METABOLISM OF CHOLESTEROL -4-C^{14} IN RESISTANT AND SUSCEPTIBLE STRAINS OF ASEPTICALLY REARED HOUSE FLIES, MUSCA DOMESTICA L.

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JOHN WILLIAM BAUER
1968

ABSTRACT

METABOLISM OF CHOLESTEROL-4-C^{14} IN RESISTANT AND SUSCEPTIBLE STRAINS OF ASEPTICALLY REARED HOUSE FLIES, MUSCA DOMESTICA L.

By John William Bauer

Six different resistant and susceptible strains of houseflies, Musca domestica L., were reared aseptically on a synthetic diet containing cholesterol-4-C^{14} as the only sterol source. The uptake of cholesterol was different for all the strains, but there was no correlation of cholesterol content between the resistant and susceptible flies. The uptake of cholesterol by the females was higher than that for the males indicating a greater requirement for the females because of oögenesis. Esterification of the 3-β-hydroxyl group accounted for an average of 28% with the free sterol fraction making up approximately 71% of the total sterols. Small amounts of radioactive polar steroids were recovered, indicating at least to a small extent, the metabolism of cholesterol to other more polar derivatives. As expected no radiolabelled hydrocarbons were found, indicating a lack of degradation of the steroid ring nucleus. Analyses of the free and esterified sterols by column chromatography, gas-liquid chromatography, ultraviolet spectroscopy, and reverse isotope dilution demonstrated that unchanged cholesterol accounted for 96% of

these fractions. Analyses by column chromatography and reverse isotope dilution showed that 0.9% behaved like 7-dehydrocholesterol.

METABOLISM OF CHOLESTEROL-4-C^{14} IN RESISTANT AND SUSCEPTIBLE STRAINS OF ASEPTICALLY REARED HOUSE FLIES, *MUSCA DOMESTICA* L.

By

John William Bauer

A THESIS

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

MASTER OF SCIENCE

Department of Entomology

1968

ACKNOWLEDGMENTS

The author would like to express his gratitude and appreciation to Dr. R. E. Monroe for his guidance and counsel throughout the course of this investigation and to the members of the graduate committee Drs. R. A. Hoopingarner, M. J. Zabik, and L. L. Bieber.

Special thanks are expressed to my wife, Elizabeth, for her continually encouraging attitude and enthusiasm throughout the period of investigation.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The development of resistance by insects to insecticides poses most serious problems in their control, and the occurrence of such resistance is not new, as this phenomenon has been known for more than 50 years. Despite the research of many investigators, the actual mechanism(s) of pesticide resistance is still not known. Although many scientists have implicated potential mechanisms and have compared compounds and metabolites among different strains of insect species, they have not as yet found a sound physiological and biochemical basis for resistance. Because of the lipoidal-like nature of pesticides, the naturally occurring lipids of insects and other animals received much attention during early resistance studies. Unfortunately, such studies were usually gross in design and early instrumentation greatly curtailed the scientist's ability to study the finer details of these investigations. One such comparison (Enan et al., 1964) made among several pesticide resistant strains of adult houseflies implicated different levels and/or absorption rates of cholesterol in these insects. Their results showed major differences between the resistant and susceptible strains, with the resistant strains containing far less cholesterol than the one strain of susceptible flies studied. These early investigations,

however, were made without the use of aseptic synthetic methods and without modern instrumentation techniques, nor were the sterol levels studied in more than one susceptible strain of flies. This investigation, therefore, was concerned with the metabolism of cholesterol-4-C^{14} in different resistant and susceptible strains of aseptically reared houseflies, to determine if sterol metabolism or absorption rates actually enters into pesticide resistance per se or whether strains of flies differ widely even if they are resistant or not.

LITERATURE REVIEW

The importance of cholesterol and related steroids in insect nutrition was first demonstrated when Hobson (1935) showed that the larvae of the blowfly, Phaenicia sericata, required a dietary source of sterol. Lipke and Fraenkel (1959) further established that insects in general require a dietary sterol, and it is now recognized that insects do not possess the complete enzymatic system necessary for the synthesis of sterols from more simple compounds such as acetate and mevalonate. This characteristic is in direct contrast to most plants and animals which are able to synthesize their sterols from more simple compounds (Block, 1965). However, there are a few insects which have been reported to be capable of some degree of cholesterol biosynthesis, such as the silkworm (Saito et al., 1963), the silverfish, Ctenolepisma sp., (Clayton et al., 1962), and the German cockroach, Blattella germanica L., (Clayton, 1960), but Clayton (1964) attributes these syntheses to the activity of symbionts.

Though insects themselves are unable to synthesize sterols, these substances are vitally important for them, because as Clark and Block (1959) have shown, sterols serve a dual purpose. They have proposed that "sparing-sterols," which can replace up to 97% of the cholesterol

requirement of Dermestes maculatus, served in a structural capacity, and the remaining irreplaceable cholesterol served in a metabolic role possibly as the basic materials for some hormones that control essential life processes. Robbins (1963) has stated that every insect studied to date has been found to require a dietary or exogenous source of sterol for normal larval growth and metamorphosis. In addition to this, sterols have been reported to play a role in several other physiological processes in insects. Kobayashi, et al. (1962) has demonstrated that certain sterols have "brain hormone" activity, and Gilbert (1963) has shown that some exhibit "juvenile hormone" activity in insects. Ecdysone, from the silkworm, Bombyx mori L., has been shown to have a steroid structure (Karlson, et al., 1963). It has also been found that sterols are involved in the initiation of ovarian development (Robbins and Shortino, 1962) and are necessary for sustained viable egg production (Monroe, 1959, 1960).

For some functions the sterols may be utilized as such by the insect, but to satisfy other requirements they must first be converted into other sterols or steroids. Much work has been done recently on the mechanisms of these conversions as this may lead to the development of specific methods in insect control. According to Kaplanis et al. (1963), the house fly, Musca domestica L., is unable to convert β-sitosterol into cholesterol or

campesterol. This seems to be an exception because many other insects such as the oriental housefly (Levinson, 1962) and the German cockroach (Robbins et al., 1962) could convert β-sitosterol into cholesterol. Both Eurycotis floridana (Clayton and Edwards, 1962) and Blattella germanica L. (Louloudes et al., 1962) were able to convert cholestanol into Δ^7-cholestenol. Also cholesterol could be dehydrogenated at the 7-position (Robbins et al., 1964) by B. germanica L. The conversion of cholesterol into 7-dehydrocholesterol has also been observed in the housefly, M. domestica, by Kaplanis et al. (1960), Monroe (1964), and Monroe et al. (1967).

Several investigators have tried to synthesize cholesterol derivatives that could act as growth-inhibiting anti-metabolites to be used as possible insect controls. Clayton (1964) has come to the conclusion, however, that these cholesterol derivatives had no real inhibitory effects on the growth of insects. Some competitive inhibition was occasionally found, but this effect was usually completely reversed by cholesterol in normal concentrations. Earle et al. (1967), however, recently has shown a partial inhibition of growth in the boll weevil by two different azasterols even in the presence of cholesterol. Also, inhibition of growth by another diazacholesterol was found in the tobacco hornworm (Svoboda et al., 1967).

As mentioned above, Karlson (1963) found that the molting hormone ecdysone was a sterol, synthesized from cholesterol by the blowfly, Calliphora erythrocephala. Many investigators concluded that it was plausible that other hormones were also produced from cholesterol by insects. After administration of radioactive cholesterol to insects, several investigators such as Kaplanis et al. (1960), Monroe (1964), Monroe et al. (1967), Robbins et al. (1961), Ishii et al (1963), and Lasser et al. (1966) have always found a certain percentage of the radioactivity in the so-called polar fractions of some tissues. So far, the nature of these radioactive components is completely unknown.

MATERIALS AND METHODS

Experimental Insects

The houseflies (Musca domestica L.) used in these tests were obtained from the Entomology Research Division, U. S. Department of Agriculture, Corvallis, Oregon, and consisted of the following strains:

1. DDT resistant. Orlando DDT strain immune to DDT with major genes for DDT dehydrochlorinase and for knockdown resistance (Kdr).
2. Malathion resistant. The "Grothe" strain resistant to malathion with about 300-fold tolerance due to the altered ali-esterase factor for organophosphate resistance.
3. Parathion resistant. Parathion "classic wing" resistant to parathion with about 30-fold tolerance.
4. Sevin resistant. A strain immune to sevin due to a major 5th chromosomal factor for Sevin® tolerance and possibly additional factors.
5. Orlando susceptible. The Orlando regular strain completely susceptible to DDT, parathion, and dieldrin.

Another strain of flies employed in these studies was an insecticide-susceptible, maximum longevity strain obtained

from the Insect Physiology Laboratory, U. S. Department of Agriculture, Beltsville, Maryland.

Diets and Rearing Methods

Pupae from each strain were placed in a large screen cage for eclosion and the adults fed a 1:1 mixture of non-fat dry milk and confectioners sugar. The larvae were routinely reared by the CSMA procedure (Anon., 1959) and kept at 26±2° C. The test pupae were separated from the medium and 600 from each strain were placed into 50 ml beakers. Paper funnels with escape exits at the top were placed over the beakers to prevent the flies from having access to the puparial cases which have been found to contain sterols (Monroe, personal communication).

The adults were allowed to emerge in large screen cages and were supplied with 10 g of a synthetic diet (Monroe, 1960) every third day. The synthetic diet, as modified by Monroe and Lamb (1968), was composed of the following constituents:[1]

---

[1]The casein was obtained from Calbiochem, Los Angeles, California; the sucrose was obtained locally as granulated cane sugar; the sodium oleate and nucleic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and the cholesterol was a purified grade received from Fischer Scientific Co., Fair Lawn, New Jersey.

	Parts
Casein (sodium salt)[2]	47.0
Sucrose	47.0
Sodium oleate	2.0
Wesson's salts	4.0
RNA	0.1
Cholesterol (final concentration 0.1%)	
B-Vitamin mixture	

The vitamin mixture[3] used was as follows:

	Mg/100g diet
Thiamine hydrochloride	50.0
Riboflavin	25.0
Nicotinic acid	100.0
Calcium pantothenate	50.0
Pyridoxine hydrochloride	25.0
Folic acid	5.0
Choline chloride	1,000.0
Inositol	500.0
Biotin	1.0

The RNA, salts, sodium oleate, sucrose, and casein were ball-milled for 3-5 hours. The cholesterol was added as

---

[2]500 g of casein was mixed with 500 g of 1% NaOH in distilled water, dried for 2-4 days, cut into small pieces, and hammer-milled to pass a 0.009 mesh screen.

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

a dichloromethane solution and the mixture stirred occasionally until the solvent had evaporated. The vitamin mixture was dissolved in distilled water with the addition of 2-4 drops of concentrated ammonium hydroxide. Ten ml of the vitamin solution was added to 100 g of diet and ground in a mortar. The diet was then fan dried for approximately 20 minutes and subsequently reground until a fine homogeneous powder was obtained and the finished diet stored at -30° C until used. This adult synthetic diet was fed to the pretest flies in order to remove phytosterols from their tissues and thus allow finite studies with cholesterol only.

Distilled water was provided in 100 ml beakers with a thin piece of styrofoam floating on the surface to prevent drowning. Dead flies were removed from the cages daily to prevent cannibalism, which may serve as a source of nutrients (Ascher and Levinson, 1956).

Petri dishes containing muslin bags soaked in ammonium carbonate solution were placed into the pretest fly cages for oviposition. After 6-7 hours the dishes were removed, and the eggs placed into 50 ml Erlenmeyer flasks. The eggs were then surface sterilized for 20 minutes in 0.1% sodium hypochlorite. Using aseptic techniques, approximately 300 eggs were then transferred by a calibrated pipette to 250 ml Erlenmeyer flasks containing 7.5 g of a larval synthetic diet with 0.1%

cholesterol -4-C^{14}. This diet was similar to that reported by Monroe (1962) except that sodium oleate was omitted. The diet was composed of the following constituents:

Nutrients[4]	Parts
Casein	70.0
Alphacel	3.0
RNA	1.0
Wesson's salts	4.0
Agar	20.0
Cholesterol -4-C^{14}	0.1%
B-Vitamins	0.75 ml/7.5 g of diet

The casein, celluflour, ribose nucleic acid, Wesson's salts, and agar were ball-milled for 4-5 hours. The cholesterol -4-C^{14} was added to the dry diet as a dichloromethane solution. The solvent was then allowed to evaporate from the diet with periodical stirring and heating in an oven at 45° C. To each of the flasks was added the vitamin solution and 50 ml of distilled water and the mixture autoclaved at 15 pounds (121° C) pressure for 20 minutes.

After the eggs were transferred to the larval synthetic diets, the flasks were placed in an incubator at 34° C for 2 days, at which time most of the larvae were

---

[4]All nutrients from Nutritional Biochemicals Corp., Cleveland, Ohio, except the casein which was obtained from Calbiochem, Los Angeles, California and the cholesterol -4-C^{14} which was purchased from Nuclear Chicago, Des Plaines, Illinois.

in second or third instar. For the next four days the larvae were held at 26°±2° C, during which time they had pupated.

Each flask was forcefully filled with distilled water, swirled, and the contents poured into a No. 20 standard sieve. Agar and other debris was washed through the sieve by distilled water until only the pupae remained. The pupae were then placed onto folded paper towels until dry. The dry pupae were put into 50 ml beakers with paper funnel escape assemblys and placed into quart jars fitted with screen lids, and the adults allowed to emerge.

The adults were supplied sucrose and distilled water by placing sugar cubes and cotton moistened with distilled water in inverted paper cups on the tops of the screen lids. After three days the flies were anesthetized with CO_2, sexed, weighed live, placed in vials, and kept at -30° C until analysed.

All of these tests were conducted in triplicate.

Analytical Standards and Radiolabelled Cholesterol

The cholesterol (m.p. 149-150° C), cholesteryl acetate (m.p. 116-117° C), and cholestane (m.p. 80-81.5° C) were received from the Insect Physiology Laboratory, U. S. Department of Agriculture, Beltsville, Maryland. A Kofler block was used to determine melting points and the values recorded are uncorrected.

The 7-dehydrocholesterol (m.p. 143-144° C), (Nutritional Biochemicals Corp., Cleveland, Ohio) used was purified by successive recrystallizations from acetone, ethanol, methanol, acetone-ethanol (1:1), acetone-methanol (1:1), ethanol-methanol (1:1), and methanol. The 7-dehydrocholesteryl acetate (m.p. 130-131° C) was made by acetylating the 7-dehydrocholesterol. Both were kept *in vacuo* in the dark in a desiccator to minimize autodecomposition.

The cholesterol-4-C^{14} was obtained from Nuclear-Chicago in two 50 μC lots. The radiolabelled cholesterol was diluted with authentic unlabelled cholesterol for a final observable specific activity of 917 counts per minute per μg. Radiochemical purity was found to be 99% as determined by paper chromatography using a distilled water-*n*-propanol (2:3) system saturated with deobase (Kaplanis *et al.*, 1960).

Radioassays were made with a windowless gas-flow counter attached to a Baird Atomic scaling unit. The weightless samples were all prepared in triplicate and redistributed with 1 ml of *n*-hexane before being counted, and all samples were counted for a sufficient time to give a maximum standard error of ±5%.

Direct Extration of Lipids

The adult flies were homogenized with distilled water in an all-glass homogenizer. The homogenate was transferred

quantitatively into a 300 ml round-bottom flask. A volume of acetone-ethanol (1:1) at 4 times the volume of aqueous used in the homogenization was then added to the reaction flask and the mixture refluxed for 90 minutes (Kaplanis et al., 1960). The hot mixture was decanted into a Büchner funnel containing two circles of Whatman No. 1 filter paper and the supernatant was filtered into a vacuum filtering flask. Several rinses were made with small aliquots of acetone-ethanol (1:1) for the assurance of a complete and quantitative transfer. The residue from the filter paper was placed into a planchet and radioassayed as a quantitation check. The solvent pair was then removed in vacuo from the aqueous extract, and the remaining aqueous quantitatively transferred to a separatory funnel to which was added approximately 0.5 ml of concentrated hydrochloric acid. The aqueous mixture was then extracted 3 times with equal volumes of peroxide-free ethyl ether. The pooled ether fractions were back extracted with water until neutral and then after drying over anhydrous sodium sulfate, the ether was removed in vacuo. The residue (total lipids) was weighed and then assayed radiometrically for the total sterol(s). The aqueous of the ether extracts was also radioassayed as a quantitative check. The extracts were then frozen in benzene in a nitrogen atmosphere at -30° C until further analysis.

Column Chromatography

The total lipids were then fractionated by column chromatography on a 1.1 cm column of 7.5 g of neutral Woelm aluminum oxide, grade I, deactivated with 1.5% water (packed in n-hexane). One to 2 g of anhydrous sodium sulfate was also added to the top surface of the alumina. The total lipids were transferred quantitatively to the column in small aliquots of benzene, and then successively washed with 100 ml of benzene, ethyl ether, and methanol. The benzene fraction was evaporated *in vacuo* and added to a second column which had been prepared in the same manner as the first column, but washed with n-hexane, benzene, and methanol. After pooling the two methanol fractions, the 4 fractions consisting of n-hexane, benzene, ethyl ether, and methanol, which elute the hydrocarbons, sterol esters, free sterols, and more polar steroids, respectively, (Kaplanis *et al.*, 1960) were assayed radiometrically. The free sterols and sterol ester fractions were kept at -30° C in benzene in a nitrogen atmosphere until further analysis.

Acetylation and Saponification of Sterols

The free sterol fractions were pooled and acetylated as reported by Johnston *et al.* (1957) with some modifications. The sterols were transferred to a glass stoppered graduated centrifuge tube with a Pasteur pipette and the

solvent removed with a stream of dry nitrogen. Approximately 1 ml of acetic anhydride and pyridine (1:1) were added to the centrifuge tube. The tube was then flushed with nitrogen, stoppered tightly, and heated in a boiling water bath for 90 minutes. The tube was then removed from the water bath, and the solution quantitatively transferred to a separatory funnel with ice water (final volume 20 ml). The aqueous mixture was extracted 3 times with ethyl ether and the pooled ether fractions back extracted with 6.25% hydrochloric acid-ice water (1:1), water until neutral, 5% sodium bicarbonate solution, and water until neutral. The ether fractions were then dried over anhydrous sodium sulfate, the ether removed in vacuo, and the residue assayed radiometrically.

The sterol esters were subsequently saponified by transferring them quantitatively to a glass stoppered graduated centrifuge tube with a Pasteur pipette and the solvent removed with a stream of dry nitrogen. The volume of lipid was noted, and 0.5 ml of 10% potassium hydroxide in 95% ethanol was added per 0.1 ml of lipid. The tube was flushed with nitrogen, stoppered tightly, and heated in a boiling water bath for 90 minutes. The solution was then quantitatively transferred to a separatory funnel containing 5 ml of ice water. The centrifuge tube was rinsed with small aliquots of ice water bringing the total volume of water in the separatory funnel to approximately 20 ml. The resulting aqueous was then extracted repeatedly

with ethyl ether, the pooled ether fractions back extracted with water until neutral, dried over anhydrous sodium sulfate, the ether removed *in vacuo*, and the residue assayed radiometrically. After radioassay, the sterol ester fractions were acetylated as described above.

Separation of Sterols by Column Chromatography

The acetates of the free sterols and saponified steryl esters were subsequently analysed separately by column chromatography (Kaplanis *et al.*, 1960). A 1.1 cm water jacketed glass column was packed with 25 g of Woelm acid aluminum oxide, grade II, in *n*-hexane. The steryl acetates were added to the column with small aliquots of *n*-hexane until a quantitative transfer had been made. The column was then wrapped with a heating coil so that the temperature of the column remained at 29° C to 31° C. The column was also wrapped in aluminum foil to prevent light from decomposing the $\Delta^{5,7}$-conjugated dienes. The column was eluted with 3 L of *n*-hexane-benzene (95:5), and 20 ml fractions were collected on an automatic fraction collector. After all of the *n*-hexane-benzene had passed through the column, 50 ml of methanol was added and collected separately. The methanol was then reduced *in vacuo* and the residue assayed radiometrically. From each of the 20 ml fractions, 200 µl aliquots were pipetted into liquid scintillation vials and radioassayed with a Nuclear-

Chicago Model 6850 "Unilux 1", an ambient temperature liquid scintillation spectrometer. Before the fluor mixture was added to the vials, the 200 µl aliquots were evaporated so that there would be no difference in quenching. The fluor mixture used (5 ml per vial) was composed of 4 g of PPO (2, 5 diphenyloxazole) and 0.05 g of POPOP (p-bis [2-(5-phenyloxazolyl)] -benzene) per liter of reagent grade toluene. Two peaks of radioactivity were observed that corresponded to two major groups of compounds which this column separates: Δ^5-steryl acetate (as cholesteryl acetate) and $\Delta^{5,7}$-steryl acetate (as 7-dehydrocholesteryl acetate). The fractions corresponding to the two peaks were pooled separately, and then radioassayed on the windowless gas-flow counter to determine total activity. These two major groups eluted from the column were frozen in benzene in a nitrogen atmosphere, at -30° C and designated as Δ^5- and $\Delta^{5,7}$-steryl acetates.

Sterol Purification

The Δ^5- and $\Delta^{5,7}$-steryl acetate fractions were saponified separately using the method described above and then purified by digitonin precipitation (Sperry and Webb, 1950). The Δ^5- and $\Delta^{5,7}$-steryl acetates were each transferred to centrifuge tubes, and the solvent removed with a stream of dry nitrogen. To each tube, 1-2 ml of acetone-ethanol (1:1) was added and heated until the residue

dissolved. Sufficient digitonin[5] to precipitate the sterols present was added as a 1% solution in 85% ethanol. The digitonin solution was added hot with a Pasteur pipette to insure mixing. The solution was stirred and then held for 3 hours in a 45° C water bath. Both tubes were removed from the bath, the sides were rinsed down with 85% ethanol, then centrifuged at 1200 rpm for 10 minutes. The supernatant of each tube was removed with a Pasteur pipette, and 3-4 ml of acetone-ethanol (1:1) was added and the mixture thoroughly stirred. The tubes were again centrifuged for 10 minutes at 1200 rpm, the supernatant removed, and the washing process repeated with acetone-ether (1:1) and finally with ether. The washed digitonin-sterol precipitates were then suspended in 3-4 ml of ether and the ether removed with a fine stream of dry nitrogen in a water bath in order to coat the precipitate on the walls of the tubes. The sterol digitonides were then broken by dissolving the precipitate in 1-2 ml of pyridine and holding the mixture overnight at room temperature. The pyridine was removed with nitrogen to a volume of 0.2-0.3 ml. Both tubes were stirred thoroughly after the addition of 4 ml of ether and centrifuged at 1200 rpm for 5 minutes. This step was repeated 3 times and each ether fraction was pooled in another centrifuge tube. The free purified sterols were then coated on the

[5]The digitonin required was 3.1 times the weight of sterols plus an excess of 20%.

walls of the tubes by evaporating the ether with a stream of dry nitrogen. Both the Δ^5- and $\Delta^{5,7}$-sterol fractions were then reacetylated using the same procedure as described above.

Sterol Identification

Analyses of the Δ^5- and $\Delta^{5,7}$-steryl acetates were made by gas-liquid chromatography on a Research Specialties "600 Series" gas chromatograph equipped with a hydrogen flame detector and employing three different columns (VandenHeuvel et al., 1961). Stainless steel columns, 4 ft x 7 mm (i.d.), were packed with a solid support of 100-120 mesh, silanized base-and acid-washed Gas Chrom P [6] coated with one of the following liquid phases: 3% SE-30, 1% QF-1, or 0.75% neopentyl glycol succinate. The SE-30 column was operated at 238° C, with the vaporizer at 214° C and a hydrogen flow rate of 60 ml per minute and air at 160 ml per minute. The flow rate for nitrogen, the carrier gas, was 125 ml per minute. The QF-1 column was operated at 218° C, with the vaporizer at 272° C and a hydrogen flow rate of 60 ml per minute and the air at 160 ml per minute. The flow rate for nitrogen was 127.7 ml per minute. The neopentyl glycol succinate column was operated at 230° C, with the vaporizer at 280° C and a hydrogen and air flow rate of 60 and 160 ml per minute, respectively. The flow

[6] Gas Chrom P obtained from Applied Science Laboratory, State College, Pennsylvania.

rate for nitrogen was 113.2 ml per minute. Both sterol fractions were diluted with benzene so that all samples injected were composed of 4 µg of steryl acetate in 1 µl of benzene. Three µl (12µg) of the samples were injected by a microliter syringe. All retention times were calculated relative to the retention time of cholestane, and peaks were identified by comparison of these retention times with those of authentic standards.

Further analyses of the steryl acetates were made by ultraviolet spectroscopy and compared with spectra of equal concentrations of cholesteryl acetate and 7-dehydrocholesteryl acetate standards. The samples were dissolved in absolute ethanol and the mixture placed in silica cuvettes. Analysis was made on a Beckman DB recording spectrophotometer.

The radiolabelled Δ^5- and $\Delta^{5,7}$-steryl acetates were admixed with 100 and 25 mg of authentic non-radioactive cholesteryl and 7-dehydrocholesteryl acetate, respectively, for reverse isotope dilution analysis. To each of the flasks, sufficient n-hexane was added to solubilize the mixtures and then evaporated to dryness. Tares were taken on two 5 ml volumetric flasks, and 10 mg of the labeled-unlabeled mixtures were added individually to the flasks. The flasks were then filled to the mark with benzene, and subsequently radioassayed. The mixtures were then returned to the flasks from which they came and the total mixtures now

transferred to small beakers with a Pasteur pipette. The benzene solvent was removed from the beakers with a stream of dry nitrogen. The beakers were then filled half full with acetone and brought to a boil on a hot plate. After total solubilization had taken place, the beakers were removed from the hot plate and placed in the freezer. As the mixtures cooled, the beakers were occasionally swirled until a good crop of crystals had formed. The crystals were collected in a sintered glass funnel under vacuum. When absolutely dry, 10 mg from each funnel were placed into the original 5 ml volumetric flasks, and the entire sequence was then repeated as described above. The mixtures were repeatedly recrystallized from ethanol (twice), methanol (twice), and ethanol-methanol (1:1, twice). After every recrystallization, radioassays were conducted for observable specific activity, and the results recorded as counts per minute per mg of steryl acetate.

RESULTS

Direct Extraction of Lipids and Sterols

The average weight per fly for all strains investigated is shown in Table 1.

TABLE 1.--Weights of resistant and susceptible strains of adult male and female houseflies fed cholesterol-4-C^{14} in a larval aseptic synthetic diet.

Fly strain	Number of flies		Weight (g)		Average weight/fly (mg)	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Beltsville susc.	277	296	2.9329	3.8846	10.6	13.1
Orlando susc.	37	69	0.4198	0.9047	11.3	13.1
Malathion resis.	172	210	1.8753	2.6124	10.9	12.4
Sevin resis.	158	193	1.8298	2.6592	11.6	13.8
DDT resis.	165	155	1.4610	1.8417	8.9	11.9
Parathion resis.	36	28	0.3828	0.4155	10.6	14.8

No significant difference in weight between the resistant and susceptible strains was observed. In all strains, the females were approximately 2.5 mg heavier than the males. The weight difference was lowest in the malathion resistant strain at 1.5 mg and highest in the parathion

resistant strain at 4.2 mg with the other two resistant and two susceptible strains falling between these two figures.

Following direct extraction of lipids, the average weight of lipids per fly for each strain was calculated (Table 2).

All strains showed approximately the same amount of lipid per mg of fly, ranging from 0.033 mg for the Sevin® resistant females to 0.057 mg for the parathion resistant males. The males in all the strains except for the malathion resistant flies contained slightly higher amounts of lipids per body weight than the corresponding females. The males of the malathion resistant strain contained 0.01 mg per mg of body weight less than the females of that strain.

The sterol content of the flies as determined radiometrically is shown in Table 3.

The DDT resistant strain had the highest radiolabelled sterol content at 0.66 µg per mg of body weight for the males and 0.71 µg per mg of body weight for the females. The Orlando susceptible strain had the lowest sterol content at 0.40 µg and 0.46 µg for the males and females, respectively. The sterol content for all the other strains was between the DDT resistant and Orlando susceptible strains. In all the strains, with the exception of the parathion resistant strain, the females

TABLE 2.--Total weight of lipids extracted from resistant and susceptible strains of adult male and female houseflies fed cholesterol-4-C^{14} in a larval aseptic synthetic diet.

Fly strain	Number of flies		Total lipids (mg)		Average weight of lipids (mg) per fly		Average weight of lipids (mg) per mg body weight	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Beltsville susc.	277	296	160.3	179.5	0.58	0.61	0.055	0.047
Orlando susc.	37	69	22.8	36.0	0.62	0.52	0.055	0.040
Malathion resis.	172	210	68.0	98.9	0.40	0.47	0.037	0.038
Sevin resis.	158	193	79.8	89.1	0.51	0.46	0.044	0.033
DDT resis.	165	155	61.4	35.8	0.37	0.43	0.042	0.036
Parathion resis.	36	28	21.6	18.7	0.60	0.67	0.057	0.045

contained a significantly higher amount of sterol per mg of body weight than did the corresponding males.

TABLE 3.--Radiolabelled sterol content of resistant and susceptible strains of adult male and female houseflies fed cholosterol-4-C^{14} in a larval aseptic synthetic diet.

Fly strain	µg-equivalent of cholesterol-4-C^{14}			
	Per insect		Per mg body weight	
	♂♂	♀♀	♂♂	♀♀
Beltsville susc.	6.8	9.3	0.65	0.69
Orlando susc.	4.5	6.0	0.40	0.46
Malathion resis.	4.5	8.7	0.40	0.70
Sevin resis.	7.0	9.6	0.60	0.69
DDT resis.	5.3	7.2	0.66	0.71
Parathion resis.	6.7	8.9	0.63	0.60

Composition of Radiolabelled Sterols

Results of column chromatographic analyses of total C^{14}-compounds extracted from the resistant and susceptible strains of houseflies are presented in Table 4.

Most of the radioactivity was eluted in the sterol ester and the free sterol fractions with a small amount in the polar steroid fraction.

TABLE 4.--Fractionation of total C^{14}-compounds from males and females of resistant and susceptible strains of houseflies reared on aseptic synthetic diets containing cholesterol-4-C^{14}.

Fly strain	Per cent of radioactive compounds							
	Hydrocarbons		Sterol esters		Free sterols		Polar steroids	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Beltsville susc.	0	0	20.5	27.4	78.4	71.3	1.1	1.3
Orlando susc.	0	0	38.1	28.3	60.1	70.2	1.8	1.5
Malathion resis.	0	0	25.4	27.3	73.2	71.6	1.4	1.1
Sevin resis.	0	0	23.3	30.4	75.5	68.5	1.2	1.1
DDT resis.	0	0	29.7	33.4	69.3	64.7	1.0	1.9
Parathion resis.	0	0	24.8	23.6	74.2	75.5	1.0	0.9

The n-hexane fraction, which elutes the hydrocarbons, contained no radioactivity, indicating an absence of degradation of the steroid ring nucleus.

Esterification of sterols ranged from a low of 20.5% in Beltsville susceptible males to a high of 38.1% in Orlando susceptible males.

The concentrations of free sterols were inversely related to sterol ester concentrations, and consequently the Beltsville susceptible males had a high of 78.4% and the Orlando susceptible males had a low of 60.1%. Therefore, in all the strains, the sterol ester concentrations ranged from approximately 1/4 to 2/3 of the free sterol concentrations.

The methanol fraction, which elutes the more polar steroids, was less than 2% in all the strains, ranging from 0.9% in parathion resistant females to 1.8% in Orlando susceptible males.

Separation of Radiolabelled Sterols

When the free sterols were acetylated and the sterol esters saponified and then acetylated and subsequently fractionated by column chromatography, two major radioactive peaks were eluted. The first peak behaved as authentic cholesteryl acetate (Δ^5-steryl acetate peak), and the second peak behaved as pure 7-dehydrocholesteryl acetate ($\Delta^{5,7}$-steryl acetate peak). After the removal of these two compounds, the more polar steroids were

eluted with methanol. The relative percentages of the Δ^5- and $\Delta^{5,7}$-sterols found in the resistant and susceptible strains of houseflies are shown in Table 5.

Free and esterified sterol fractions had nearly equal amounts of Δ^5- and $\Delta^{5,7}$-steryl acetates in all the strains studied.

The Δ^5 fraction was the major compound in all the strains, ranging from 98.3% in DDT resistant females to 94.5% in Beltsville susceptible females.

Very small amounts of $\Delta^{5,7}$-steryl acetates were found in all strains with 0.1% in the Beltsville susceptible males to 1.7% in Orlando susceptible females.

The polar steroids were slightly more predominant than the $\Delta^{5,7}$-steryl acetates with an average of 2.7% of the radioactive compounds.

Identification of Radiolabelled Sterols

The Δ^5- and $\Delta^{5,7}$-steryl acetate fractions were pooled, saponified, purified by digitonin precipitation, reacetylated, and analysed by gas-liquid chromotography (Table 6).

When the SE-30 column was employed, the relative retention time of the Δ^5-steryl acetate fractions was identical with that of pure cholesteryl acetate (2.9 minutes). When the QF-1 and the NGS columns were employed, the relative retention times of the Δ^5-steryl acetate

TABLE 5.--Fractionation of the acetylated free and sterol ester fractions from males and females of resistant and susceptible strains of houseflies reared on an aseptic synthetic diet containing cholesterol-4-C^{14}.

	Per cent of radioactive compounds											
	Free sterols						Sterol esters					
	Δ^5		$\Delta^{5,7}$		Polars		Δ^5		$\Delta^{5,7}$		Polars	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Beltsville susc.	98.0	97.2	0.1	0.2	1.9	2.6	95.0	94.5	0.9	0.4	4.0	5.1
Orlando susc.	97.1	95.8	0.8	0.9	2.1	3.3	95.3	96.0	1.6	1.7	3.1	2.3
Malathion resis.	98.2	97.3	0.3	0.4	1.5	2.3	95.7	97.7	1.6	0.6	2.7	1.7
Sevin resis.	98.1	96.7	0.2	0.5	1.7	2.8	95.4	----	0.7	---	3.9	---
DDT resis.	97.4	98.3	0.3	0.3	2.3	1.4	98.1	96.8	0.6	0.9	1.3	2.3
Parathion resis.	95.8	94.8	0.7	0.9	3.5	4.3	96.8	95.9	1.4	1.0	1.8	3.1

fractions were again identical with those of pure cholesteryl acetate (5.1 and 6.3 minutes, respectively).

TABLE 6.--Results of gas-liquid chromatographic analyses of pooled steryl acetate fractions from resistant and susceptible strains of houseflies reared on an aseptic synthetic diet containing cholesterol-4-C^{14}.

Compound	Relative retention time to cholestane		
	SE-30	QF-1	NGS
Cholesteryl acetate	2.9	5.1	6.3
Δ^5-steryl acetate from houseflies	2.9	5.1	6.3
7-Dehydrocholesteryl acetate	3.2	5.7	8.2
$\Delta^{5,7}$-steryl acetate from houseflies	---	---	---

The $\Delta^{5,7}$-steryl acetate fraction was very small and contained an unknown viscous substance which when injected into the chromatograph, yielded many breakdown products which masked the $\Delta^{5,7}$ peak.

The Δ^5-steryl acetate fraction was dissolved in absolute ethanol, placed in silica cuvettes, and analysed by ultraviolet spectroscopy. An ultraviolet spectrum was obtained with an equal concentration of pure cholesteryl acetate for comparison, which gave a single peak at 261 mμ. The spectrum for the pooled Δ^5-steryl acetate

was identical with that of the pure cholesteryl acetate (261 mμ). The yellow viscous substance in the $\Delta^{5,7}$-steryl acetate fraction that masked the results during gas-liquid chromatography also obscured the four major absorption peaks found in the pure 7-dehydrocholesteryl acetate (262, 272, 282, and 294 mμ).

The Δ^{5}- and $\Delta^{5,7}$-steryl acetate fractions were analysed by reverse isotope dilution (Table 7).

TABLE 7.--Reverse isotope dilution and recrystallization of the pooled Δ^{5}- and $\Delta^{5,7}$-steryl acetate fractions from resistant and susceptible strains of houseflies reared on an aseptic synthetic diet containing cholesterol-4-C^{14}.

Procedure	Observable specific activity counts/min per mg	
	Δ^{5}	$\Delta^{5,7}$
Dilution with cholesteryl acetate	53,460	---
Dilution with 7-dehydrocholesteryl acetate	---	320
Recrystallization from:		
(1) Acetone	53,440	160
(2) Ethanol	53,240	180
(3) Ethanol	52,880	160
(4) Methanol	53,220	140
(5) Methanol	53,380	160
(6) Ethanol-methanol (1:1)	52,940	140
(7) Ethanol-methanol (1:1)	53,120	---

When the Δ^5-steryl acetate fraction was admixed with pure non-radioactive cholesteryl acetate and the mixture repeatedly recrystallized with 7 solvents, no significant change in observable specific activity could be detected, indicating that the Δ^5-steryl acetate was identical with the cholesteryl acetate standard. When the $\Delta^{5,7}$-steryl acetate fraction was admixed with authentic non-radioactive 7-dehydrocholesteryl acetate, the first recrystallization resulted in a 50% decrease in specific activity. During the next 5 recrystallizations, however, the observable specific activity remained constant, indicating that this 50% was indeed identical with the 7-dehydrocholesteryl acetate standard.

DISCUSSION

The houseflies of all six resistant and susceptible strains appear to be approximately equal in size and weight with the females averaging about 2.5 mg heavier than the males. Also the amount of lipid per mg of body weight was approximately the same in all strains studied. The females, however, with the exception of the malathion resistant flies, contained a smaller percentage of lipids per body weight than the corresponding males.

The amount of radiolabelled sterol varied in the different strains but there seemed to be no correlation of sterol content between resistant and susceptible strains, which is contradictory with the results of Enan et al. (1964). Not only was there a resistant strain of flies with a higher sterol content than the Belteville susceptible flies, the Orlando susceptible strain actually contained the least amount of sterols of any strain tested, indicating that the sterol content, per se, has no direct correlation with pesticide resistance.

The females of all the strains studied, except for the parathion resistant flies, contained higher quantities of radiolabelled sterols than the males which indicated that the females possibly retained more sterols because of oögenesis.

The amount of esterification of the 3-β-hydroxyl group was only about 28% of the total sterols with the free sterol fraction making up approximately 71%, as similarly reported by Monroe et al. (1967). Sterol esters were relatively low in the adult stages because esterification, as reported by Kaplanis et al. (1960), was correlated with oögenesis. Therefore, sterol esters were highest in the egg stage where they accounted for 60% of the sterols present (Robbins, 1963). All strains contained a small amount of polar steroids, indicating at least to a small extent, the metabolism of cholesterol to other more polar deravitives. There was a lack of degradation of the steroid ring nucleus because no radiolabelled hydrocarbons were found in any strain.

Upon fractionation by column chromatography, two major radioactive peaks were eluted which behaved as cholesteryl acetate and 7-dehydrocholesteryl acetate. The Δ^5-steryl acetate peak accounted for 94.8% to 98.3%, and the $\Delta^{5,7}$-steryl acetate fraction accounted for only 0.1% to 1.7% of the total radiolabelled sterols. The nearly equal percentages of the $\Delta^{5,7}$-sterols in both ester and free sterol fractions indicated no enzyme selectivity in esterification, as similarly reported by Monroe et al. (1967).

The Δ^5-steryl acetate fraction was identified by gas-liquid chromatography, ultraviolet spectroscopy, and reverse isotope dilution and found to be unchanged

cholesterol. Due to the very small quantity and contamination of the $\Delta^{5,7}$-sterols by a yellow, viscous substance, identification by gas-liquid chromatography and ultraviolet spectroscopy was not feasible. However, using the reverse isotope dilution technique, 50% of the fraction was identical to 7-dehydrocholesterol. It is likely that the other 50%, which separated with the first recrystallization might be breakdown products as it is known that 7-dehydrocholesterol is very unstable even when extreme care is exercised.

SUMMARY

Cholesterol-4-C^{14} absorption and metabolism was studied in six resistant and susceptible strains of houseflies reared on an aseptic synthetic diet. The results showed that there are differences in sterol content in the six strains of houseflies tested, but no correlation of sterol content existed between the resistant and susceptible strains. Therefore, it was concluded that cholesterol uptake and metabolism per se did not directly enter into pesticide resistance, but that strains of flies differed in their steroid metabolism regardless of whether they were resistant or susceptible.

A small percentage of 7-dehydrocholesterol was formed by the dehydrogenation of cholesterol. The conversion of cholesterol to a $\Delta^{5,7}$-diene as an initial step in the metabolism of cholesterol during its utilization as a hormone precursor in insects was discussed. There was some radioactivity present in the polar fraction which indicated that cholesterol was metabolized to other more polar derivatives.

LITERATURE CITED

LITERATURE CITED

Anon. 1959. The Peet-Grady method. Official method of the Chemical Specialties Manufactures Association for evaluating liquid household insecticides, latest revision. Soap and Chem. Specialties, Blue Book and Catalog, 32nd year, pp. 219-220.

Ascher, K. R. S. and Z. H. Levinson. 1956. The Influence of protein Addition to the Larval Diet on Oviposition of the House Fly. Riv. Parassitol. 17:217-222.

Block, K. 1965. The Biological Synthesis of Cholesterol. Science. 150:19-27.

Clark, A. J. and K. Block. 1959. Function of Sterols in Dermestes vulpinus. J. Biol. Chem. 234:2583-2594.

Clayton, R. B. 1960. The Role of Intestinal Symbionts in the Sterol Metabolism of Blatella germanica. J. Biol. Chem. 235:3421-3425.

_____. 1964. The Utilization of Sterols by Insects. J. Lipid Res. 5:3-17.

Clayton, R. B. and A. M. Edwards. 1962. A Novel Biological Desaturation of the Steroid Nucleus: Conversion of Cholestanol to Δ^7-Cholestenol in Cockroaches. Federation Proc. 21:297.

Clayton, R. B., A. M. Edwards, and K. Block. 1962. Biosynthesis of Cholesterol in an Insect, Silverfish (Ctenolepisma sp.). Nature. 195-1125-1126.

Earle, N. W., E. N. Lambremont, M. L. Burks, B. H. Slatten, and A. F. Bennett. 1967. Conversion of β-Sitosterol to Cholesterol in the Boll Weevil and the Inhibition of Larval Development by Two Aza Sterols. J. Econ. Entomol. 60:291-293.

Enan, O., R. Miskus, and R. Craig. 1964. A Comparison of the Sterols in Resistant and Susceptible House Flies, Musca domestica. J. Econ. Entomol. 57: 364-366.

Gilbert, L. I. 1963. Endocrine Control of Growth. Science. 139:928-929.

Hobson, R. P. 1935. On a Fat-Soluble Growth Factor Required by Blow-Fly Larvae. Biochem J. 29:1292-1296.

Ishii, S., J. N. Kaplanis, and W. E. Robbins. 1963. Distribution and Fate of 4-C^{14}-Cholesterol in the Adult Male American Cockroach. Ann. Entomol. Soc. Amer. 56:115-119.

Johnston, J. D., F. Gautschi, and K. Bloch. 1957. Isolation of lanosterol from "iso-cholesterol." J. Biol. Chem. 224:185-190.

Kaplanis, J. N., R. E. Monroe, W. E. Robbins, and S. J. Louloudes. 1963. The Fate of Dietary H^3-β-Sitosterol in the Adult House Fly. Ann. Entomol. Soc. Amer. 56:198-201.

Kaplanis, J. N., W. E. Robbins, and L. A. Tabor. 1960. The Utilization and Metabolism of 4-C^{14}-Cholesterol by the Adult House Fly. Ann. Entomol. Soc. Amer. 53:260-264.

Karlson, P. and H. Hoffmeister. 1963. Zur Biogenese des Ecdysons - I. Umwandlung von Cholesterin in Ecdyson. Hoppe-Seyler's Z. physiol. Chem. 331:298-300.

Karlson, P., H. Hoffmeister, W. Hoppe, and R. Huber. 1963. Zur Chemie des Ecdysons. Ann. Chem. Justus Liebigs 662:1.

Kobayashi, M., J. Kirimura, and M. Saito. 1962. The "Brain" Hormone in an Insect, Bombyx mori L. (Lepidoptera). Mushi. 36:85-92.

Lasser, N. L., A. M. Edwards, and R. B. Clayton. 1966. Distribution and Dynamic State of Sterols and Steroids in the Tissues of an Insect, the Roach Eurycotis floridana. J. Lipid Res. 7:403-412.

Levinson, Z. H. 1962. The Function of Dietary Sterols in Phytophagous Insects. J. Insect Physiol. 8: 191-198.

Lipke, H. and G. Fraenkel. 1956. Insect Nutrition. Ann. Rev. Entomol. 1:17-44.

Louloudes, S. J., M. J. Thompson, R. E. Monroe, and W. E. Robbins. 1962. Conversion of Cholestanol to Δ^7-Cholestenol by the German Cockroach. Biochem. Biophys. Res. Commun. 8:104-106.

Monroe, R. E. 1959. Role of Cholesterol in House Fly Reproduction. Nature. 184:1513.

_____. 1960. Effect of Dietary Cholesterol on Housefly Reproduction. Ann. Entomol. Soc. Amer. 53:821-824.

_____. 1962. A Method for Rearing Housefly Larvae Aseptically on a Synthetic Medium. Ann. Entomol. Soc. Amer. 55:140.

_____. 1964. Thesis, Metabolism and Utilization of Cholesterol-4-C^{14} for Growth and Reproduction of Aseptically-reared House Flies, Musca domestica L., Kansas State University.

Monroe, R. E., and N. J. Lamb. 1968. Effect of Commercial Proteins on House Fly Reproduction. Ann. Entomol. Soc. Amer. 61:292-296.

Monroe, R. E., T. L. Hopkins, and S. A. Valder. 1967. Metabolism and Utilization of Cholesterol-4-C^{14} for Growth and Reproduction of Aseptically Reared Houseflies, Musca domestica L. J. Insect Physiol. 13:219-233.

Robbins, W. E. 1963. Radiation and Radioisotopes Applied to Insects of Agricultural Importance. Proc. of Symposium at Athens, Greece. April 22-26:269-280. (International Atomic Energy Agency).

Robbins, W. E., J. N. Kaplanis, R. E. Monroe, and L. A. Tabor. 1961. The Utilization of Dietary Cholesterol by German Cockroaches. Ann. Entomol. Soc. Amer. 54:165-168.

Robbins, W. E., M. J. Thompson, J. N. Kaplanis, and T. J. Shortino. 1964. Conversion of Cholesterol to 7-Dehydrocholesterol in Aseptically Reared German Cockroaches. 4:635-644.

Robbins, W. E., R. C. Dutky, R. E. Monroe, and J. N. Kaplanis. 1962. The Metabolism of H^3-β-Sitosterol by the German Cockroach. Ann. Entomol. Soc. Amer. 55:102-104.

Robbins, W. E. and T. J. Shortino. 1962. Effect of Cholesterol in the Larval Diet on Ovarian Development in the Adult House-Fly. Nature. 194:502-503.

Saito, M., M. Yamazaki, and M. Kobayashi. 1963. Steroid Biosynthesis in the Silkworm. Nature. 198:1324.

Sperry, W. M. and M. Webb. 1950. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem. 187:97-106.

Svoboda, J. A. and W. E. Robbins. 1967. Conversion of Beta Sitosterol to Cholesterol Blocked in an Insect by Hypocholesterolemic Agents. Science. 156: 1637-1638.

VandenHeuvel, W. J. A., E. O. A. Haahti, and E. C. Horning. 1961. A New Liquid Phase for Gas Chromatographic Separations of Steroids. Jour. Amer. Chem. Soc. 83:1513-1514.