



Biosynthesis of hydrocarbons in the American cockroach, *Periplaneta americana*
by Charles Wayland Conrad

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Chemistry
Montana State University
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Abstract:

Hydrocarbons are biosynthesized from acetate, propionate, and fatty acid substrates in *P. americana* and are spread over the surface throughout adult life. The total amount of surface hydrocarbon is approximately the same in teneral and fully-tanned adult roaches.

Since acetate-1-¹⁴C, propionate-1-¹⁴C, and linoleate-1-¹⁴C are not incorporated into hydrocarbons in teneral adults, while other fatty acid substrates are incorporated, hydrocarbon biosynthesis via an elongation-decarboxylation pathway does not occur at this time. An alternative mechanism for hydrocarbon biosynthesis is head-to-head condensation of fatty acids. Propionate, and not isoleucine or the S-methyl group of methionine, is thought to be the source of the methyl side-chain in branched hydrocarbons. Linoleate is believed to be the source of the unsaturated chain in diene hydrocarbons.

BIOSYNTHESIS OF HYDROCARBONS IN THE AMERICAN
COCKROACH, PERIPLANETA AMERICANA

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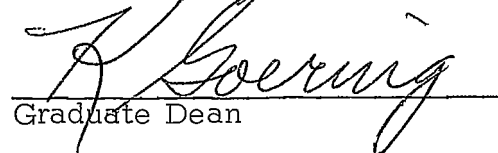
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ABSTRACT

Hydrocarbons are biosynthesized from acetate, propionate, and fatty acid substrates in *P. americana* and are spread over the surface throughout adult life. The total amount of surface hydrocarbon is approximately the same in teneral and fully-tanned adult roaches. Since acetate-1-¹⁴C, propionate-1-¹⁴C, and linoleate-1-¹⁴C are not incorporated into hydrocarbons in teneral adults, while other fatty acid substrates are incorporated, hydrocarbon biosynthesis via an elongation-decarboxylation pathway does not occur at this time. An alternative mechanism for hydrocarbon biosynthesis is head-to-head condensation of fatty acids. Propionate, and not isoleucine or the S-methyl group of methionine, is thought to be the source of the methyl side-chain in branched hydrocarbons. Linoleate is believed to be the source of the unsaturated chain in diene hydrocarbons.

INTRODUCTION

It is often the case in scientific research that the function of a biochemical is found long before the identity or the mechanism of synthesis of the chemical is discovered. Such is the case of the "waxy" substances which cover the cuticles of many plants and animals. Among the animals with lipid cuticular coverings are the insects. Dusham (1) reported that "oily" substances were secreted by wax glands in cockroaches, but such glands have not been observed by other workers. Dusham found that at least part of the secretion of the wax glands was true wax (esters of fatty acids and fatty alcohols) which coated the surfaces of the cockroaches. In 1935 Ramsay (2) demonstrated that water loss from the cuticle of the American cockroach, Periplaneta americana, was due to passive physical factors, rather than to any active physiological phenomenon. Subsequent to these two discoveries, interest in determining the identities and metabolic functions of these cuticular lipid materials has grown.

Physiology of the Insect Cuticle

Literature concerning the structure and functions of insect cuticular lipids has been recently reviewed by Jackson and Baker (3). Although Dusham's (1) discovery of wax glands as the site of synthesis for cuticular lipids in two species of cockroaches was made many years ago, no site of cuticular lipid synthesis common to all insect species

has been found. Piek (4) concluded that hydrocarbon biosynthesis in Apis mellifera occurred in oenocytes.

In the past decade research has centered around the controversy over the mechanism by which lipids are transported to and laid down upon the surface of the insect body. Based on experimental data that the water transpiration rate through the insect cuticle can be altered by temperature and adsorptive dusts, Hurst (5) and Beament (6,7) had postulated a protein-bound base lipid layer upon which are layered either an ordered or an unorganized covering of other lipid materials which constitute the main barrier to water loss by the insect in an environment of low relative humidity (8-11). Recent electron microscopic studies by Locke (12,13) and others (14,15) have shown the existence of wax canals extending from near the monocellular hypodermis layer to the external cuticular surface. According to this model, lipophilic and hydrophilic channels in these wax canals are responsible for transport of lipid materials and water, respectively.

Holdgate and Seal (16) found that the cuticle of teneral (newly-molted) Tenebrio molitor was more highly permeable to water than the cuticle of an insect at a later stage in its adult life. They also observed that the surface wax of a teneral insect melted at a lower temperature than did that of an older insect. Based on these observations, they concluded that a very thin layer of lipid covered the cuticle and rapidly

spread over it immediately after ecdysis; a heavier, more permanent lipid layer was secreted subsequently.

Cuticular Lipids of Insects and Other Organisms

Compositions of cuticular lipids and the identities of several individual compounds have been determined for several insects. Among these are bees (4), scale insects (17-20), silkworms (21), crickets (22, 23), stoneflies (24), ants (25), and cockroaches (3,26-31). These lipids consist of fatty acids, alcohols, aldehydes, esters, sterols, and hydrocarbons in varying amounts (3,4,17-31), depending upon species and, in some cases, sex of the insect (28). Several recent reviews indicate that the types of surface lipids of animals (32), plants (33), bacteria (34), and insects (3,35) are similar.

Biosynthesis of Fatty Acids

Since the pathways of hydrocarbon synthesis which have been proposed in the literature involve fatty acids as precursors, the literature concerning fatty acid biosynthesis has been investigated.

Normal Fatty Acids. Fatty acid biosynthesis has recently been reviewed (36), and the basic steps are presented in Figure 1 to provide a basis for discussion. Almost all organisms are capable of synthesis of normal fatty acids; however, only plants and some microorganisms,

none of the animals so far studied, are capable of synthesis of poly-unsaturated fatty acids.

Branched Fatty Acids. Kaneda (37) has proposed pathways for incorporation of some branched amino acids into tobacco fatty acids, which were then considered to be substrates in the synthesis of branched hydrocarbons. His proposed pathway for incorporation of isoleucine into branched fatty acids is shown in Figure 2. Albro and Dittmer (38) have investigated incorporation of amino acids into fatty acids in the micrococcus Sarcina lutea. They determined that isoleucine was incorporated into anteiso (methyl branch on third carbon from the methyl end) branched fatty acids, and valine was incorporated into iso (methyl branch on second carbon from the methyl end) branched fatty acids, probably by a mechanism similar to that proposed by Kaneda.

Another possible mechanism of synthesis of branched fatty acids has been offered by Saz and Weil (39), who found that in Ascaris, propionic acid was incorporated into anteiso branched fatty acids by the proposed mechanism shown in Figure 3. This mechanism was supported by the results of Kaneda's experiments involving several microorganisms (40).

Still another common source of methyl groups in biological systems is the S-methyl group of methionine. Although Jaureguiberry et al. (41) demonstrated incorporation of this group into the internal branch of 10-methyloctadecanoic acid, Albro and Dittmer (38) could not show that

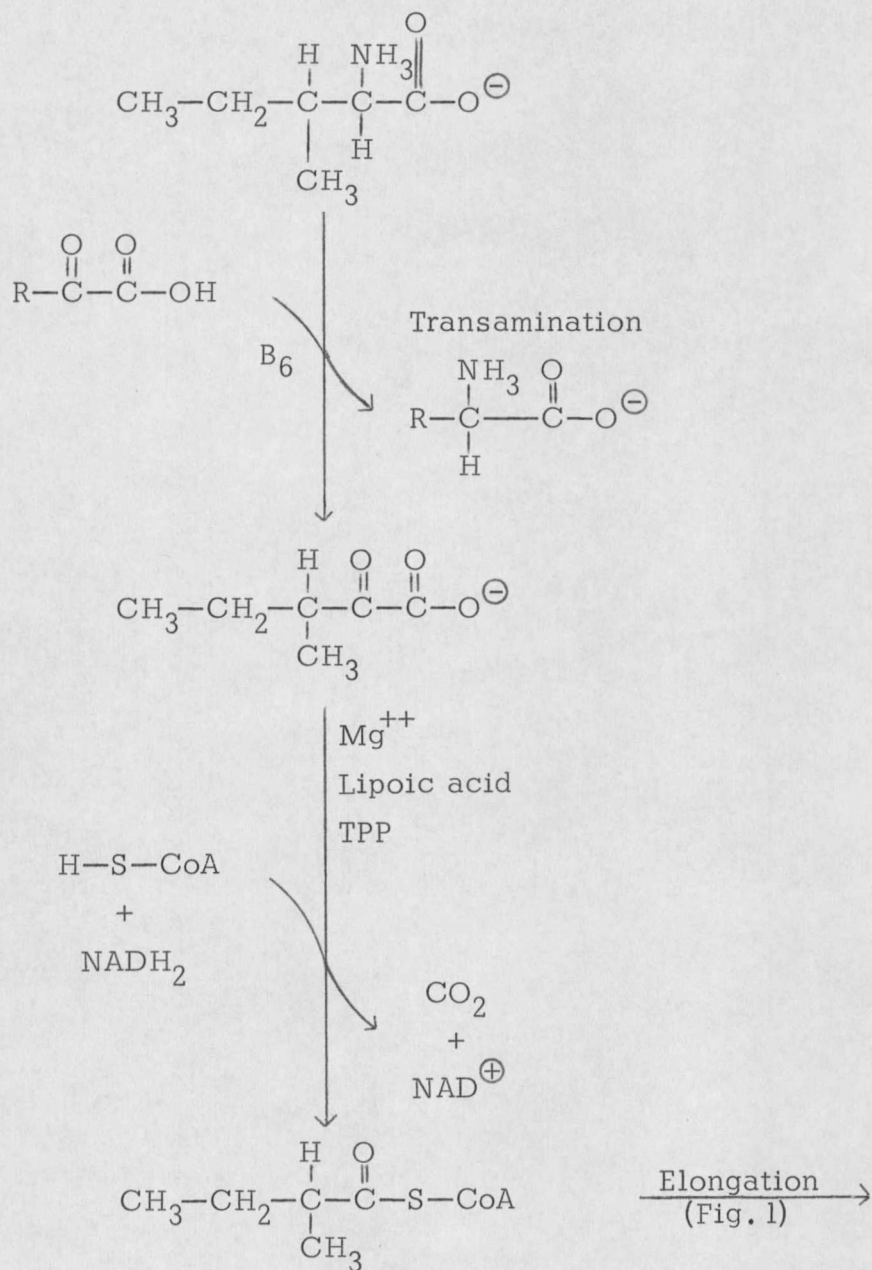
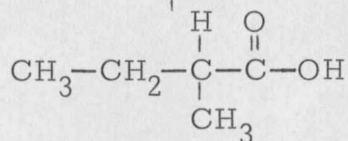
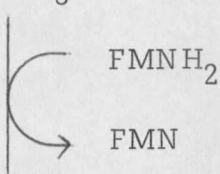
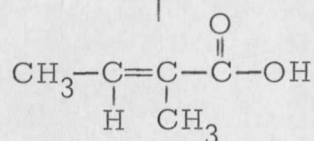
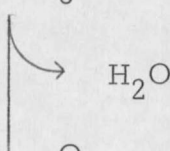
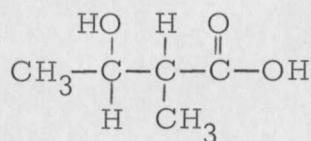
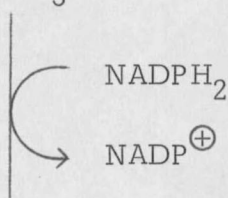
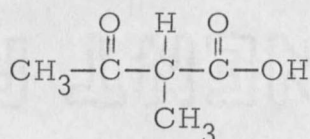
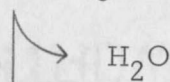
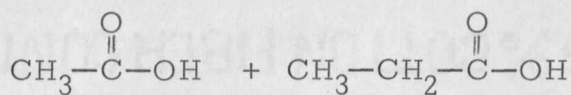


FIG. 2. Proposed pathway for incorporation of isoleucine into branched fatty acids in tobacco (37).



Elongation
(Fig. 1) →

FIG. 3. Proposed mechanism for synthesis of branched fatty acids from acetic acid and propionic acid (39).

the S-methyl group of methionine was incorporated into the branched hydrocarbons of S. lutea. Jackson and Baker (3) have demonstrated that the S-methyl group of methionine was not incorporated into either the saturated or unsaturated fraction of P. americana hydrocarbons.

Biosynthesis of Hydrocarbons

Of the possible pathways for biosynthesis of hydrocarbons, two have received serious consideration. These are (a) head-to-head condensation of two fatty acids, for which the outline of a possible pathway is shown in Figure 4, and (b) elongation of a fatty acid with subsequent decarboxylation.

Head-to-head Condensation Pathway. Channon and Chibnall (42) proposed head-to-head condensation of two fatty acids in hydrocarbon biosynthesis, based on their discovery of 15-nonacosanone in broccoli. According to their theory, this ketone was an intermediate decarboxylation product which was reduced to nonacosane, the predominant hydrocarbon found in broccoli. In more recent work, Kaneda (37,43) with tobacco, and Albro and Dittmer (33,38,44-46) with S. lutea have indicated that several organisms may form hydrocarbons via such a condensation pathway.

Gastambide-Odier and Lederer (47) proposed a condensation mechanism for production of corynomycolic acid (Fig. 5) in Corynebacterium diphtheriae. This acid, they suggested, might be an

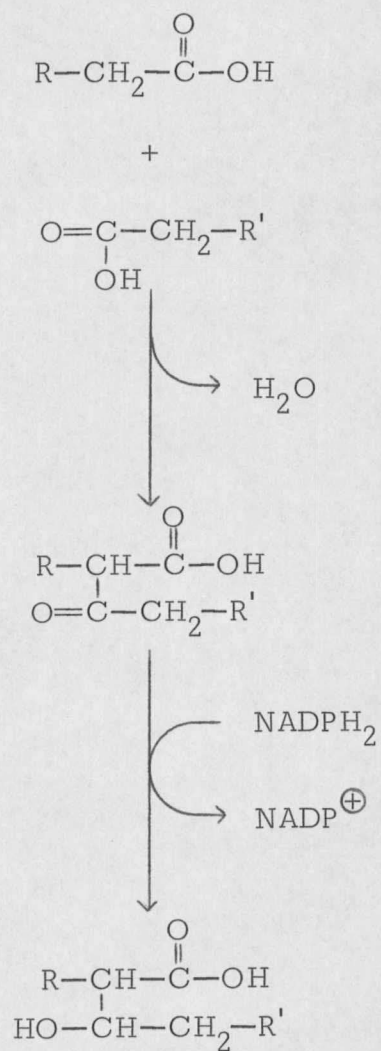


FIG. 5. Proposed mechanism for formation of corynomycolic acid in C. diphtheriae (47).

intermediate in hydrocarbon biosynthesis via the condensation pathway.

A variation of the condensation pathway has been proposed by Albro and Dittmer (34) in their studies of *S. lutea*. They suggest reduction of fatty acids to aldehydes which are then incorporated, as their vinyl ethers, into neutral plasmalogen structures. Upon release from these plasmalogens, each molecule of vinyl ether was proposed to condense with a fatty acid molecule to form a hydrocarbon unsaturated at the position of the double bond in the vinyl ether.

Elongation-decarboxylation Pathway. As was shown in Figure 1, fatty acids, whether normal or branched, saturated or unsaturated, may be elongated by successive addition of two-carbon units from malonate. The theory of the elongation pathway is that formation of a long-chain fatty acid is followed by its decarboxylation to form a hydrocarbon (Fig. 6). Work by Kolattukudy and coworkers (48-55), following the work of Channon and Chibnall (42), demonstrated that long-chain ketones were not precursors of hydrocarbons in broccoli. Kolattukudy (48,49) suggested that these hydrocarbons were, in fact, synthesized via an elongation-decarboxylation pathway, rather than via condensation of fatty acids as had been proposed by Channon and Chibnall (42). This conclusion was based, in part, on the fact that trichloroacetic acid inhibited both elongation of short-chain fatty acids (16 carbons or

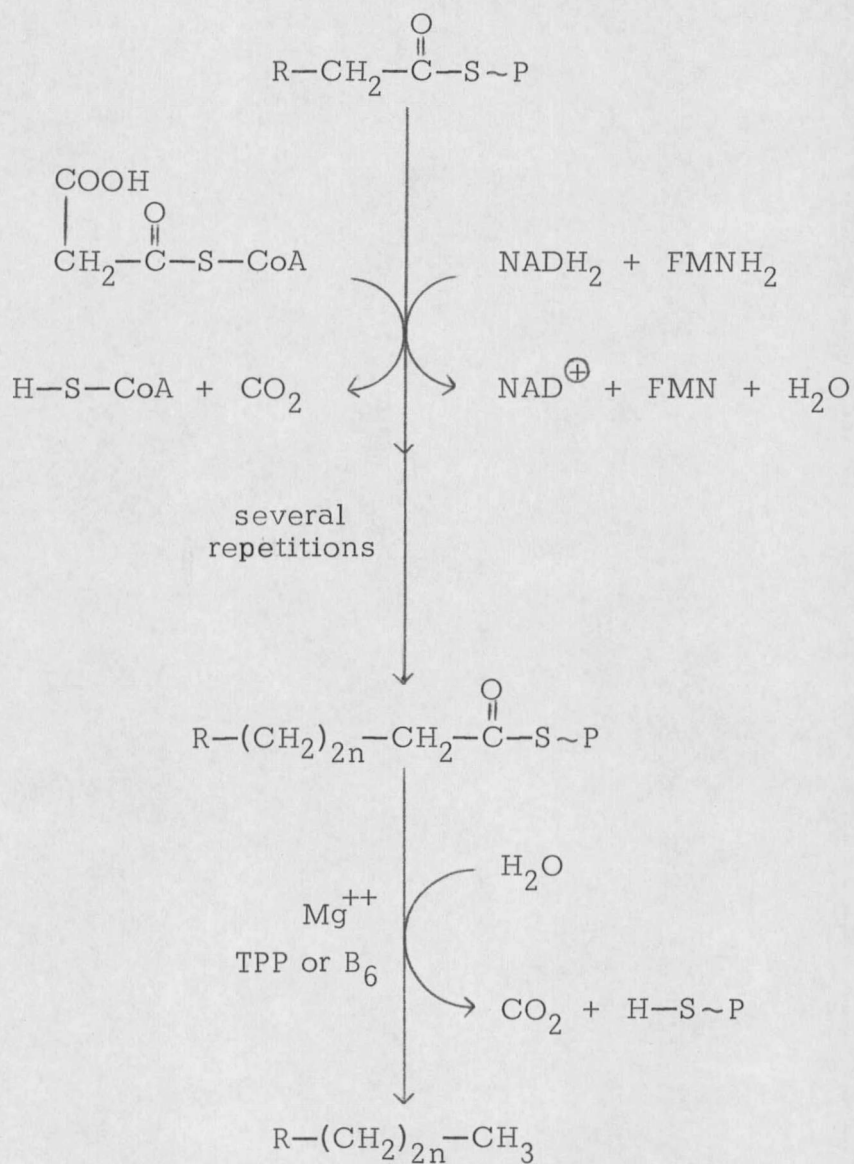


FIG. 6. Proposed elongation-decarboxylation pathway to form a hydrocarbon molecule from a fatty acid molecule.

shorter) and their incorporation into hydrocarbons, while incorporation of stearic acid into hydrocarbons was not affected.

Occurrence of Both Pathways in the Same Organism. Albro and Dittmer (46) believe that head-to-head condensation may be the preferred mechanism of hydrocarbon biosynthesis in S. lutea; however, their data indicate that elongation-decarboxylation may be responsible for some of the hydrocarbon synthesis. They based this conclusion on the fact that different patterns of incorporation of labeled fatty acids were observed when cells were grown under different conditions of media formulation, cell concentration, and cofactor presence.

Hydrocarbons of Periplaneta americana

Periplaneta americana, the American cockroach, was chosen for study because its three principal hydrocarbons (n-pentacosane, 12%; 3-methylpentacosane, 20%; cis,cis-6,9-heptacosadiene, 65%) (26, 30, 31) represent three distinctly different structural types (Fig. 7). In addition, minor quantities of other hydrocarbons have been found, including monounsaturated hydrocarbons of 41- and 43-carbon chain lengths (29). Hydrocarbons of both male and female P. americana are almost identical, although more is present in females (26, 29, 56). Since only three compounds comprise 97% of the hydrocarbons of this insect, study of the pathways leading to their biosynthesis was particularly attractive. Presumably, biosynthetic pathways for each of these hydrocarbons can be

$\text{CH}_3-(\text{CH}_2)_{23}-\text{CH}_3$	<u>n</u> -pentacosane
$\text{CH}_3-(\text{CH}_2)_{25}-\text{CH}_3$	<u>n</u> -heptacosane
$\text{CH}_3-(\text{CH}_2)_{27}-\text{CH}_3$	<u>n</u> -nonacosane
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{CH}_2-\text{CH}-(\text{CH}_2)_{21}-\text{CH}_3 \end{array}$	3-methylpentacosane
$\text{CH}_3-(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_{16}-\text{CH}_3$	<u>Cis,cis</u> -6,9-heptacosadiene
$\text{CH}_3-(\text{CH}_2)_{13}-\text{CH}=\text{CH}-(\text{CH}_2)_{24}-\text{CH}_3$	15-hentetracontene
$\text{CH}_3-(\text{CH}_2)_{13}-\text{CH}=\text{CH}-(\text{CH}_2)_{26}-\text{CH}_3$	15-tritetracontene

FIG. 7. Structures of hydrocarbons which have been reported for P. americana (26,29,30).

determined by investigation of the metabolism of precursor molecules with similar partial structures.

Investigation of hydrocarbon biosynthesis in insects has been very limited. Lambremont and coworkers (57) have demonstrated incorporation of acetate- ^{14}C into both fatty acids and hydrocarbons of the boll weevil. Similar observations have been made by Lamb and Monroe (58) with the cereal leaf beetles, Bloch et al. (59) with hide beetles, and Robbins et al. (60) with the housefly. Work by Nelson (61) has demonstrated in vivo incorporation of radioactivity from both acetate- ^{14}C and palmitate- ^{14}C into hydrocarbons in the fat body and integument of P. americana. Based on in vitro incorporation of radioactivity into

hydrocarbons of the integument, but not the fat body, Nelson concluded that the epidermal cells were the site of hydrocarbon synthesis.

Loulouides et al. (62) had previously found incorporation of acetate-1-¹⁴C into both saponifiable and unsaponifiable lipids of P. americana.

Previous work in this laboratory (3) has demonstrated the incorporation of several different substrates into saturated and unsaturated fractions of the hydrocarbons of P. americana. These results showed that in live roaches acetate and malonate were incorporated into both fractions equally, while palmitate and propionate were incorporated preferentially into the saturated fraction, and linoleate was incorporated more into the unsaturated fraction. Studies of substrates applied to excised integuments of this insect showed the same pattern of incorporation of substrates, although not nearly as much incorporation was observed as in vivo, except in the case of palmitate. Subsequent to this publication, it was found that the palmitate substrates which had been used in these studies were contaminated with labeled hydrocarbons from some unknown source, and data from these experiments involving palmitate must be disregarded. These workers also showed that trichloroacetate injected into the roaches had an inhibitory effect upon incorporation of acetate, and chloroform anesthetization reduced incorporation of malonate into hydrocarbons.

In addition to hydrocarbons, which make up about 75% of the surface lipids of P. americana (26,29), other lipid species have been

identified. Both Baker et al. (26) and Gilby and Cox (30) have observed fatty acids and long-chain alcohols and some of their esters. In addition, Gilby and Cox (30) have reported the presence of long-chain aldehydes in substantial quantities. This observation has not been made in our laboratory, leading to the conclusion that aldehydes may possibly be formed as products of autoxidation of the unsaturated hydrocarbons of the cockroach cuticle. Atkinson and Gilby (63) have determined that this phenomenon may be related to the hardening of lipid material on the cuticle and oötheca.

Biosynthesis of Hydrocarbons in *Periplaneta americana*

Fatty acid substrates participate not only in hydrocarbon biosynthesis, but also in catabolic and storage pathways. Formation of glycerides and other esters diminishes the pool of free fatty acids, making them less available for hydrocarbon biosynthesis. Production of acetyl-coenzyme A units which can participate in a number of well-known pathways (the "acetate effect") results from β -oxidation of fatty acids, and the related elongation of fatty acids.

The purpose of this research was to investigate the fate of labeled fatty acids and other substrates, particularly in their relation to the pathway or pathways leading to the biosynthesis of the major hydrocarbons of *P. americana*. The approach was to allow lyophilized integument homogenates and living cockroaches to metabolize various labeled

substrates and to study the incorporation of these substrates into product hydrocarbons. Studies were carried out using teneral (newly-molted) adult roaches and adults whose cuticles had tanned completely. Furthermore, attempts were made to block competing pathways by administering several compounds which were expected to demonstrate inhibitory effects.

HYPOTHESIS

Hydrocarbons of Periplaneta americana are biosynthesized from fatty acid precursors via a pathway of elongation and decarboxylation of fatty acids and/or a pathway involving head-to-head condensation of two fatty acids. Biosynthesis of hydrocarbons can be studied through the pattern of incorporation of fatty acids and their precursors into different hydrocarbon fractions.

EXPERIMENTAL

Growth Conditions

Periplaneta americana were reared in covered garbage cans equipped with electric fences to prevent escape of the roaches. No attempt was made to keep different developmental stages separated, and adults were allowed to breed freely. Food and water were placed in the cages with the cockroaches. Diet was ground Gravy Train dog food. Water was provided in the form of an agar-water (1:99) gel. In order to determine metabolic differences during the life cycle, adult cockroaches were collected at various times following the imaginal ecdysis. One series of experiments was performed using teneral adults which had not yet begun to tan. These were collected and injected with substrate solutions within two hours following ecdysis. Another group used for experimentation was collected from a few days after the imaginal ecdysis through several weeks following this ecdysis. Still a third group of adults was collected at least two months following the imaginal ecdysis.

Attempts to Prepare a Cell-free Biosynthetic System

It is recognized that in a whole animal, indeed in a whole cell, several metabolic systems compete for substrate molecules. In order to study one particular metabolic system, therefore, it is desirable to isolate that system from other enzymes. To that end, an attempt was made

to prepare in vitro a culture capable of hydrocarbon biosynthesis, without competing enzymes.

Several cockroaches were frozen at -25 C for 20 min; their heads and appendages were removed with forceps, and the internal organs were removed by dissection. These excised integuments (which Jackson and Baker (2) had determined were capable of synthesizing hydrocarbons) were then placed in a small amount of cold phosphate buffer, pH 7.2 (previously determined to be the pH of cockroach integument homogenate). In an effort to destroy as few protein molecules as possible, disruption of the excised integuments was done at high speed in a Virtis homogenizer for 10 sec and in a motorized mortar and pestle for 2 min. The slurry, which was not checked for hydrocarbon synthetic activity, was then transferred to a centrifuge tube with a small amount of additional buffer, and centrifuged at $5000 \times g$ for 15 min to remove cell fragments. Up to this point, all work was done at 4 C in order to preserve enzyme integrity. The supernatant, which was thought to contain most soluble enzymes, was then coated on the inside of a flask and quickly frozen in a dry ice-butanol bath. The flask was attached to a vacuum apparatus and lyophilized over a period of several hours. This method provided a convenient means of storage for the preparation. Approximately 35 to 50 mg of powder was prepared from each integument. In each experiment 25 mg of lyophilysate, which had been stored at -25 C , was suspended in 2 ml of fresh buffer, and incubation was

allowed to proceed at room temperature for 6 hr. Controls containing only buffer and substrates were run concurrently.

Injection of Substrates and Inhibitors

In each experiment 1.0 μ l of a solution of a substrate in water or toluene solution (depending upon solubility) was injected into the thoracic haemocoel of each cockroach. Insects did not seem to suffer any toxic effects from toluene. Radiobiochemicals were purchased from Volk and Amersham-Searle. In all cases the injection consisted of approximately 0.25 μ mole of a substrate with specific activity approximately 1 μ C/ μ mole. Analytical thin-layer chromatography was used to determine the purity of fatty acid substrates, and preparative thin-layer chromatography was used to remove labeled hydrocarbon contaminants which were found in palmitate-9,10-¹⁴C and palmitate-16-¹⁴C. Care was taken to inject at a point lateral to the ventral nerve cord through the ventral conjunctival membrane between the metathorax and the first abdominal segment. No significant loss of substrate due to bleeding was observed. In experiments in which inhibitors were used, water solutions of these compounds were injected in the same manner 30 min prior to substrate injection.

Incubation of Injected Cockroaches

After injection, each cockroach was placed into a round-bottom flask, which was then covered with aluminum foil in which several

small holes had been made. The insect was then kept in this flask for 18 hr at room temperature in order to allow adequate circulation and metabolism of the substrates. Incubation time of 18 hr was chosen, since the total amount of radioactivity from several substrates incorporated into both the saturated and unsaturated hydrocarbons appeared to show no increase after this time (Fig. 8).

Extraction of Surface Lipids

After the incubation period, about 150 ml of hexane was poured into each flask. The hexane both killed the roaches and extracted their surface lipids. A time study (Fig. 9) showed that after a period of 30 min in hexane almost all of the surface hydrocarbons had been extracted from the roaches. Therefore an extraction time of 30 to 35 min, during which time essentially all of the surface hydrocarbons and very little internal (non-hydrocarbon) lipid was recovered, was used in all experiments.

Chromatographic Purification of Hydrocarbon Fractions

Hexane was removed from the sample by rotary evaporation under aspirator vacuum. The residue was transferred to a Pasteur pipette column containing 50 mg of column silica gel (Adsorbosil 100-140 mesh) and eluted into a vial with 7 ml hexane. The column retained the polar lipids and allowed all hydrocarbons (1.0 to 1.7 mg per insect. Cf. Jackson (29)) to pass through together in the hexane (Fig. 10A).

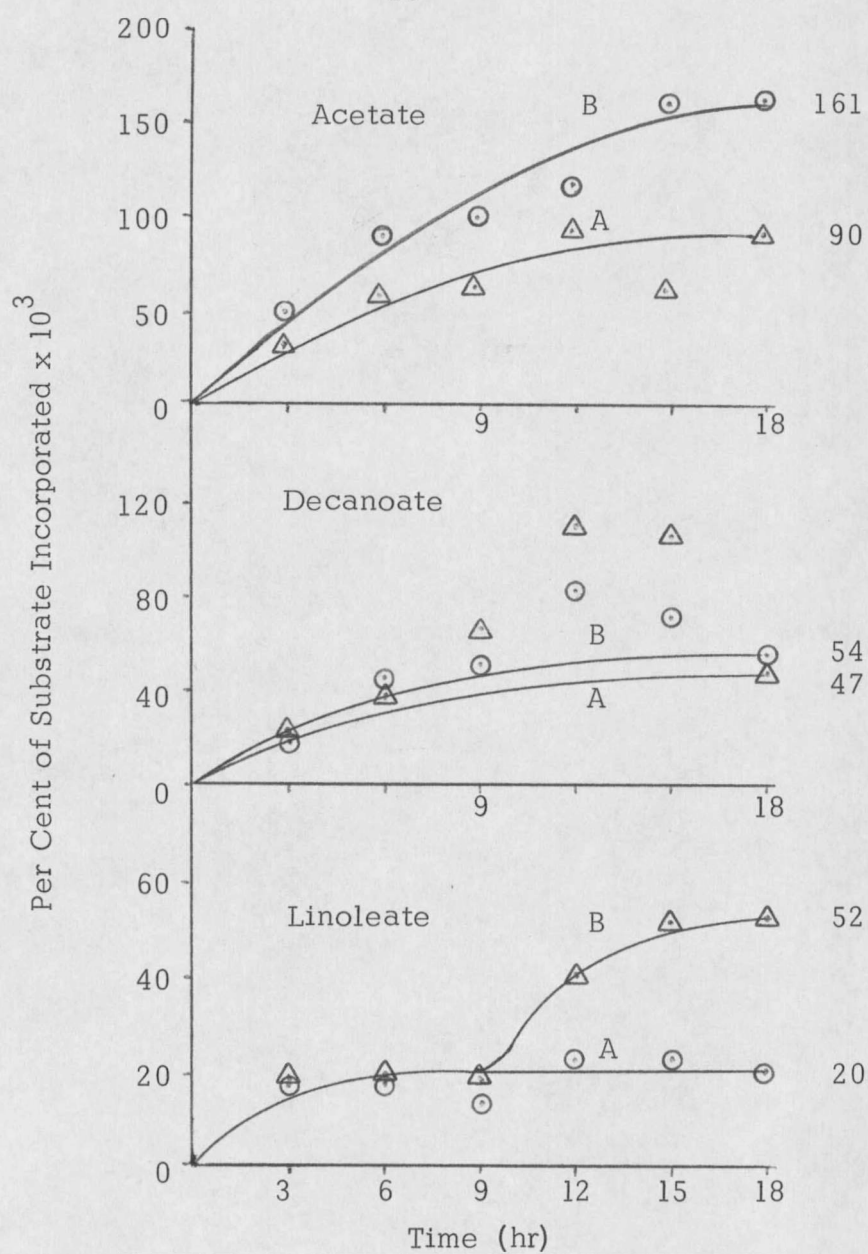


FIG. 8. Time-course of incorporation of acetate-1-¹⁴C, decanoate-1-¹⁴C, and linoleate-1-¹⁴C into saturated (A) and unsaturated (B) hydrocarbons of male *P. americana*.

PERMANENT RECORD

SOUTHWORTH, SC. U.S.A.

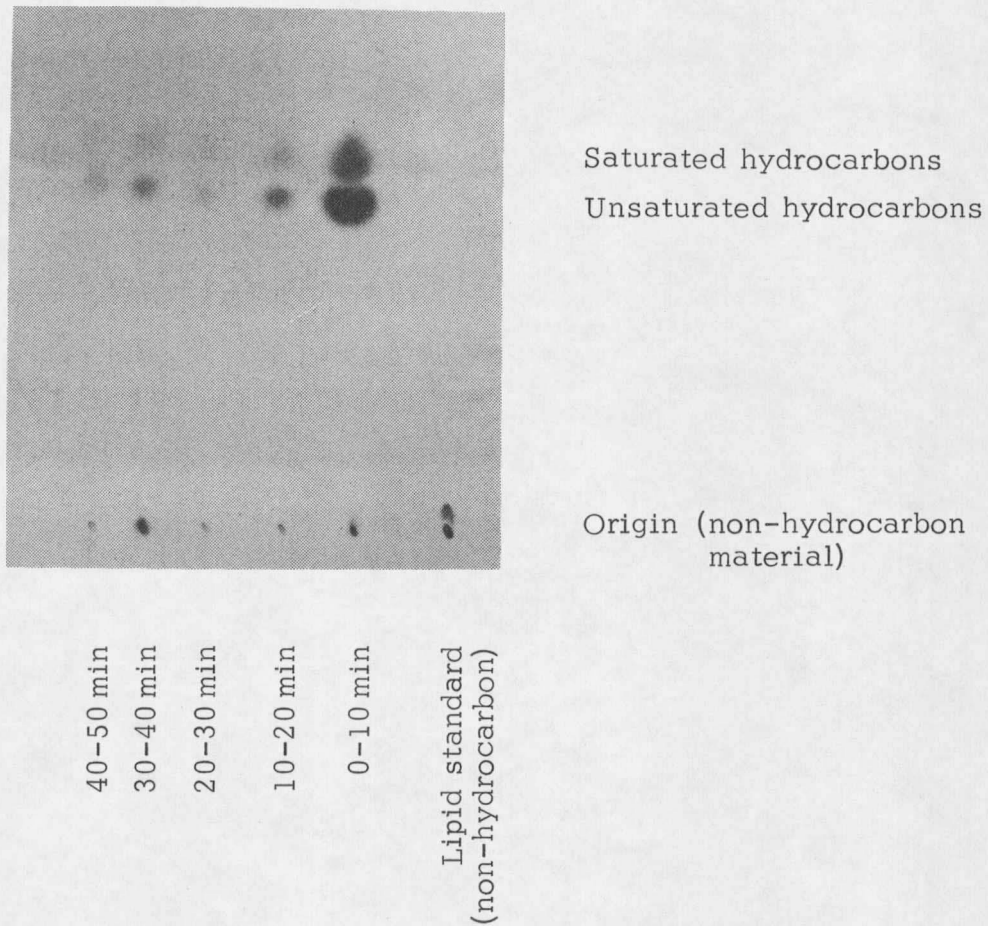


FIG. 9. Analytical thin-layer plate of lipids extracted with hexane from the surface of female *P. americana*. Five roaches were serially extracted for ten-minute periods. Plate is silica (Adsorbosil-3) developed in hexane and charred after spraying with a solution of chromic and sulfuric acids.

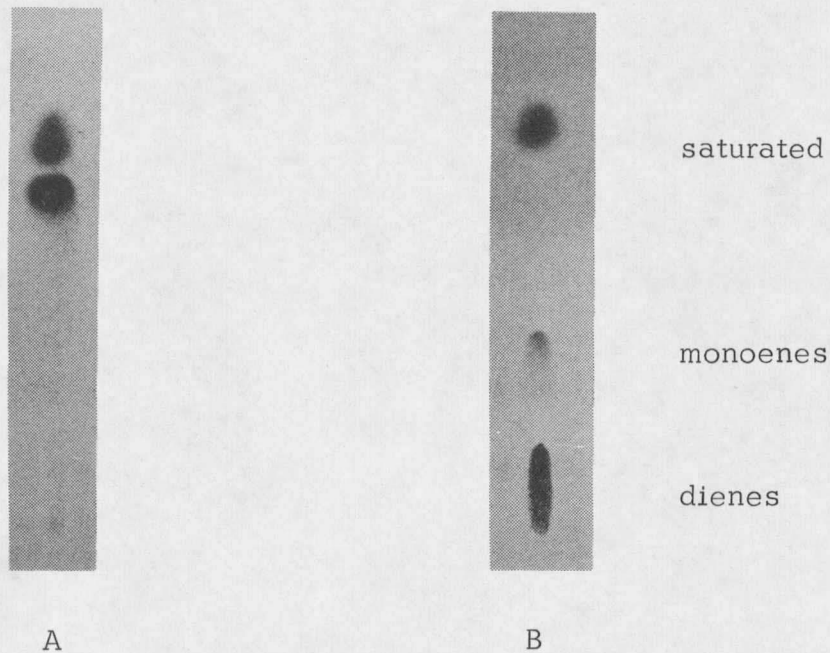


FIG. 10. Analytical thin-layer plate of hydrocarbons from the surface of *P. americana* after separation from other surface lipids on a silica gel (Adsorbosil 100-140 mesh) column. Chromatographic conditions were the same as in Fig. 9. Sample B was sprayed with a solution of silver nitrate in ethanol before developing the plate.

Hexane was evaporated under a nitrogen sweep. The total hydrocarbon sample was transferred with a small amount of hexane to a silica gel (Adsorbosil 3 impregnated with silver nitrate) thin-layer plate, on which it was preparatively chromatographed with hexane as the developing solvent (24,64). The hydrocarbons were thus separated into three fractions: saturated, monounsaturated, and diunsaturated (from top of plate) (Fig. 10B). Separation of fractions was visible under ultraviolet light. The silica had been impregnated with rhodamine 6-G in order to facilitate visibility.

Each fraction was then scraped into a medium porosity sintered glass Büchner funnel and eluted from the silica gel with 100 ml toluene. After rotary evaporation of the toluene, each sample was transferred to a liquid scintillation vial with hexane, which was then evaporated off under a nitrogen sweep. In order to separate normal from branched saturated hydrocarbons, the saturated fraction was then dissolved in a highly-branched solvent, 2,2,4-trimethylpentane, and shaken with Linde molecular sieve 5A for 3 hr. Straight-chain compounds passed into the sieve, while branched compounds, including the solvent, were excluded (65). This solution containing branched hydrocarbons only was transferred to separate counting vials, leaving the sieve containing normal hydrocarbons in the original vial. The 2,2,4-trimethylpentane was evaporated off under nitrogen from the vial containing the branched

hydrocarbon fraction. Figure 11 is a reproduction of gas chromatograms obtained in analysis of the contents of these hydrocarbon fractions.

Analysis of Internal Lipid Fractions

After hexane extraction of surface lipids, roach carcasses were homogenized in a motorized mortar and pestle in chloroform. A few drops of hydrochloric acid were added in order to ensure that all fatty acids were in the soluble free acid form, rather than in the salt form, which is insoluble in chloroform. After removal of particulate material by filtration through glass wool, the chloroform was removed by rotary evaporation. The residue was transferred to a vial with chloroform which subsequently was removed by evaporation under nitrogen. A small portion of the sample was transferred with chloroform to a thin-layer plate. The plate was eluted first with hexane to separate hydrocarbons, which moved to the top of the plate in hexane, from polar compounds, which remained near the origin. Next, the plate was eluted with hexane: diethyl ether: acetic acid (85:15:1, v/v/v) in order to separate the polar fractions. Three fractions were scraped from the plate: hydrocarbons, glycerides, and other esters, and origin, which contained free fatty acids and other highly polar lipids. The compounds were eluted from the silica gel with toluene, which in turn was removed by rotary evaporation. Samples were transferred to liquid scintillation vials in the same manner as surface hydrocarbon fractions.

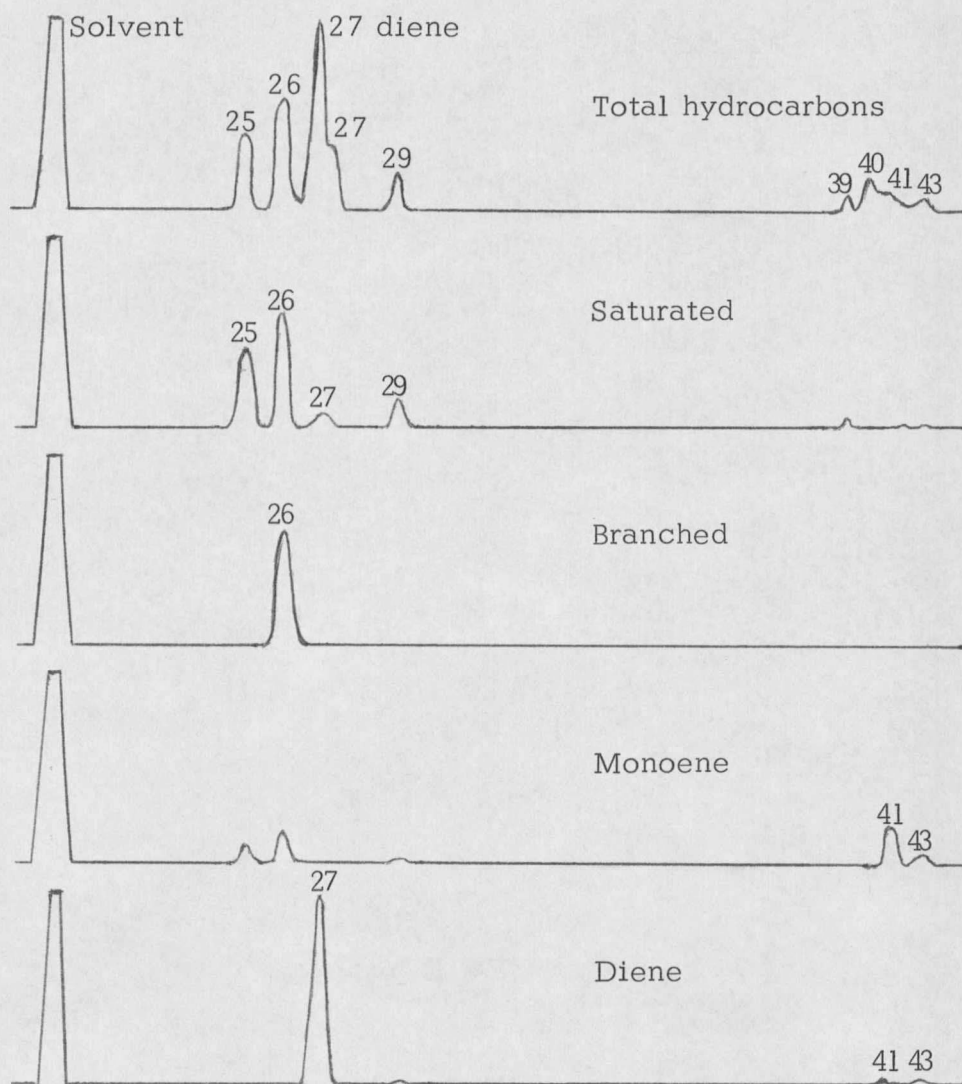


FIG. 11. Gas chromatographic analysis of hydrocarbon fractions from a surface extract of *P. americana*. Complete assignments of structure have been done by other workers (26,29).

Liquid Scintillation Counting of Lipid Fractions

A 15-ml aliquot of fluor solution (0.4% PPO in toluene) was added to each of the counting vials containing lipid fractions. Counting for 10 min was done in the ^{14}C channel of the Beckman LS-100 liquid scintillation counting system. By external standard calibration, counting efficiency was determined to be about 90%. Since direct counting of samples contained in molecular sieve did not give constant efficiency, the counting rates for fractions of normal hydrocarbons were determined by subtracting the counts in the branched fraction from the total number of counts in the saturated hydrocarbon fractions. Data have been converted to the fraction of administered substrate radioactivity incorporated into lipid fractions. Since the resulting numbers were very small, they have been multiplied by 10^3 to convert them to whole numbers for presentation in the Results section.

Oxidative Degradation of Branched Saturated Hydrocarbons

In order to determine the position of incorporation of the radioactivity from propionate into the branched saturated hydrocarbons, oxidative degradation of the branched fraction was performed (25,66). The sample (0.3 mg) was oxidized with 7 mg chromic anhydride (CrO_3) in 0.5 ml glacial acetic acid in a test tube for 4 hr at 65 C. Acetic acid was removed by evaporation under a stream of nitrogen. Methylation of the fatty acids produced was accomplished by refluxing the residue in 3 ml

of a 14% solution of boron trifluoride in methanol for 10 min on a water bath. After the solution had cooled, it was diluted with two volumes of water and washed with hexane three times, and the hexane solution was concentrated under nitrogen and transferred to a thin-layer plate, which was then eluted with hexane:diethyl ether:acetic acid (85:15:1). The silica gel was divided into two portions by a line drawn below the hydrocarbon spot; each portion was then scraped into a sintered glass funnel and the lipids extracted with toluene. Hydrocarbons and fatty acid methyl esters were identified by comparing their gas chromatograms with those of known standards.

RESULTS

Table I contains results of experiments in which palmitate-9,10-³H was incorporated into saturated and unsaturated hydrocarbons. No appreciable difference was observed between cell-free preparations and controls. The low radioactivity could have been due entirely to the presence of contaminating hydrocarbons in this substrate. These results indicate that the cell-free preparations were not capable of hydrocarbon biosynthesis. Therefore, these attempts to prepare a cell-free hydrocarbon biosynthetic system were abandoned in favor of in vivo experiments. Since Jackson and Baker (3) had reported that live cockroaches constituted a better system for biosynthesis of hydrocarbons than did excised integuments, living adult roaches were used in the remaining experiments.

TABLE I
Incorporation of Palmitate-9,10-³H into Hydrocarbons by Cell-free Preparations of Male P. americana and Buffer Controls

Preparation	Percent of Substrate Incorporated x 10 ³	
	Saturated	Unsaturated
Cell-free lyophilysate	12	11
Control	9	14

Integument Hydrocarbons of Teneral Cockroaches

It has been found previously (29) that hydrocarbons of the American cockroach comprise about 75 to 85% of the external lipids (1.7 to 2.6 mg per insect). In Table II data are given demonstrating that the total amount of hydrocarbon material on the cuticles of teneral and older adult roaches was approximately the same. Gas chromatographic analysis showed the hydrocarbon fractions of teneral adults to be qualitatively and quantitatively the same as those of older adults (Fig. 11).

TABLE II

Weights of Surface Hydrocarbons in Newly-molted and Two-month
Older Adult P. americana

Age	Male	Female
Teneral	1.40 mg	1.39 mg
Two-month	1.27 mg	1.65 mg

Incorporation of Labeled Substrates into Surface Hydrocarbons

Results of experiments concerning the incorporation of substrates into cuticular hydrocarbons of P. americana at various adult ages are shown in Table III. A wide variation was observed in the data (see Appendix) which were averaged to the values shown in the table. It did not seem reasonable, therefore, to attempt to observe subtle differences in incorporation of different substrates. However, large differences in these values were considered to represent true differences in metabolism.

Acetate was incorporated less into the four types of hydrocarbon structures in teneral cockroaches than in either group of older cockroaches. Although propionate also was not incorporated into hydrocarbons in teneral roaches, it was incorporated appreciably into the branched hydrocarbons in older adults. Neither isoleucine nor the S-methyl group of methionine seemed to be incorporated into branched hydrocarbons. Normal saturated fatty acids were incorporated into most hydrocarbon fractions in roaches of all ages tested, indicating that hydrocarbons were being formed continuously during adult life. In both groups of tanned adults, few major qualitative differences in the incorporation of substrates were observed. Stearate was well-incorporated into hydrocarbons in the teneral roaches. Linoleate was not incorporated into hydrocarbons in teneral cockroaches, while linoleate incorporation into diene hydrocarbons was high in the tanned adults.

TABLE III

Incorporation of Labeled Substrates into Surface Hydrocarbon Fractions
in Adult P. americana

Roaches were allowed to digest substrates for 18 hr after administration. Numbers represent percent of administered substrate incorporated $\times 10^3$. N = normal saturated hydrocarbons. B = branched saturated hydrocarbons. M = monoene hydrocarbons. D = diene hydrocarbons. t = less than 10.

Substrate	Adult time	Males				Females			
		N	B	M	D	N	B	M	D
2:0-1- ¹⁴ C (acetate)	0	t	t	t	22	19	t	12	30
	1-4 wk	35	54	47	113	66	74	29	188
	2+ mo	77	36	16	159	53	39	12	132
3:0-1- ¹⁴ C (propionate)	0	t	t	t	t	t	t	t	t
	1-4 wk	100	90	t	t	28	85	t	t
	2+ mo	33	138	t	t	18	56	t	t
8:0-1- ¹⁴ C (octanoate)	0	t	t	t	26	11	16	29	47
	1-4 wk	20	20	42	68	59	55	15	144
	2+ mo	73	55	28	92	74	31	12	60
10:0-1- ¹⁴ C (decanoate)	0	13	t	t	30	27	t	19	50
	1-4 wk	42	22	13	46	40	46	32	108
	2+ mo	26	53	14	35	46	30	t	62
12:0-1- ¹⁴ C (laurate)	0	t	16	t	15	24	t	20	54
	1-4 wk	78	25	20	38	81	65	43	98
	2+ mo	43	27	22	39	52	27	15	105

TABLE III (continued)

Substrate	Adult time	Males				Females			
		N	B	M	D	N	B	M	D
14:0-1- ¹⁴ C (myristate)	0	t	t	t	t	16	t	t	95
	1-4 wk	82	16	36	47	60	43	45	53
	2+ mo	29	13	15	19	51	20	15	64
16:0-1- ¹⁴ C (palmitate)	0	11	t	t	24	15	t	t	39
	1-4 wk	23	19	36	37	19	19	14	53
	2+ mo	24	t	17	21	16	t	t	24
16:0-16- ¹⁴ C (palmitate)	0	t	17	22	19	24	19	24	19
	1-4 wk	t	33	45	64	131	29	31	70
	2+ mo	85	15	25	26	85	33	23	46
18:0-1- ¹⁴ C (stearate)	0	48	47	23	40	58	36	29	36
	1-4 wk	t	16	25	17	34	t	11	38
	2+ mo	25	t	t	20	23	t	t	32
18:0-18- ¹⁴ C (stearate)	0	35	t	13	66	28	12	14	44
	1-4 wk	t	17	47	22	19	12	38	42
	2+ mo	41	t	t	29	68	t	t	56
18:2-1- ¹⁴ C (linoleate)	0	10	t	t	12	t	t	t	t
	1-4 wk	t	t	10	31	t	12	12	75
	2+ mo	28	14	t	71	30	21	11	136
Isoleucine-UL- ¹⁴ C	0	t	t	t	55	40	t	18	142
	1-4 wk	16	29	t	19				
Methionine-CH ₃ - ¹⁴ C	1-4 wk					t	t	t	t

Incorporation of Labeled Substrates into Internal Lipid Fractions

Teneral adult P. americana did not incorporate a significant proportion of the administered substrates into internal hydrocarbons, as shown by the data in Table IV. On the other hand, adult roaches of two months or longer incorporated a significant portion of labeled acetate and propionate into internal hydrocarbons. The pattern of incorporation of fatty acids into the other two fractions — esters, including glycerides, and origin, which contained fatty acids and phospholipids — was similar in some cases and different in others. In general, however, almost all of the label from fatty acid substrates remained in the fatty acid fractions.

Degradation of Branched Hydrocarbons

Oxidative degradation of branched hydrocarbons was attempted after injection of older roaches with propionate (25,66). Following this procedure, the hexane extract was chromatographed to separate hydrocarbons from fatty acid methyl esters. Other branched hydrocarbons, previously treated this way in our laboratory, were not found to be degraded (67). In this case, no spot other than the hydrocarbon fraction was identified by gas chromatography as having 26 carbons; no fatty acid methyl ester was identified when the extract of the lower portion of the plate was subjected to gas chromatography. Thus, this attempt to degrade branched hydrocarbons was unsuccessful.

TABLE IV

Incorporation of Labeled Substrates into Internal Lipids of
Adult Male *P. americana*

Numbers indicate percent of internal label incorporated into each fraction.

Substrate	Adult time	Hydrocarbons	Esters	Origin
2:0-1- ¹⁴ C	0	3	68	30
	2+ mo	25	34	41
3:0-1- ¹⁴ C	0	6	60	35
	2+ mo	40	46	14
8:0-1- ¹⁴ C	0	6	41	53
	2+ mo	6	21	73
10:0-1- ¹⁴ C	0	16	34	50
	2+ mo	3	51	46
12:0-1- ¹⁴ C	0	2	83	14
	2+ mo	2	96	2
14:0-1- ¹⁴ C	0	1	77	22
	2+ mo	1	46	53
16:0-1- ¹⁴ C	0	2	42	56
	2+ mo	2	76	22
16:0-16- ¹⁴ C	0	2	31	67
	2+ mo	1	3	96
18:0-1- ¹⁴ C	0	0	8	92
	2+ mo	0	36	64
18:0-18- ¹⁴ C	0	2	30	68
	2+ mo	6	93	1
18:2-1- ¹⁴ C	0	6	92	1
	2+ mo	1	98	2

Inhibition with Trichloroacetic Acid

Kolattukudy (48) has suggested that trichloroacetic acid (TCA) is an inhibitor of fatty acid elongation. When several different concentrations of TCA were administered to P. americana 30 min prior to injection with acetate-1-C¹⁴, hydrocarbons were labeled as shown in Table V. It was observed that TCA caused reduction of hydrocarbon biosynthesis to about half the normal level when concentrations of 8% TCA or less were used. When the level of 10% TCA was used, further inhibition was noted. Unfortunately, this concentration of inhibitor caused the death of all the roaches before the end of the 18-hour incubation period; some of the roaches which had been injected with only 5 to 8% TCA died within this period.

TABLE V

Trichloroacetate Inhibition of Acetate Incorporation into Hydrocarbons in Adult Male P. americana 1-4 Weeks after the Imaginal Ecdysis

TCA Solution (Per Cent)	TCA Solution (μ l)	Substrate Incorporated (Per Cent $\times 10^3$)	Inhibition (Per Cent)
0	10	264	--
2	10	138	48
5	10	140	47
6	10	138	48
8	10	178	33
10	10	106	60

Inhibition with Sodium Citrate

To eliminate the acetate effect, sodium citrate, a well-known inhibitor of β -oxidation (68), was injected prior to incubation with linoleate-1- ^{14}C . If the expected inhibition actually had occurred, a decreased incorporation of ^{14}C from linoleate into the saturated fractions relative to the diene fraction would have been observed. Treatment with citrate resulted in no reduction of this relative incorporation, as shown by the data in Table VI.

TABLE VI

Sodium Citrate Inhibition of Linoleate Incorporation into Hydrocarbons in Adult Male *P. americana* 1-4 Weeks after the Imaginal Ecdysis

Sodium Citrate Solution	Per Cent of Substrate Incorporated into Hydrocarbons $\times 10^3$		
	Saturated	Monoenes	Dienes
None	20	14	38
10 μ l 10%	27	12	26

Inhibition with Sodium Acetate

A further attempt to eliminate the acetate effect was made by using product inhibition of β -oxidation. Since acetate is the major end product of fatty acid catabolism, this was injected into roaches prior to incubation with substrates. In several experiments, acetate inhibition was not shown to alter the pattern of incorporation of substrates into hydrocarbons appreciably (Table VII).

TABLE VII

Inhibition by 2 M Sodium Acetate of Incorporation of Labeled Substrates into Hydrocarbons of Adult Male *P. americana* 1-4 Weeks after the Imaginal Ecdysis

Substrate	NaOAc' (μ l)	Per Cent of Substrate Incorporated into Hydrocarbons x 10^3				Total
		Normal	Branched	Monoene	Diene	
Malonate-1,3- 14 C	0	11	26	9	49	95
	10	8	22	17	66	113
	20	6	21	18	44	89
16:0-1- 14 C	0	22	19	35	31	107
	10	8	21	47	40	116
18:0-1- 14 C	0	6	16	25	19	66
	10	6	6	11	26	49

DISCUSSION

Several assumptions must be made before the results presented herein are discussed. First, it is assumed that most of the radioactivity from labeled substrates incorporated into each fraction was incorporated into the major component of that fraction. This is probably a valid assumption since a single compound comprises the bulk of each of the most abundant fractions. Next, it is assumed that the biosynthetic pathways for each hydrocarbon were the same in both male and female roaches. It is unlikely that different pathways have evolved by which members of each sex produce the same compounds in the same ratios. Finally, it is assumed that, since cockroaches seem incapable of synthesis of polyunsaturated fatty acids (36), linoleate is the only source of unsaturated hydrocarbon chain to be incorporated into the diene hydrocarbon fraction. It is noted that the distances of the double bonds from the terminal methyl end of linoleate are the same as in cis,cis-6,9-heptacosadiene.

During the period in the life cycle of P. americana 1-4 weeks after the imaginal ecdysis, metabolic changes occur related to the reproductive function of these insects (69). A higher rate of metabolism is known during this time than at other times in the life cycle. As a result, incorporation of fatty acid substrates into hydrocarbons was expected to be more pronounced in females than in males.

Incorporation of Acetate and Fatty Acids into Hydrocarbons

Acetate was incorporated into both integument and internal hydrocarbons of fully-tanned adult P. americana. This incorporation was not found in teneral adults. This suggests two possibilities: either acetate was not incorporated into precursors of hydrocarbons during the teneral period, or the precursors were not metabolized to form the hydrocarbons. On the other hand, many of the fatty acid substrates were incorporated into hydrocarbons almost as well during this teneral period as they were at later periods during the adult stage. This indicates that fatty acid synthesis was inhibited during the teneral period. It indicates further that, although hydrocarbons were formed to almost the same extent during the period immediately following ecdysis as later in adult life (as indicated both by the comparable amounts of cuticular hydrocarbons of both groups and by the incorporation of fatty acid substrates), this synthesis in the teneral period was not due to incorporation of acetate units in an elongation-decarboxylation pathway.

Incorporation of Linoleate into Diene Hydrocarbons

Teneral cockroaches were capable of synthesis of diene hydrocarbons from labeled normal saturated fatty acids, but did not incorporate the labeled carboxyl group of linoleate. In older adults, this carboxyl group of linoleate was incorporated well into dienes. It will be recalled that the positions of unsaturation in the major diene hydrocarbon

(cis,cis-6,9-heptacosadiene) of P. americana are the same as those in linoleate, counting from the methyl end. In older adult cockroaches, as well as in those in the teneral period, saturated fatty acids were also incorporated into dienes to a large extent.

It is possible to explain incorporation of linoleate by either elongation to form a 28-carbon fatty acid which was then decarboxylated to the 27-carbon hydrocarbon (elongation-decarboxylation pathway) or condensation of linoleate or one of its elongation products with a shorter, normal fatty acid to form the hydrocarbon. In teneral adults, however, there was neither incorporation of acetate (no elongation pathway) nor incorporation of the carboxyl carbon of linoleate. Thus, no hydrocarbon formation from linoleate during the teneral period could have occurred except by means of a condensation in which the carboxyl group of linoleate was lost.

Precursors of Branched Hydrocarbons

Propionate was incorporated into branched hydrocarbons in tanned adults, but not in teneral adults. Neither isoleucine nor the S-methyl group of methionine was incorporated into branched hydrocarbons in the groups of older adults. From these observations, it can be concluded that propionate probably was incorporated into fatty acids in tanned adults by a mechanism similar to that already mentioned (Fig. 3). However, since free branched fatty acids have not been reported in

P. americana (3), branched fatty acids, without release from hydrocarbon biosynthesis enzymes, then might have been converted to hydrocarbons by either the elongation-decarboxylation pathway or the condensation pathway. Alternatively, propionate could have been incorporated as the penultimate unit during elongation of a normal fatty acid (Fig. 12). To investigate this possibility degradation of branched hydrocarbons was attempted. Results from the amino acid experiments showed that incorporation of propionate was the only mechanism of synthesis of branched hydrocarbons observed. Therefore, it appears that branched hydrocarbons were not formed during the teneral period.

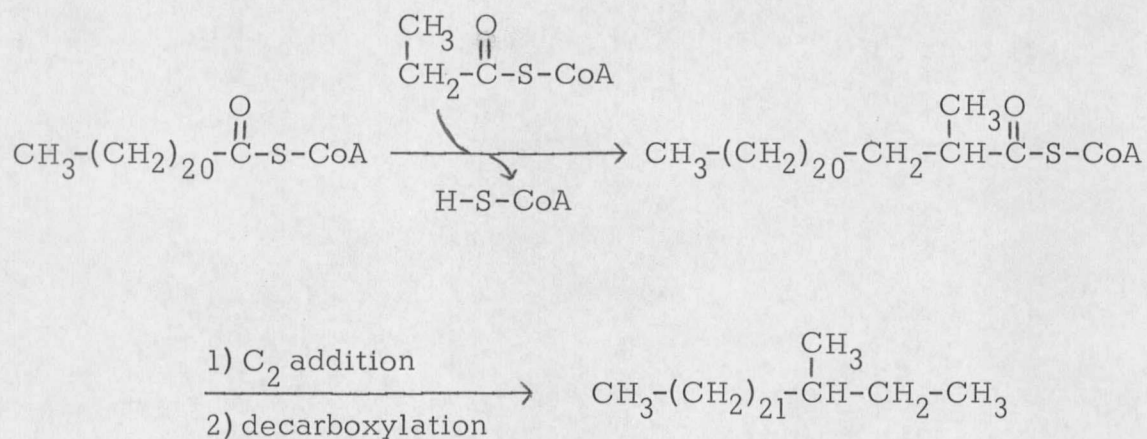


FIG. 12. Incorporation of propionate as the penultimate unit in elongation to form 3-methylpentacosane.

Pathways of Hydrocarbon Biosynthesis

It has been observed that acetate was not incorporated into hydrocarbons during the teneral period of adult P. americana, while fatty acid substrates were incorporated. This rules out elongation as a pathway of hydrocarbon biosynthesis, at least during the teneral period. Thus, the major pathway of hydrocarbon biosynthesis during this period must have been by way of condensation of fatty acids (Fig. 13). However, the data are inconclusive as to which of several possible condensations occurred.

Of the possible condensations, one in which linoleate condensed with decanoate, with loss of the carboxyl group from the linoleate, may have been responsible for formation of diene hydrocarbons. On the other hand, the carboxyl carbon of linoleate may have been incorporated in older roaches by elongation of the acid, followed by either condensation with another fatty acid or an elongation-decarboxylation pathway (Fig. 13). The lack of incorporation of the carboxyl carbon into the diene hydrocarbons of the teneral adult roaches suggests that a condensation had occurred (Fig. 14).

Other classes of hydrocarbons probably were formed also by condensation of fatty acids during the teneral period. Fatty acid substrates were incorporated into these other hydrocarbons in teneral cockroaches, although acetate was not. The large incorporation of stearate relative to other fatty acids suggests that it may have been the main fatty acid

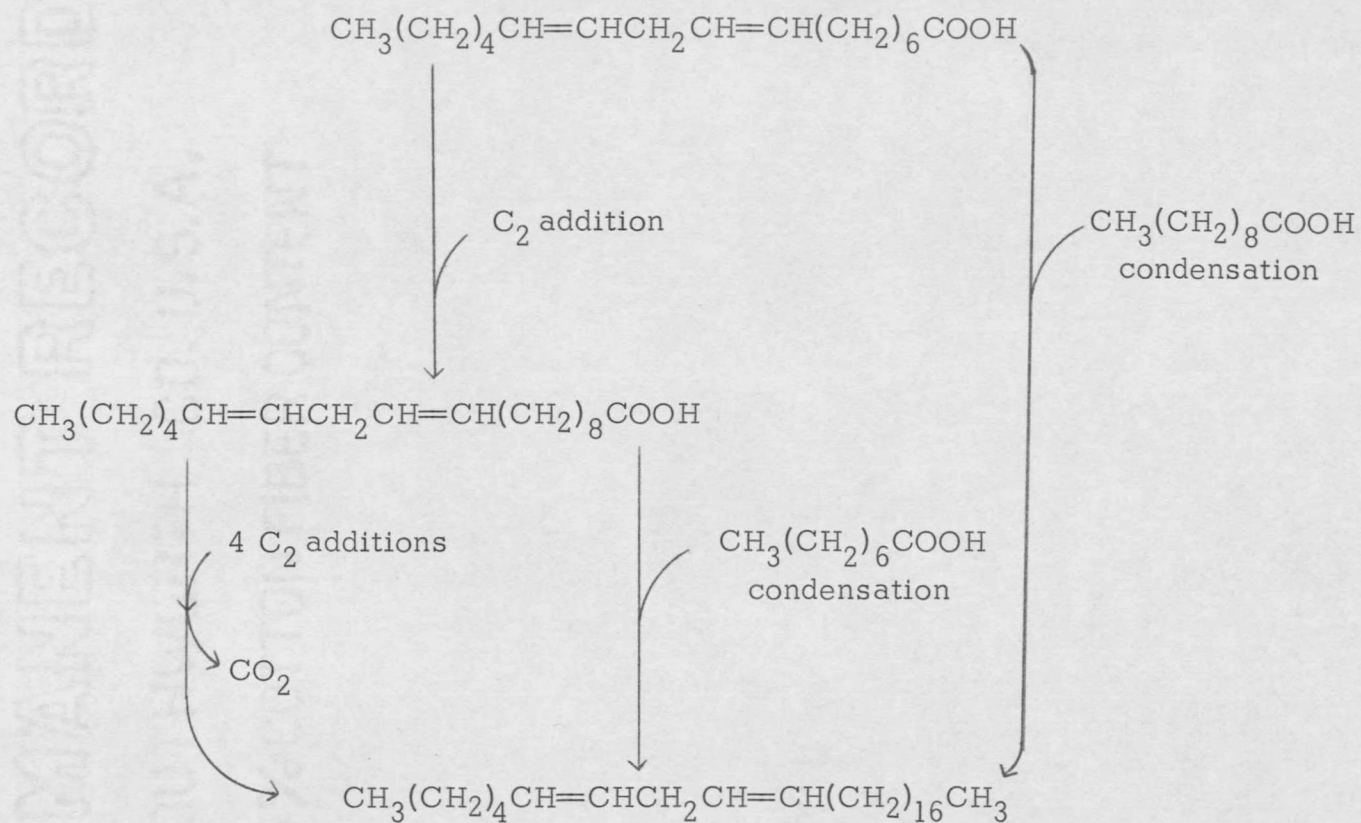


FIG. 13. Possible means of biosynthesis of diene hydrocarbons in fully-tanned adult P. americana.

