-γ cleavage, respectively, from an α-methyl methyl ester. The typical spectrum of a β-methyl methyl ester gives a base peak at *m/e* 74 for the McLafferty ion an a large ion at *m/e* 101 for the β-γ cleavage (169). The ions at *m/e*'s 88 and 101 were seen in the

FIGURE 25. Total Ion Intensity Chromatogram of Fatty Acid Methyl Esters after Catalytic Hydrogenation of the Saturated Fraction and Normalized Bar Graph of Scan 99

Mass spectra were recorded as described in the legend to Figure 21. Fatty acid methyl esters are identified by chain length and degree of unsaturation. The ions of interest in scan 99 are noted and discussed in the text.


relative intensity (%)
100
80
60
40
20
74
87
88
101
143
205
241
284
scan 99
10
Σ
40
80
120
160
200
240
280
m/e

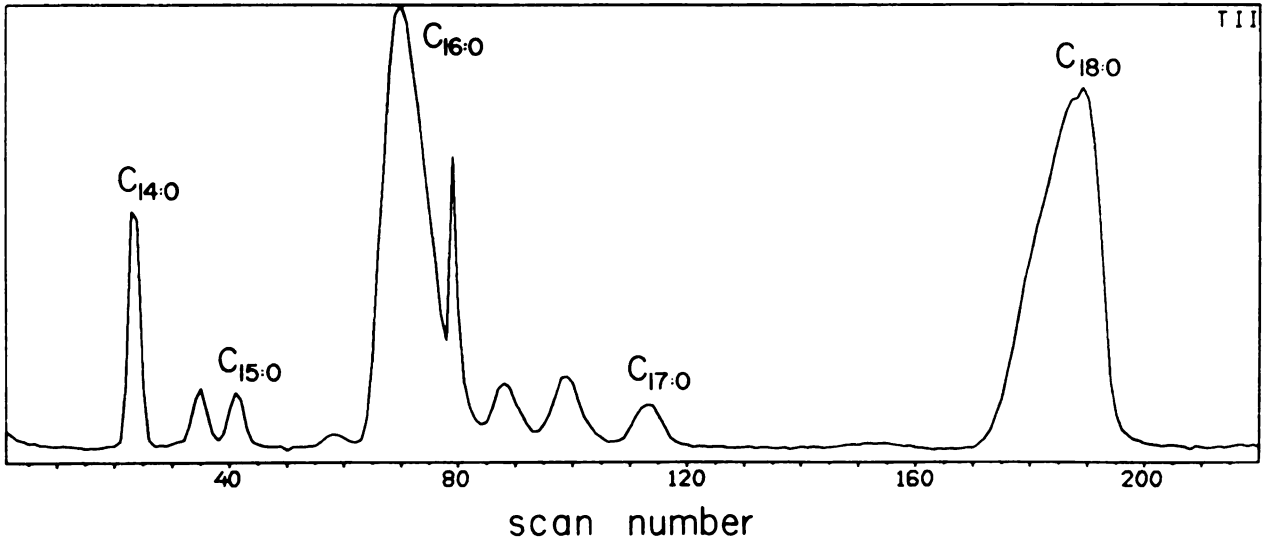
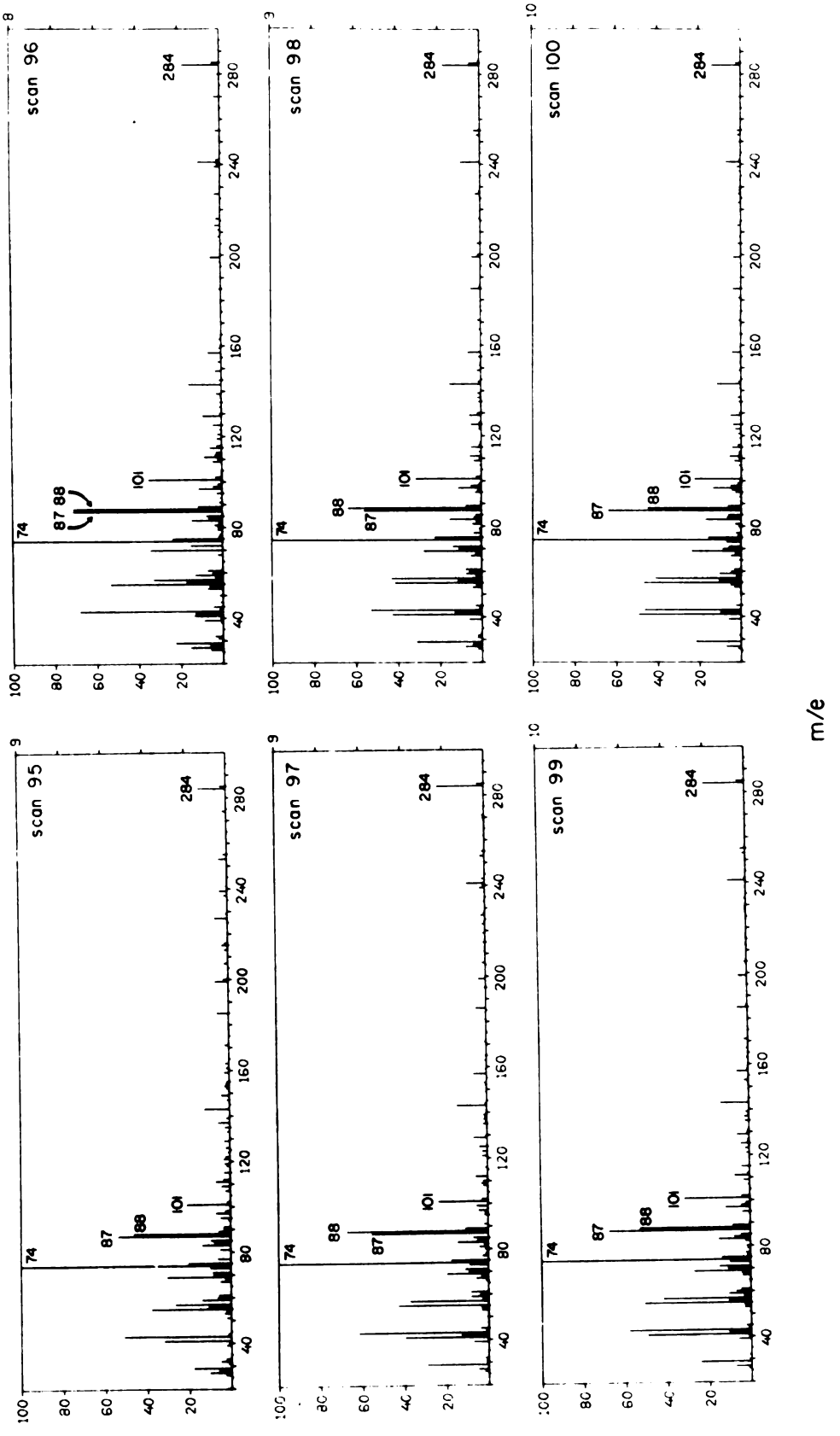

C14:0
C15:0
C16:0
C17:0
C18:0
TII
40
80
120
160
200
scan number

FIGURE 26. Mass Spectra of Scans 95-100

The ions of interest are noted and discussed in the text.


scan 95
scan 96
scan 97
scan 98
scan 99
scan 100
relative intensity (%)
m/e

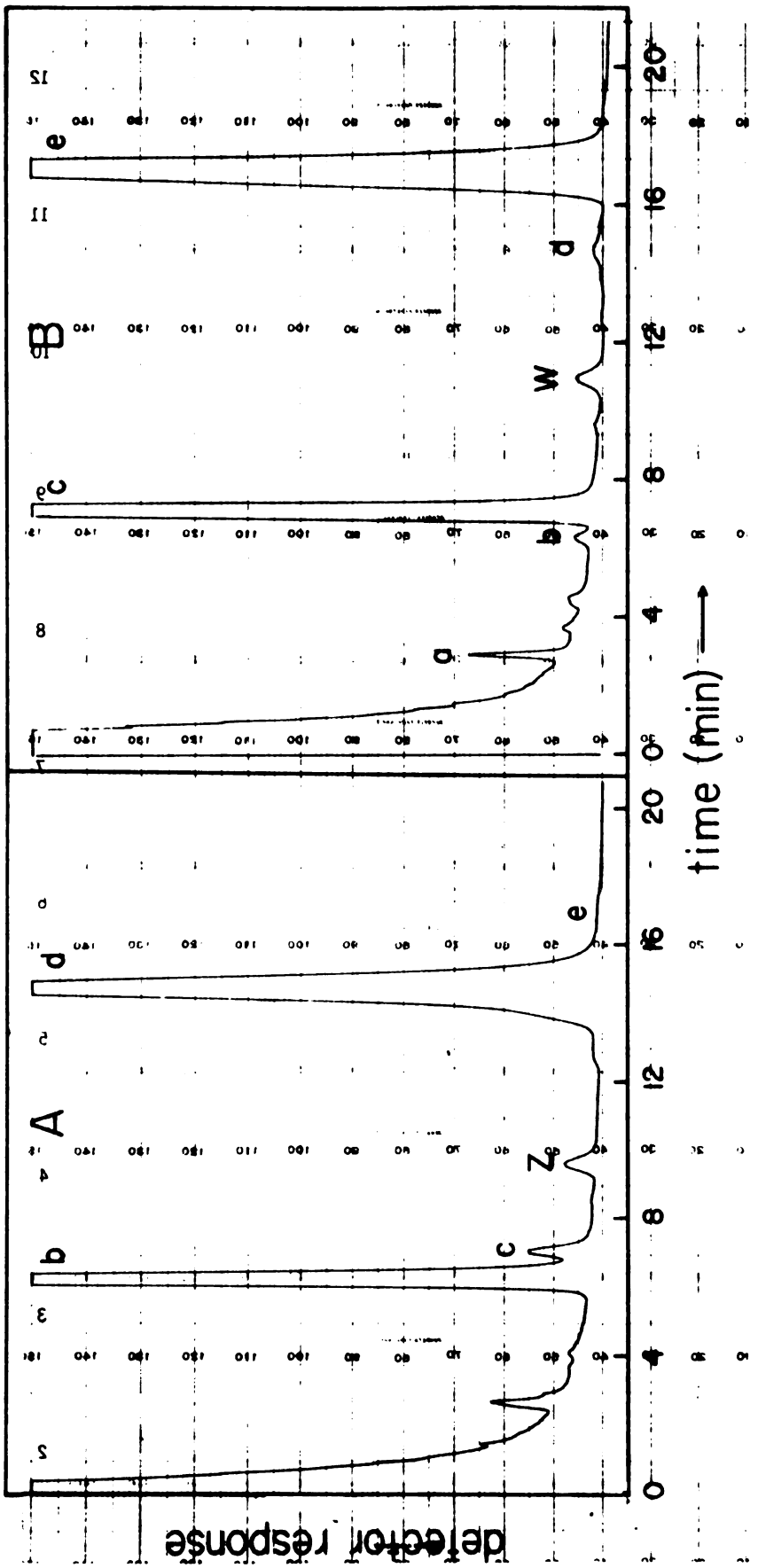
spectra of fatty acid ethyl esters (see Figure 11) however there was no significant ion at $[M-45]^+$ or $[M-46]^+$ for loss of ethoxy or ethanol radicals. The possibility that compound Y contained an ethyl ester was therefore ruled out. The relative proportions of α-methyl and β-methyl methyl ester is less than that of the anteiso fatty acid methyl ester since the ions at *m/e* 88 and at *m/e* 101 showed smaller relative intensities than those at *m/e* 74 and at *m/e* 87. It was also evident that the mixture of α- and β-methyl methyl esters eluted slightly before the anteiso methyl ester from the GLC column (1% SE-30).

The spectrum of compound Y (Figure 23) did not indicate the presence of either an α- or β-methyl methyl ester. These products probably resulted, therefore, from catalytic hydrogenation, which is reasonable if it can be assumed that compound Y was a mixture of a saturated anteiso C_{17} methyl ester and an α-β cyclopropanoid C_{16} fatty acid methyl ester which was opened up upon hydrogenation. In the spectrum of Y (Figure 23) a small ion at *m/e* 282 was present which would account for the molecular ion of a C_{16} cyclopropanoid methyl ester. The X and Y components were therefore tentatively identified as methyl 15-methyl hexadecanoate and a mixture of methyl 14-methyl hexadecanoate and methyl 2,3-methylene-hexadecanoate, respectively.

Although much information was gained from catalytic hydrogenation of the saturated fraction, this did not help solve the identity of the monounsaturate, Z. Additional monoene fraction was obtained from argentation TLC and a gas chromatogram of the resultant fractions gave results that were for all intensive purposes identical to those obtained previously. Figure 27A, a gas chromatogram of an

FIGURE 27. Gas Chromatograms of Fatty Acid Methyl Esters after Argentation Thin-Layer Chromatography and Catalytic Hydrogenation

Gas-liquid chromatography was carried out as described in Figure 24. A and B are the monoene fraction and the catalytic hydrogenation product of this fraction, respectively. See Figure 24 for explanation of the letter designation of the methyl esters.

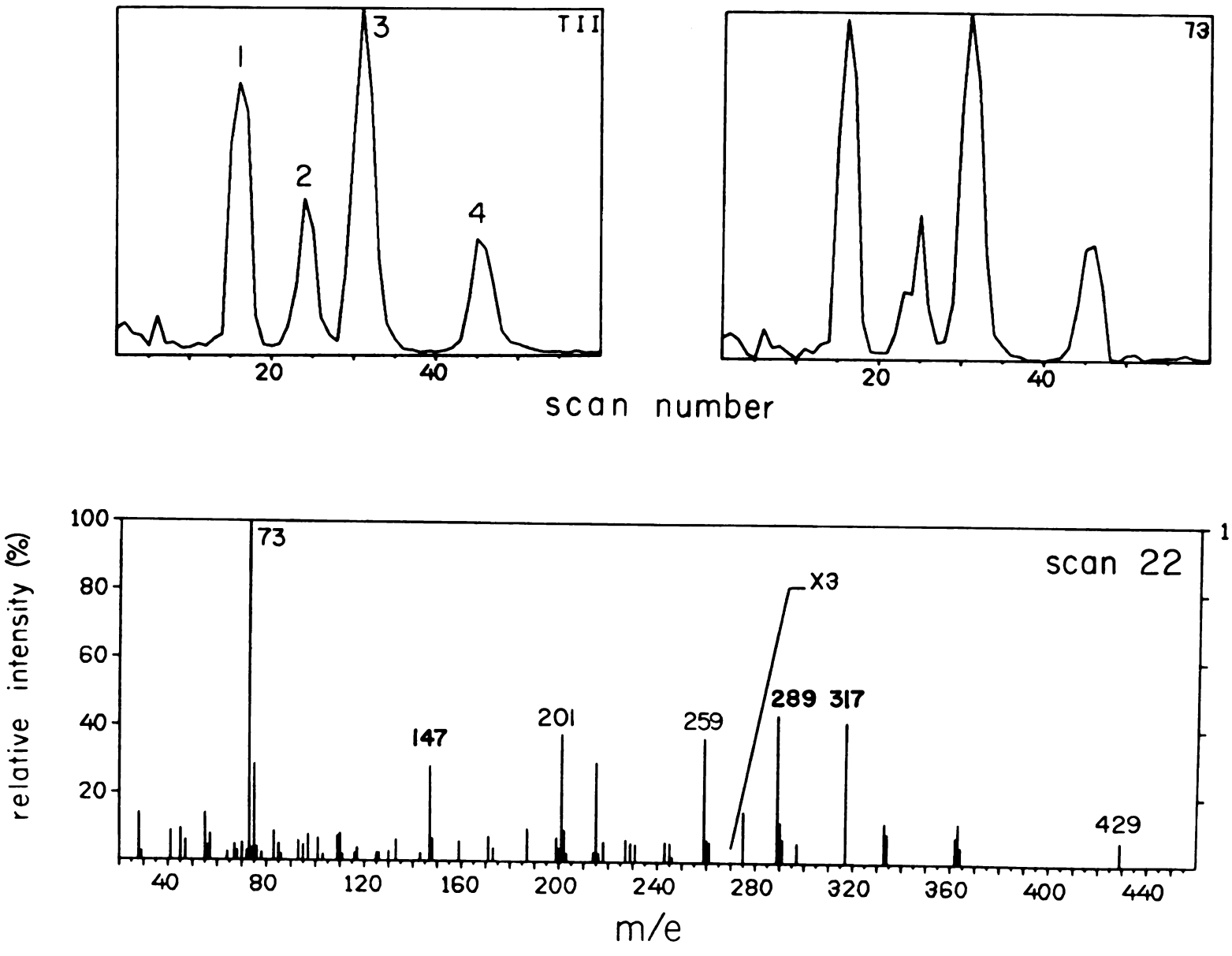

detector response
time (min)
A
B
Z
W
a
b
c
d
e

aliquot of the monoene fraction is similar to that shown in Figure 24B. A GLC tracing of an aliquot of the catalytic hydrogenation product of this fraction is shown in Figure 27B. This fraction was subjected to mass spectral analysis and spectra were recorded continuously over a mass range of *m/e* 6-400 at intervals of 6 seconds. Due to minor complications with the mass spectrometer-computer system, a total ion intensity chromatogram of the run was not obtained. The spectrum of the hydrogenated product of compound Z, labeled W, was for all intensive purposes identical to that of methyl heptadecanoate. It gave a molecular ion at *m/e* 284 and the spectrum was entirely consistent with that expected for a long-chain fatty acid methyl ester (162).

To determine the location of the double bond in compound Z, osmium tetroxide oxidation was carried out with subsequent GC-MS of the trimethylsilyl derivatives of the polyhydroxy fatty acid methyl esters obtained. One half (approximately 1 mg) of the total monoene fraction was treated thusly and, after recovery of the product and derivatization, GC-MS was carried out by continuous repetitive scanning over a mass range of *m/e* 6-700 at intervals of 10 seconds. The total ion intensity chromatogram as well as a mass chromatogram of *m/e* 73 is presented in Figure 28. The mass chromatogram of *m/e* 73 indicated those compounds in the mixture that had incorporated a trimethylsilyl group ($Si(CH_3)_3$). From this mass chromatogram it was concluded that the peak seen in scans 20-28 was a mixture of two compounds. Upon analysis of the mass spectra obtained at the apex of each of the major peaks, it was concluded that the peaks labeled 1, 2, 3, and 4 were

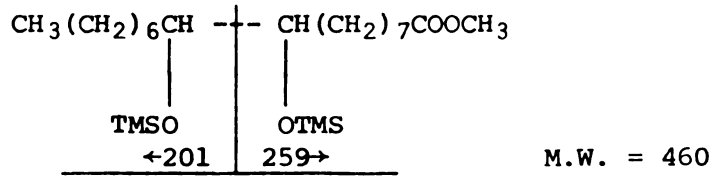
FIGURE 28. Total Ion Intensity and Mass Chromatogram of *m/e* 73 after Osmium Tetroxide Oxidation; Mass spectrum of Scan 22

Mass spectra were recorded continuously over a range of *m/e* 6-700 at intervals of 10 seconds at an ionizing potential of 70 eV. The GLC inlet consisted of a coiled glass column (1.4 m x 3 mm) packed with 1% SE-30. The *O*-TMS ethers were separated isothermally at 220°. Peaks are numbered and identified in the text. Ions of interest in scan 22 are noted and discussed in the text.


TII
1
2
3
4
20
40
73
20
40
scan number
relative intensity (%)
100
80
60
40
20
73
147
201
259
289 317
X3
scan 22
429
17
Σ
40
80
120
160
200
240
280
320
360
400
440
m/e

Δ^9 $C_{16:1}$, $\Delta^{4,7}$ $C_{14:2}$, Δ^9 $C_{18:1}$, and $\Delta^{4,7}$ $C_{16:2}$, respectively. This indicated the presence of methyl palmitoleate and methyl oleate, as expected from GLC retention times and previous mass spectral analysis, and two unusual dienoic fatty acid methyl esters not previously identified.

The mass spectrum of scan number 22 (Figure 28) was the only one consistent with that of a C_{17} monounsaturated fatty acid methyl ester. The ions at *m/e* 201 and at *m/e* 259 represent cleavage at the vicinal *O*-TMS ether groups and are indicative of a molecule with a molecular weight of 460. This locates the double bond between C-9 and C-10. The ion at *m/e* 429 ($[M-31]^+$) is consistent with this molecular weight however those at *m/e*'s 363, 317, 289, and 215 were due to the dienoic C_{14} compound eluting from the GLC column directly after the C_{17} monoene. The structure of the *O*-TMS component and its main fragment ions are shown below:



This established compound Z as being consistent with the structure methyl 9,10-heptadecenoate.

Although the mass spectrometric analyses yielded tentative identification of compounds X, Y, and Z, additional gas-liquid chromatography was undertaken to add an independent confirmation of the results obtained by means of mass spectrometry. Gas-liquid chromatography of known authentic standards of various iso, anteiso,

saturated, and monounsaturated (ω-9) fatty acid methyl esters from C_{14} to C_{21} was accomplished using 15% EGA; chromatographic conditions were adjusted so that authentic methyl heptadecanoate would have a retention time of approximately ten nimutes. The actual retention time of the methyl heptadecanoate was 9.1 minutes under the conditions chosen (170°, isothermal; column, 6' x 3/8"; carrier gas, N_2 at 40 ml/min). An EGA column was chosen for these analyses for its highly polar characteristics and similarity to EGSS-X since a column packed with EGSS-X was not available at the time.

Semi-logarithmic plots were made of retention time versus chain length according to the method of Woodford and van Gent (199). Since linear relationships exist for homologous families of fatty acid methyl esters, the plots yield a series of parallel lines specific for those fatty acid methyl esters under the same operating conditions. The data is presented in Table 9 as relative retention times using authentic methyl heptadecanoate as the standard of 1.00. Since authentic standards were not available for methyl 15-methyl hexadecanoate and methyl heptadecenoate, their relative retention times were predicted from the semi-logarithmic plots obtained. As can be seen from the table the relative retention times of compounds X, Y, and Z are in good agreement with those either predicted or found from the standards. These relative retention times are in very good agreement with those obtained by Ackman (200) using an open tubular column with BDS as a liquid phase. Both liquid phases (BDS and EGA) are very similar in their GLC behaviors. Independent confirmation of the identity of compounds X, Y, and Z as being those predicted from

TABLE 9. Comparison of Gas Chromatographic Relative Retention Times

Compound	Retention Time Relative to Methyl Heptadecanoate[a]	
	Predicted[b] or Actual	Observed
methyl 15-methyl heptadecanoate	0.84	
methyl 14-methyl heptadecanoate	0.90	
methyl heptadecanoate	1.00	
methyl heptadecenoate	1.14	
X		0.85
Y		0.90
Z		1.12

[a] 15% EGA, 170°, retention time of methyl heptadecanoate=9.1 minutes.

[b] Predicted from semi-logarithmic plots of retention time versus chain length of authentic standards as described by Woodford and van Gent (199).

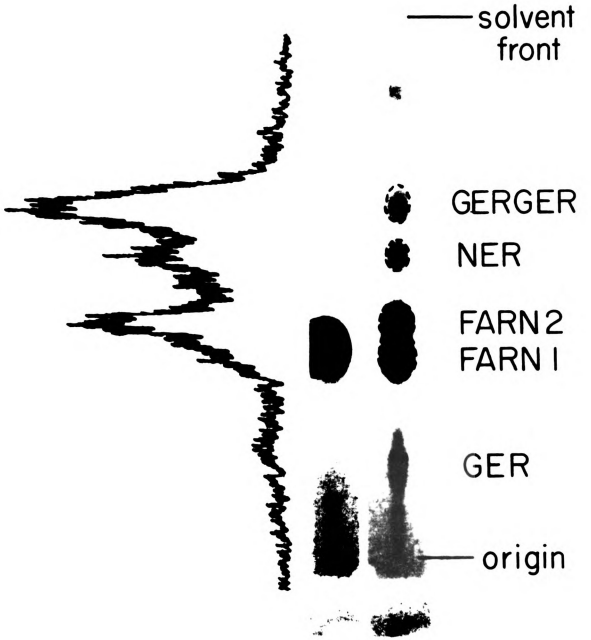
mass spectrometric analysis was therefore accomplished. Authentic standards of 2,3-methylene or α- or β-methyl methyl esters were not obtained and therefore the tentative identification of these compounds as part of Y was not independently confirmed.

An estimation of the specific activity of the iso fatty acid (X) was calculated to be 7.1 nCi/nmole or 7.1 mCi/mmole. Known amounts of methyl palmitate were injected onto the gas chromatographic column and the area responses obtained were compared with those obtained when known amounts (2 µl) of the saturated fraction were injected to get an estimation of the weight of compound X present. Duplicate samples (10 µl each) were taken for liquid scintillation counting. Since the specific activity of the substrate, DL-[2-^{14}C]mevalonic acid was 7.2 mCi/mmole, there was very good agreement with a hypothesis that one mole of mevalonic acid had been incorporated into the 15-methyl hexadecanoic acid.

Prior to the elucidation of the structure of this fatty acid by all of the above procedures, part (one-tenth) of the original extract was subjected to total reduction using lithium aluminum hydride for analysis of the free alcohols by TLC and GLRC. Thin-layer radiochromatography of the free alcohols was expected to have yielded one radioactive band representing the fatty alcohols made from the fatty acids. However, as seen in Figure 29, at least three and probably five radioactive bands were realized in the solvent system used (benzene-ethyl acetate, 4:1). This solvent system is the one of choice for separation of isoprenoid alcohols (see Table 4) and the radioactive bands co-chromatographed with the standards shown having R_f values of 0.43, 0.55, and 0.65. Mass, as determined by iodine

FIGURE 29. Thin-Layer Radiochromatogram of the Product after Lithium Aluminum Hydride Reduction

Alcohols were chromatographed on silica gel G (250 μm, Analtech) in benzene-ethyl acetate (4:1). GER=geraniol, FARN 1=*t,t*-farnesol, FARN 2=*c,t*-farnesol, NER=nerolidol, GERGER=geranylgeraniol.


solvent front
GERGER
NER
FARN 2
FARN I
GER
origin

vapor, was only detected in the area of the non-isoprenoid fatty alcohols (e.g. cetyl alcohol, oleyl alcohol). The identity of these radioactive bands, however, cannot be made on the basis of this co-chromatography alone. Since no mass was detected using iodine vapor (lower limit of sensitivity approximately 6 μg lipid), the specific activity of the compounds was probably very high. This would be expected if these compounds were indeed isoprenols.

The alcohols or their acetylated derivatives were subjected to analysis by GLRC. The results were entirely negative in that no radioactive responses were obtained for any of the samples, injected either as free alcohols or the acetyl derivatives. In final desperation up to 10,000 dpm of each sample (at least 10 mg) was injected onto the column and still no radioactive responses were seen above background. Mass responses were consistent with those expected for such overloads on the column and lauryl, palmitoleyl, cetyl, oleyl, and stearyl alcohols were identified by GC-MS as being present in the sample migrating with the authentic fatty alcohols. Directly after each GLRC analysis that gave the negative results, less than 1000 dpm of the standard methyl [1-^{14}C]palmitate was injected and radioactive and mass responses indicated an overall efficiency of at least 70% thus showing that the system was in usual and proper working order. This phase of the project was therefore discontinued. Possible explanations for these observations and postulation as to the identity of the radioactive alcohols will be discussed later in the dissertation.

DISCUSSION

Quantitative Determination of Juvenile Hormone

The reverse isotope dilution/carrier technique used in this study was originally devised by David Rittenberg and co-workers (170,171) for the quantitative determination of palmitic acid and glutamic acid in various biological tissues. In their experiments, an excess of the appropriate stable isotopically labeled species was added and then the mixture of protium and deuterium forms was purified by classical techniques such as fractional steam distillation and various precipitation procedures. They developed this method since total quantitative recoveries were next to impossible using the methods available and they assumed that there would be no differential loss of either form during the purification. A drawback was that totally pure compounds were needed for mass spectral analysis. In Rittenberg's case this was several hundred milligrams of pure compound since combustion was necessary for the mass spectral analyses. Modern instrumentation, the development of micro techniques, and the coupling of a gas chromatograph to a mass spectrometer has reduced the sample size to several micrograms of highly enriched compound. The reverse isotope dilution/carrier technique has recently gained wide acceptance with its "rediscovery" by Samuelsson, Hamberg, and Sweeley (172) in their application using prostaglandin E_1. Now other compounds occurring in very low amounts in biological systems, such as prostaglandin E_2,

prostaglandin F_{2_α}, 9,11-dihydroxy-15-keto-prost-5-enoic acid, 9,11,15-trihydroxy-prost-5-enoic acid (151,173,174), homovanillic acid (175), 5-hydroxyindole-3-acetic acid (176), chenodeoxycholic acid (177), dimethyltesterone (174), androst-16-ene-3-one (178), acetylcholine (179) and, in this case, insect juvenile hormone (180), are being determined by this method. There are many reasons for believing that the assumptions made in using the reverse isotope dilution/carrier technique for determination of juvenile hormone were valid and that the JH molecule and not another species was being measured by the AVA technique. These will be discussed in the following paragraphs.

There was no evidence from trial runs with synthetic labeled and unlabeled hormone that the protium and deuterium forms did not co-purify. None of the techniques used, with the exception of GLC, have ever been implicated to display selectivity between stable isotopic forms of the same compounds, expecially with a 3 amu difference.

An indication that the protium form of JH was being determined was the slight shift seen in retention time for the ion envelopes of the protium form. As seen in Figure 4, the solid line (*m/e* 117) is shifted to a shorter retention time, indicative of a deuterated compound. The deuterium isotope effect observed in GLC was noted by Sweeley *et al.* (181) in their development of multiple ion detection using the AVA. This phenomenon is unexplained.

There were definite, discreet, and repeatable increases over the blank value of the deuterated form at both ion pairs studied when purification was carried out in the presence of an insect lipid extract. These increases were internally consistent with each other,

which implied that the JH molecule and not another species was being measured. When the purification was carried out without added lipid or in the presence of lipids extracted from human plasma, no increase was observed at either of the ion pairs, again implying that the increase was due to JH and not an artifact.

A further implication that the actual JH molecule was being measured was the close agreement of the values obtained for the level of JH in a single male cecropia abdomen with those of Roller and Dahm (8), determined by totally independent methods using classical techniques on a micro scale. As a parenthetical note, this investigator had completed the first analyses while his major professor was on leave and believed that the level of JH determined by the AVA technique was almost twice as high as that previously reported for male cecropia. Only after further investigation with the major professor was the deuterium isotope effect in fragmentation discovered and the purity of the synthetic deuterated form considered, which together lowered the figure obtained to that presented in Table 2. This "after the fact" re-evaluation of the data to come up with a value in close agreement with that previously determined added credence to the technique and assurance that JH was being measured.

There are many advantages to using the reverse isotope dilution/ carrier technique for quantitative determination of juvenile hormone; the most important is that fact that this is one of the only ways to quantitatively measure small amounts of this naturally occurring compound by a physical method. A biological assay is not needed and could not be used with this type of purification. This alleviates

many problems, inaccuracies, and time-consuming periods while waiting (at least one week) for bioassay results. The final preparation does not need to be 100% pure, only highly enriched in the compound of interest so that no other GLC peaks are present in the area of elution of the compound of interest, in this case JH. Another distinct advantage, as discovered in Rittenberg's lab, is that quantitative recoveries are not necessary and therefore more precision can be gained in the purification, *i.e.*, portions of the TLC plates could be viaualized with iodine vapor and discarded and GLC monitoring of the fractions was possible. A further advantage is that if indeed a molecule is ubitiquous, it is a relatively straight forward method to measure it. There are, however, several disadvantages in carrying out this operation.

Probably the greatest disadvantage is that the stable isotopically labeled form must be chemically or biochemically synthesized, with the label in a location in the molecule that will lend itself to mass spectral analysis. One must also have access to a combined gas chromatograph-mass spectrometer with multiple ion monitoring. Another disadvantage is that only the compound specifically labeled can be determined and therefore if more than one compound is of interest, other labeled species must be synthesized and more ions must be followed. A further disadvantage is using a GC-MS without an on-line data system for multiple ion detection. The majority of the work done with the insect JH was carried out manually and operator bias can influence the results. It is difficult to avoid this especially in cases where the result is known in advance as in the determination of a standard curve.

The future of the technique for measurement of compounds occurring

in low concentration is unlimited. Once an appropriately labeled form is made or purchased and its mass spectral behavoir studied in relation to that of the protium form, analyses can be carried out. Stable isotopes can be used in man for metabolic experiments and it can be predicted that the reverse isotope dilution/carrier technique will lend itself to human research. The method can also be used to confirm values obtained from other sensitive methods of assay, such as use of fluorescent antibodies and radioimmunoassay of compounds occurring in low levels in biological systems.

Although the quantitative determination of JH was developed to be used in conjunction with biosynthetic experimnets, in which both radioactivity and amounts of JH could be measured simultaneously, the biosynthetic experiments did not prove to be successful. A wealth of information was gained, however, in a survey of insects to determine their levels of JH. This is the first report of JH levels determined chemically in some of the insects studied. Earlier reports of these levels were based solely on bioassay data in comparison to the standard of cecropia "golden oil". Many of the bioassays, some of which were carried out over twelve years ago on insect lipid extracts that had been subjected to arsh conditions such as sitting *in vacuo* for an hour at 60° prior to bioassay, have since been shown to be inaccurate (10). It is interesting to note that in one of Roller's earlier purifications of the JH molecule, molecular distillation was one of the steps (48,49,50).

The purification procedure for isolating the mixture of protium and deuterium forms of JH was acceptable. Although it may not be the

quickest purification (2) and probably does not give the highest yields of JH possible (11), it was successful and well characterized by this investigator and therefore was continued to be used throughout this investigation. Developing an alternate purification procedure would have required much time and was not thought to be necessary. There were few problems encountered; the only difficulties were in the selection of JH in fractions eluting from the LH-20 column. If fractions were included that contained less JH in relationship to contaminating lipid, more TLC plates were needed and therefore more losses were incurred. A typical purification usually took five days in addition to the growth time of the insect. Mass spectral analysis usually required thirty minutes per run to record the ions and calculate the results. Since triplicate analyses of the sample were usually performed and duplicate analyses of the blank were obtained before and after a run, about three hours to mass spectral time was needed for each sample at the selected ion pair. This time has now been reduced with the aid of the on-line computer system by approximately one-half since the system has control of fine focus and data reduction.

The results of quantitative determination of JH (Table 3) show that a single male cecropia abdomen contains approximately 1.8 μg and a female abdomen, about 0.6 μg. As previously noted, Roller's group reported that the average amount of JH in a 7-day old male cecropia was approximately 1.6 μg (8); these two results are in good agreement within experimental error. The results with the female showed a level of hormone that is approximately three times higher than that predicted from bioassay results (182).

Gilbert and Schneiderman (182) conducted biological assays on other saturniid moths and concluded that the male and female cynthia moth contained approximately 25% and 8%, respectively, of the biological activity found in male cecropia. These results were expressed on a per organism basis. The study reported in this dissertation revealed that male and femal cynthia moths contain approximately 10-11% of the JH of male cecropia when the results are expressed on the basis of μg JH per g lipid. Sexual dimorphism is only seen when the results are expressed on a per organism basis and these are more similar to those of Gilbert and Schneiderman (182). Gilbert and Schneiderman (182) also predicted that male and female promethea moths had similar amounts of JH in which the levels were approximately 5% and 4%, respectively, of male cecropia activity. The results of the present study are in good agreement with those of Gilbert and Schneiderman (182) for amounts of JH observed in male and female promethea moths.

Juvenile hormone bioassay data have not been reported for housefly larvae, housecricket, or cereal leaf beetle. One reason for this may be that relatively large amounts of lipid would have to be applied. For example, in the case of the housefly larvae, it would have been necessary to topically apply about 10 mg lipid in a volume of not more than 10 μl, using a sensitive bioassay such as the wax test (42) to elicit a positive response. Other insects studied in this investigation would not have required such high concentrations of lipid, however they probably have not been studied since these insects were not the ones of high interest after the discovery of JH was made and the techniques of bioassay perfected. Subsequently, the emphasis of

JH research has changed to finding and bioassaying analogs for use in pest control (98).

Although the results indicate the presence of the C_{18} JH molecule, it is unknown whether this molecule is the true active hormone in the insects tested, since other closely related molecules such as the 7-desmethyl homolog, Bowers' compound, or the ethyl ester of JH may be present and posses a higher biological activity in that particular insect. This seems to be true for the tobacco hornworm where all three JH methyl esters seem to be present but Bowers' compound seems to be the major active hormone molecule (201). The synthetic ethyl ester, although not found at a level of 100 ng in male cecropia, gives a biological response approximately eight times that of the methyl ester in the *Tenebrio* bioassay (8).

Future work on the determination of JH levels in insects could include an extended survey of insects and also a developmental study of JH titers as has been carried out for the cecropia moth (182) to expand and more accurately quantitate these results. The technique, of course, lends itself perfectly to help in biosynthetic experiments. The other known JH molecule, the 7-desmethyl homolog, could be quantitatively determined in the same preparation if the proper deuterated carrier were synthsized. Although the mass spectrum of this molecule gives a peak at *m/e* 114 (2,51), the ion envelope for it was not observed when the analyses were carried out. This is probably since it comprises not more than 20% of the activity of the C_{18} hormone in the male cecropia and much of it may have been lost due to manipulative procedures. The GLC elution volume of the 7-desmethyl JH is approximately

10% less than that of the C_{18} JH under the conditions used (52). The failure to find an ion envelope at *m/e* 114 at this retention time points up the importance of using a deuterated molecule for carrier and internal standard in carrying out quantitative determination when only microgram amounts of compound are present in several hundred milligrams of lipid extract.

Occurence and Levels of Fatty Acid Methyl and Ethyl Esters in Housefly Larvae

The validity of the experimental approach and the proof of structure of methyl and ethyl esters by mass spectral analysis have already been presented and will not be discussed here. The only alternative interpretation of the spectrum of scan 59 (Figure 11) would be that on an α-methyl methyl ester since the ions at *m/e* 88 and at *m/e* 101 are present in approximately the same relative abundance in these compounds (169). The ion at $[M-45]^+$, however, is absent in the spectra of α-methyl methyl esters (169) and can only arise from the loss of the ethoxy radical from an ethyl ester.

Although the multiple ion detection technique used in the determination of JH was carried out manually, the quantitative determination of fatty acid methyl and ethyl esters by single ion monitoring was carried out with the aid of on-line real-time computer control of the entire mass spectrometric process. The computer system was developed in this laboratory by Dr. Jack Holland and Richard Teets with the aid of this investigator (148), and allowed computer control of fine focus, thus eliminating problems encountered with magnet instability caused by excessive heat. The computer program also included automatic

integration of all peaks recorded, thus eliminating operator bias in the selection of baseline and integration parameters. The computer system also enable more rapid analyses to be made, since results were available within seconds after the end of a run. The further expansion of this GC-MS-AVA-computer system to include independent monitoring of up to eight ions and computer-assisted focusing of selected ions using a bipolar power supply is now in progress in the laboratory.

Studies on a biosynthetic origin of the methyl and ethyl esters were not carried out, but precedence for enzymatic formation of fatty acid methyl esters is clear. Akamatsu and Law (74) characterized an enzymatic reaction from *M. phlei* which uses S-adenosylmethionine as the methyl donor and oleic acid as the preferred substrate. S-Adenosylethionine was inactive as an ethyl donor and S-adenosylhomocysteine was found to be inhibitory to the reaction. Metzler *et al.* (11,53) observed a similar reaction in the esterification *in vivo* of JH in newly emerged cecropia moths. It would be interesting to discover the means by which the ethyl esters are formed enzymatically and what biochemical controls exist for their regulation. A host of ethyl donors could be tested in crude homogenates using radiolabeled fatty acids and subsequent thin-layer radiochromatography to determine incorporation.

The significance of these long chain esters in the housefly larvae is obscure. The juvenile hormones are methyl esters and methyl esters have been found to be present in the cuticle of some beetles (124). Methanol has been shown to be released from S-adenosylmethionine by a hydrolase (183) and McMannus *et al.* (184) have reported the presence

of endogenously produced ethanol in several tissues. Production of fatty acid esters of these alcohols by an as yet undefined mechanism could represent one method of detoxification of alcohols in biological systems. This is possible, but not probable, since flies seem to grow on diets that contain small amounts of ethanol (185). Calam (126) has pointed out that physiological roles for ethyl esters in insects have been suggested since they have been identified in the volatile territory-marking scents of bumblebees (121,122,123) and the greater part of the "assembling scent" in certain beetles (125). He also stated (126): "It may be conjectured that ethyl esters, possessing advantages of volatility and of specificity as natural products, may be used widely in the insect world as compounds modifying behavoir, that is, 'pheromones' in the broadest sense. It is also likely that rather specific, possibly unusual, biosynthetic mechanisms are operating."

In vivo Studies in Housefly Larvae

Failure to Find Radiolabeled Juvenile Hormone: The biosynthesis of JH is a problem that seemed would "fall out" without much difficulty after the elucidation of structure since the structure so closely resembled farnesol, a naturally occurring sesquiterpenoid found in insects. For example, the JH molecule(s) could be made by several methylations, oxidations, and an epoxidation, starting with farnesol. This simple problem has not been resolved within the past eight years and few positive results are known. Two likely precursors, mevalonic acid and methionine, were tested in the housefly. Although housefly larvae seem to be a poor choice to carry out these biosynthetic studies, they were used for several reasons. These reasons can only justify

the hope that housefly larvae could be a good system for such studies. Since the results were negative, the choice in retrospect, may not have been so wise.

The reasons for choosing housefly larvae are simple. If a cryptic precursor is involved in JH biosynthesis, it would probably be synthesized during the larval stage of growth and completed to JH in the newly emerged adult. A larval system, therefore, was chosen. Housefly larvae are readily available in large numbers and it was shown previously that they present an excellent system for incorporation of radioactive substrates included in the diet (166,185). The larvae were grown aseptically and axenically; if the results had been positive, the chance that JH was being synthesized by the larvae and not a symbiote would be very good. A final reason for choosing housefly larvae was that at the time these experiments were undertaken, only small numbers of cecropia, cynthia, or promethea cocoons were available and they were relatively expensive. Facilities for growing these moths in the laboratory or on campus were non-existent. In any case, JH biosynthesis is being studied in male cecropia by several major research groups headed by Meyer, Riddiford, Roller, and Siddal and the results obtained would probably only have confirmed their observations. If JH biosynthesis had been demonstrated in housefly larvae, it would have been of great impact in delineating its biosynthetic pathway.

The incorporation of radioactivity into total lipids after growth on labeled mevalonic acid was quite high; that from growth on labeled methionine was much lower. The reason for less incorporation of radioactivity from methionine was probably that the diet used had

not been depleted of methionine, as was the case in experiments carried out by Moulton *et al.* (166) where incorporation reached a level of approximately 7-8% of the total radioactivity contained in the diet (185).

It was not totally surprising that radiolabeled JH was not found after growth on mevalonic acid. Methionine, however, has been shown to be incorporated into the methyl ester methyl group of JH both *in vivo* (11) and *in vitro* (201) and there should have been no reason that this was not detected by the methods used. One major consideration must be an evaluation of the *in vivo* system used in this study. On a theoretical basis, assuming no dilution, the amount of radioactive incorporation into the hormone molecule would have been at least 3200 dpm. However, there definitely was dilution and alternate uses of the radioisotope as evidenced by the incorporation of label into the phospho- and neutral lipid fractions. Moulton *et al.* (166) also found label in the nucleic acid fractions in a similar experiment using housefly larvae. It is known that most insects can carry out selected methylations. The finding of radiolabeled phosphatidylethanolamine and phosphotidylserine arising from labeled methionine in this study substantiates this fact and serves as an internal control that the larvae were growing and incorporating label normally. Methionine could also serve as a precursor of the methyl group in the naturally occurring fatty acid methyl esters found in another phase of this study.

Methionine has been found to be an essential amino acid for most insects studied (202). Studies in *M. domestica* have shown this to be true and cysteine cannot spare for methionine in this species (203). Enzymatic formation of methionine from homoserine or homocysteine through

folic acid mediated reactions has been well characterized in microorganisms (204), however, this pathway has never been observed in any insect (202). Re-utilization of the methyl group of methionine probably does not occur and therefore once the dietary methionine has undergone a reaction, the methyl group has been lost as a metabolic precursor.

Taking the above findings into consideration, the theoretical level of radiolabeled JH calculated from knowing the total incorporation of label and level of JH present as determined in the previous study presented in this dissertation should probably be amended by a factor of at least two. This would mean that no more than 1600 dpm of JH would have been biosynthesized from the radiolabeled methionine. Since at most, 90% of the added deuterated carrier was recovered, no more than 1400 dpm could have been obtained. One half of the final fraction was injected for analysis by gas-liquid radiochromatography (e.g. 700 dpm, at most) and therefore the lower levels of sensitivity were being reached. Any physical problems with the system during the analysis (i.e., changes in voltage, incomplete combustion of the sample, incorrect or changing quench gas flow) would eliminate the possibility of realizing a peak for any radiolabeled JH with the instrument. It seems that the lower levels of detection were being challenged with the design of the experiment.

The failure to find radiolabeled JH in the larvae after growth on methionine could also have been attributed to the fact that the larvae were collected after they had entered the third instar, and, according to dogma (28,45), the JH titer should decrease after the last larval or

nymphal molt. Studies have shown that the half-life of exogenously injected JH in male cecropia is in the order of several hours at most (77), while that biosynthetically produced has a half-life of at least several days (75,78) and there is good evidence that it is complexed with a specific lipoprotein carrier (76,201). It is conceivable, therefore, that the JH was metabolized and excreted as a glucoside or glucuronide (78.79), or changed into a storage form for later use in the adult by the time that the analyses were made. A final explanation could be that not enough insects were studied since only one 2.5-l Fernbach flask was grown with 50 μCi methionine added in the diet. It may have been wiser to add less radioactivity to more flasks in which the diet had been depleted of methionine.

It is suggested that these experiments not be repeated or continued until more is known about the pathway of JH biosynthesis. This larval system may prove to be ideal for confirmation of the biosynthetic pathway once it is known, for many of the reasons previously presented. It will probably only be a matter of time until the secret of JH biosynthesis is unlocked.

Product Identification from Growth on DL-[2-^{14}C]Mevalonic Acid: It was surprising to find that the radiolabeled mevalonic acid was incorporated into at least five different radioactive regions on the TLC plate (Figure 18) representing at least three different neutral lipid fractions other than the terpenoid alcohols since these prenols all migrate to the same area in the neutral lipid solvent system used (Table 4). The hope, therefore, that a JH precursor molecule could be found seemed to pose an intriguing problem.

A hypothesis that the mevalonic acid had been incorporated into a particular fatty acid that was present in several of the neutral lipid fractions seemed attractive. The radiolabeled fatty acid isolated from triglycerides was ultimately postulated to be 15-methyl hexadecanoic acid on the basis of mass spectral and gas chromatographic data. Although it was not specifically identified in the free fatty acid or sterol ester fractions, the possibilities that this iso fatty acid was present in these fractions seems likely.

Reports of mevalonic acid incorporation into neutral lipids of housefly (134), *Neurospora crassa* (135), and cardiovascular tissue homogenates (136) have been published, however no specific identifications were made. Sridhara and Bhat (137) reported on the incorporation of [2-^{14}C]mevalonic acid into the lipids of the silkmoth, *Bombyx mori*, and tentatively identified radioactive palmitic stearic, and oleic acids. They found that radioactive incorporation reached a maximum level within four hours after isotope administration and reported incorporation varying between 8.5% and 11.8% of the total radioactivity injected, which is in good agreement with the values observed in both studies presented in this dissertation (Table 7). All of the above investigators seemed to conclude that mevalonic acid was being incorporated into esterified fatty acids, especially in the triglyceride fraction. In a 1958 symposium sponsored by the Ciba Foundation, it was suggested that mevalonic acid could serve as a precursor for branched-chain fatty acid biosynthesis, however no data were presented (186). The biosynthetic origin of the iso fatty acid identified from one mole of mevalonic acid and a fatty acid synthesizing system can be readily

explained in a consideration of the previously published findings about the biochemistry of mevalonic acid metabolism.

Mevalonic acid is made enzymatically by the irreversible reduction of hydroxymethylglutaryl CoA, a reaction which has been studied in detail (186-191). It seems unlikely that this reaction can be reversed through mevaldic acid; however, if this could happen the dicarboxylic acid could be activated to a CoA derivative and incorporated into fatty acids. If the mevalonic acid had been catabolized as such, or further to the level of acetyl CoA, all of the fatty acids would have been labeled, and this was not found. Evidence for the catabolism of mevalonic acid is scarce. Fifty hours after administration, 0.15% of the label from mevalonic acid was evolved as respiratory CO_2 in *N. cataris* plants (192) and Coon *et al.* (186) reported that mevalonic acid gave rise to radiolabeled acetone after incubation in a liver homogenate; however, less than 1% of the label was present in the acetone and they thought that this was of minor importance to mevalonic acid metabolism. When viewed in the overall scheme of incorporation and the generally accepted view that mevalonic acid is dedicated to synthesis of isoprenoid compounds, catabolism is probably insignificant, if at all present in the housefly.

The enzyme responsible for the first phosphorylation of mevalonic acid to mevalonic-5-phosphate, mevalonate kinase, has been reported to occur in another species of dipteran, the flesh fly, *Sarcophoga bullata* (131,132); the enzyme has been purified and its characteristics studied. It resembles the mammalian enzyme in most respects. Although the other enzymes responsible for conversion of mevalonate-5-phosphate

to isopentenyl pyrophosphate have not been specifically characterized in insects, they must be present since biosynthesis of terpenoids from mevalonic acid such as farnesol and farnesal (7,11) and citral and citronella (193), has been documented. In a recent report, four prenols were identified as being biosynthesized from mevalonic acid (133). Geraniol, farnesol, nerolidol, and geranylgeraniol were identified from the flesh fly by means of infra-red spectroscopy, mass spectrometry, and co-chromatography in various TLC and GLC systems. These four prenols were not specifically sought in the lipid extracts obtained from the experiments presented here; however co-chromatography of labeled products with these prenols in one TLC system after total reduction to alcohols lends support to the fact that they could have been present in the lipid extract (see Figure 29). Gas-liquid radiochromatography of the alcohol fractions gave negative results. The reasons for this are totally unknown.

In a continuing exploration of the fates of mevalonic acid and prenols, Popjak and his group characterized an unusual radioactive fraction arising after incubation of mevalonic acid with a crude liver preparation followed by incubation with a microsomal pellet and added co-factors (194). They found that the allyl pyrophosphates fromed from mevalonic acid were hydrolyzed by a microsomal phosphatase and then subsequently oxidized to the corresponding acids by a liver alcohol dehydrogenase and aldehyde dehydrogenase present in a soluble fraction of the homogenate. The oxidation of these allyl pyrophosphates to prenoic acids in mouse liver was found to be a significant pathway of mevalonic acid metabolism since 23% of the radioactivity of

[2-^{14}C]mevalonic acid was found in this fraction after 70 minutes. A recent report on farnesol metabolism in *Drosophila melanogaster* (195) showed that these insects possess a potent octanol dehydrogenase and an aldehyde oxidase which readily use long chain as well as shorter chain primary alcohols to make the corresponding acids. Lambremont (196) showed that oxidation of injected or dietary [1-^{14}C]hexadecanol and [1-^{14}C]octanol occurs rapidly in the tobacco budworm. Forty-eight hours after injection, 84% of the alcohol was oxidized to fatty acid and, when given in the larval diet, oxidation to acid was virtually complete.

It is very conceivable, therefore, that the mevalonic acid was phosphorylated and decarboxylated to yield isopentenyl pyrophosphate, which was isomerized to dimethylallyl pyrophosphate and this subsequently dephosphorylated and oxidized to form dimethylacrylic acid. The dimethylacrylic acid could have then been activated to its CoA derivative and incorporated into the fatty acid. From the work of Horning, Martin, Karmen, and Vagelos (197,198) in the early 1960's on the enzymatic synthesis of branched-chain fatty acids from various CoA precursors such as isobutryl-CoA, isocaproyl-CoA, and isovaleryl-CoA, it is quite conceivable that either dimethylacryl-CoA or isovaleryl-CoA arising from the mevalonic acid was incorporated. The step when hydrogenation of the dimethylacryl-CoA occurs was not studied. It seems likely however from the studies of Horning *et al.* (197,198) that isovaleryl-CoA is preferentially incorporated into iso fatty acids and therefore the hydrogenation probably occurs at the CoA level before incorporation into the fatty acid.

This molecule is not likely to be a precursor of juvenile hormone

since it probably could not undergo the necessary alkylations. The iso fatty acid may represent an alternate pathway of mevalonate metabolism that has not been seen before. Studies on a possible physiological role for this fatty acid were not undertaken and its significance is obscure. It is present in low amounts when compared to total triglyceride fatty acids found in the larvae (see Figure 21) and represents no more than 1% of the fatty acids released by lipase.

All of the enzymes needed for formation of this iso fatty acid are present in insects and it may be that housefly larvae show a preference for making prenoic acids from dimethylallyl pyrophosphate as opposed to geranyl or farnesyl pyrophosphates. It is interesting to note that the conversion of farnesyl pyrophosphate to farnesoic acid is analogous to that of isopentenyl pyrophosphate to dimethylacrylic acid. Farnesoic acid could serve as a precursor molecule for JH and it was almost expected that this was the esterified radiolabeled fatty acid. No evidence for the existence of farnesoic acid was obtained in these investigations.

The elucidation of 15-methyl hexadecanoic acid in housefly larvae as arising from radiolabeled mevalonate does not preclude the existence of other radiolabeled fatty acids or molecules such as hydrocarbons; however, they were not found. The specific activity calculated for the fatty acid was consistent within experimental error for the hypothesis that one mole of mevalonic acid had been incorporated.

Prenol formation was not specifically sought in the lipid extract. It seems, however, that it would be a simple task to find

these if they are there since excellent methods have been published for handling these compounds (12,133). It is suggested that further characterization of the fatty acid or the biosynthesis of prenols in housefly larvae would add little to the scientific literature and open few avenues for stimulating research, especially in the area of juvenile hormone formation. These results are interesting, but not exciting.

SUMMARY

A reverse isotope dilution/carrier technique for the quantitative determination of C_{18}-*Cecropia* juvenile hormone was devised to survey the occurence and levels of this molecule in a variety of insects. After addition of a deuterium-labeled form of the juvenile hormone to an insect extract, the mixture of deuterium and protium forms was purified and the resultant fraction was analyzed by combined gas chromatography-mass spectrometry. The deuterium/protium ratios were obtained by multiple ion detection using the accelerating voltage alternator accessory of an LKB 9000 combined GC-MS. Levels of hormone in abdomina of both adult male and female cecropia, cynthia, and promethea moths, as well as in male and female housecrickets, worker honeybees, mixed male and female cereal leaf beetles and housefly larvae were determined. The results confirmed the level of approximately 1.6 μg reported for the male cecropia moth and also indicated sexual dimorphism. Female moths contained less juvenile hormone than their male counterparts; female crickets, however, contained much more hormone than male crickets. When the results are presented on the basis of amount of lipid extracted, housefly larvae and cereal leaf beetles stand out for their relatively low and high levels, respectively. This is the first time that juvenile hormone levels have been measured chemically in these insects with the exception of male cecropia and cynthia moths. As a sidelight, the elution profile of five classes of neutral lipids on Sephadex LH-20 in benzene-methanol (1:1) was determined by use of thin-layer chromatography

and direct probe mass spectrometry of selected fractions.

A variety of naturally occurring fatty acid methyl and ethyl esters was found in late third instar housefly larvae reared aseptically and axenically. The fatty acid esters were identified by combined GC-MS with the aid of an on-line computer system and their levels quantitatively determined by computer-controlled mass fragmentography. The amounts of methyl and ethyl esters found represented approximately 0.007% and 0.019%, respectively, of the total lipid extracted. These esters were not localized to any extent in the cuticle and the possibility that they arose as artifacts of extraction or purification was ruled out by control experiments. Neither the biosynthetic origin nor physiological role of these esters in the larvae was studied, however biosynthesis of fatty acid methyl esters and physiological roles for other long-chain esters has been demonstrated in other insect systems.

Attempts to elucidate the pathway of juvenile hormone formation in housefly larvae, grown aseptically and axenically, on diets containing DL-[2-^{14}C]mevalonic acid and L-[*Me*-^{14}C]methionine, proved negative in that no radiolabeled JH was found. A thin-layer radiochromatogram of a portion of the lipid extracted after growth on the labeled mevalonic acid revealed at least five radioactive bands, three of which co-migrated with authentic neutral lipid standards. This finding would be consistent with a hypothesis that the mevalonic acid was incorporated into a fatty acid that was present in these three fractions. The triglyceride fatty acids were therefore studied in detail after enzymatic hydrolysis by hog pancreatic lipase. The acids were esterified with diazomethane and subjected to analysis by gas-liquid radiochromatography.

These analyses indicated that a radioactive fatty acid methyl ester was present in the region of C_{17} methyl esters. Preparative gas-liquid chromatography was carried out to enrich this radioactive component and through mass spectral analysis and comparative gas chromatographic data the radioactive unknown was tentatively identified as an iso fatty acid methyl ester, methyl 15-methyl hexadecanoate. It had a specific activity of 7.1 mCi/mmole which is consistent with the incorporation of one mole of mevalonic acid (specific activity= 7.2 mCi/mmole). Three other GLC peaks eluting in the same area were characterized by mass spectrometric techniques and tentatively identified as a mixture of methyl 14-methyl hexadecanoate and methyl 2,3-methylene hexadecanoate, methyl heptadecanoate, and methyl 9,10-heptadecenoate. The labeled fatty acid identified is probably not a precursor of juvenile hormone. A possible biosynthetic scheme for its formation from mevalonic acid has been presented.

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APPENDIX

APPENDIX

List of Publications

Publications

M.A. Bieber, C.C. Sweeley, D.J. Faulkner, and M.R. Petersen, Purification and Quantitative Determination of *Cecropia* Juvenile Hormone, *Anal. Biochem.* 47 (1972) 264.

J.F. Holland, C.C. Sweeley, R.E. Thrush, R.E. Teets, and M.A. Bieber, On-Line Computer Controlled Multiple Ion Detection in Combined Gas Chromatography-Mass Spectrometry, *Anal. Chem.* 45 (1973) 308.

In Manuscript:

M.A. Bieber and C.C. Sweeley, Occurrence and Levels of Fatty Acid Methyl and Ethyl Esters in *Musca domestica* Larvae, *Lipids.*

M.A. Bieber, C.C. Sweeley, D.J. Faulkner, and M.R. Petersen, Levels of Juvenile Hormone in Various Insects, *Ann. Ent. Soc. Amer.*

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J.F. Holland, R.E. Teets, M.A. Bieber, and C.C. Sweeley, A Proposed Standardization of the Mathematical Methods Used in the Calculations and Presentation of Data from Isotope Dilution Experiments, *Anal. Chem.*

Abstracts

M.A. Bieber, C.C. Sweeley, D.J. Faulkner, and M.R. Petersen, Quantitative Determination of Juvenile Hormone, Entomological Society of America, Los Angeles, 1971.

C.C. Sweeley, N.D. Young, R.E. Teets, M.A. Bieber, and J.F. Holland, Analysis of Stable Isotopic Abundance by an Automated Gas Chromatograph-Mass Spectrometer System, Eastern Analytical Symposium, Atlantic City, 1972.

C.C. Sweeley, N.D. Young, M.A. Bieber, and J.F. Holland, Utilization of Mass Chromatography for the Estimation of Stable Isotopic Abundance by Combined Gas Chromatography-Mass Spectrometry, Conference on Stable Isotopes in Biology and Medicine, Argonne National Laboratory, 1973.

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C.C. Sweeley, R.E. Teets, N.D. Young, M.A. Bieber, and J.F. Holland, Evaluation of Mass Chromatography and Multiple Ion Detection for Quantitative Analysis of Lipids and Estimation of Stable Isotopic Abundance, 9th International Congress of Biochemistry, Stockholm, 1973.