Distribution and dynamic state of sterols and steroids in the tissues of an insect, the roach *Eurycotis floridana*

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ABSTRACT The total concentrations of sterols in the tissues of the roach, *Eurycotis floridana*, reared under aseptic conditions and on semisynthetic diets, are similar to, but somewhat lower than, those of tissues of vertebrates.

Total concentrations of tissue sterols are relatively independent of dietary concentration of sterols whether the diet contains 0.1% cholesterol as the sole sterol, or a "minimal cholesterol" mixture (0.1% cholestanol together with 0.005% cholesterol). Under the latter conditions the cholesterol is incorporated preferentially into most tissues and remains almost exclusively unesterified, while the cholesterol-sparing sterol is esterified to varying degree, depending upon the tissue.

The turnover of tissue sterols has been studied. Cholesterol of the tissues of adult insects grown on a diet containing this sterol alone may be displaced by cholestanol fed as 5% of the total diet, initially at an appreciable rate but later much less rapidly. In growing insects that have received a diet containing cholestanol together with minimal cholesterol, the unesterified cholesterol turns over slowly in all tissues and immeasurably slowly in some. The unesterified sparing sterol, on the other hand, turns over at a much greater rate. The turnover of sterols during growth is accompanied by a shift of sterols from the unesterified to the esterified pool in all tissues.

The fat body of the growing insect stores sterols (apparently as their esters) that have been displaced from other tissues. The fat body of the adult does not show evidence of sterol storage.

Polar derivatives of sterols are present in minor amount in all tissues of the insect, most abundantly in the mid-intestine and gastric caeca. These compounds seem likely to be C_{27} steroids.

The results are discussed in terms of the concept that the sterols incorporated into the tissues of this insect function primarily as components of subcellular membranous structures. It seems likely that unesterified cholesterol is a tightly bound and highly specific component of such structures, while the unesterified cholesterol-sparing sterol is less firmly bound into the same structures or into others of lesser general stability.

KEY WORDS sterols · steroids · turnover · roach · tissues · concentration · cholesterol · cholestanol · Δ^7-cholestenol · dietary requirement · membrane · sterol esters · storage · fat body

WITHIN THE LAST two decades the metabolism of cholesterol to steroid hormones and bile acids has been extensively explored and largely elucidated, but the role of sterols per se in maintaining normal cellular function is still far from clearly understood, though their ubiquitous occurrence in the tissues of animals and green plants leaves little doubt that this role is vital.

A major difficulty in investigating this role has been the ability of almost all normal tissues that have been examined to synthesize sterol. In the vertebrate red blood cell and myelinated nerve fiber, tissues which have yielded the most significant data from which reasonable concepts of the structural role of cholesterol have been derived, there is an absence either of synthesis of cholesterol (red cell) (1) or of both synthesis and turnover (mature myelinated nerve) (2). These peculiarities have been exploited by many investigators with the implicit assumption that the quantity of sterol present in these tissues reflects its essential function, and this approach

Abbreviations: cholestanol, 5α-cholestan-3β-ol; Δ^7-cholestenol, 5α-cholest-7-en-3α-ol; GLC, gas–liquid chromatography.

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has led to the derivation of schematic models depicting the mode of incorporation of cholesterol into the structures of the myelin sheath (3, 4) and the red cell membrane (5). These models suggest the probable manner of involvement of the cholesterol molecule in cellular and subcellular membrane structures of other tissues where, however, a direct analytical approach is complicated by concurrent synthesis and turnover of the sterol.

Unlike the vertebrates, the insects as a class are probably incapable of sterol synthesis, since all species so far examined [except one (40)] have a dietary requirement for a sterol which can be satisfied by cholesterol (6). The insects thus seem to be ideal experimental animals for studies aimed at the elucidation of the role of cholesterol in maintaining normal cellular function, since it may be assumed that when the dietary sterol is provided in amounts minimal for growth, it will become incorporated into the tissues in concentrations which are strictly dictated by functional requirements.

The use of insects for such a study offers a further analytical advantage. Clark and Bloch (7) found that the hide beetle, *Dermestes vulpinus*, an insect which normally shows a strict requirement for cholesterol, could be reared on artificial diets in which all but a few per cent of the normal cholesterol requirement was replaced ("spared") by any of a number of other sterols which were not adequate as the sole dietary sterol. A similar "cholesterol-sparing" effect has been found to apply in the case of the aseptically reared roaches *Blattella germanica* and *Eurycotis floridana*[1] which will grow normally on diets containing 0.1% cholesterol but fail to grow on diets containing 0.1% cholestanol unless supplemented with at least 0.005% of cholesterol (referred to below as "minimal" cholesterol concentration). This concentration of cholesterol is less than one-tenth of the normal requirement for the growth of these species when this is the sole dietary sterol. Cholesterol, when fed in minimal concentrations together with a sparing sterol, has been found in preliminary studies to accumulate in the tissues of *Dermestes*,[2] *Blattella*,[1] and *Eurycotis* (8) in preference to the sparing sterol. Moreover, preliminary evidence has been given (8) that in *Eurycotis* the cholesterol incorporated into the tissues under these conditions remains almost wholly unesterified while the sparing sterol is found in both free and esterified form. It has also been reported that the housefly, *Musca domestica*, when it is fed on a diet containing mixtures of sterols, will preferentially concentrate into its tissues either cholesterol or the sterol most closely resembling cholesterol in structure, though no data have so far been presented concerning the state of esterification of the different sterols in these insects (9).

These observations suggest that the cholesterol-sparing phenomenon may be used as a tool for the study of the distribution and dynamic behavior of functionally distinct sterol pools in insect tissues, and this has been the main object of the present work. The results of such a study cannot be used as conclusive evidence for particular functions (though these may be suggested by the results), but firmer evidence may be forthcoming through the correlation of these results with complementary data for the distribution and behavior of other tissue components.

When this work was begun, the distribution of sterols in the tissues of one other insect—the roach *Periplaneta americana*—had been studied, though in limited detail (10), but there was an almost complete lack of data concerning the dynamic state of sterols in insect tissues. For our purposes a detailed study of these aspects of sterol utilization in one species of insect was therefore essential and its principal results are reported in this paper. Preliminary reports of some aspects of this work have been presented (8, 11) and since this work was completed a study of the turnover of cholesterol in *P. americana* has been described (12).

EXPERIMENTAL PROCEDURES

Insects

Stock colonies of *Eurycotis floridana* were descended from insects kindly furnished by Dr. Louis Roth of the U.S. Army Quartermaster Corps. They were reared in 220 × 250 mm battery jars on a diet of Kasco dog food and were supplied with water from 150 × 25 mm test tubes via a cotton plug. Insects were grown aseptically (13) from oothecae collected from the nonsterile cultures and sterilized by washing in turn with 0.2% mercuric chloride in 50% ethanol (2 min), 70% ethanol (2 min) and two changes of sterile water (2 min each). Both sterile and nonsterile insects were reared under 60–70% relative humidity and at 30°C.

Experimental Diets

All insects studied were reared aseptically on the synthetic diet of Noland and Baumann (14). Dietary concentrations of the sterols were varied according to the needs of the experiment, the compounds being added to the diet in an organic solvent which was then allowed to evaporate, with stirring. The diets were then introduced into 32-oz culture bottles and autoclaved as described (13). The diets contained either an optimal concentration (0.1%) (14) of cholesterol-4-^{14}C as the sole sterol, or a minimal concentration of 0.005% cholesterol-4-^{14}C supplemented with 0.1% cholestanol-7α-^{3}H. Specific

[1] Clayton, R. B., unpublished results.
[2] Lasser, N. L., unpublished results.

activities of the sterols are given in the tables. Insects grown on the optimal cholesterol diet generally reached maturity after about 6 months. Those reared on the "minimal" cholesterol diet grew more slowly, matured at about 10 months, and achieved an adult weight generally about 20% less than the insects on the optimal cholesterol diet.

Radioactive sterols, cholesterol-4-^{14}C, cholesterol-26-^{14}C, cholesterol-3α-^{3}H, and cholesterol-7α-^{3}H, were obtained from New England Nuclear Corp. (Boston, Mass). The cholesterol-^{3}H was purified via the dibromide (15). Cholestanol-7α-^{3}H was the only sparing sterol used in these experiments; it was prepared from cholesterol-7α-^{3}H by catalytic hydrogenation of the acetate with platinum in 10% acetic acid in ethyl acetate, followed by overnight treatment with excess monoperphthalic acid, hydrolysis and reduction with lithium aluminum hydride, and purification by chromatography on deactivated alumina. The sterols were diluted to various specific activities with nonradioactive cholesterol [purified via the dibromide (15)] and with cholestanol recrystallized to mp 140–141°C. The purity of all sterols used in the diets was established by GLC of their methyl ethers on columns of diethylene glycol succinate polyester, as previously described (16), with radioassay of the effluent material.

Isolation of Lipids

Each insect was analyzed individually. The insect was inactivated by immersion in ice water and dissected immediately in cold saline (17) with the use of a dissecting microscope. The alimentary canal was freed from food by longitudinally incising the gut and cleaning it with a camel's hair brush. The fresh weights of the various tissues were determined by blotting the tissue briefly with filter paper and weighing it rapidly on a glass slide with a Mettler semimicro balance. Weights of individual tissue samples and organs are not given in the tables but some approximate, representative weights are as follows: Malpighian tubules, 4.0 mg; ventral nerve cord, salivary gland, rectum, and mid-intestine 5.0–7.0 mg; crop, proventriculus, gastric caeca, and hind intestine, 10.0–16.0 mg; muscle (coxae of all six legs), 50–70 mg; fat (almost entire fat body) 200–250 mg; cuticle (dorsal exoskeleton) 25–35 mg. These amounts of tissue, if handled quickly, could be weighed with sufficient accuracy for the purpose of these studies. Lipid extracts consisted of combined material from successive 10–15 min extractions of the tissue samples with absolute ethanol, acetone, and ethyl acetate.

Analysis of Sterols

Free and esterified sterols were separated by chromatography on 2 g of deactivated alumina (18) in columns 8–10 mm in diameter. The lipid extracts were applied to the column in distilled Skellysolve B (petroleum ether bp 60–80°C). Sterol esters were eluted with 60–75 ml of 2% benzene in Skellysolve, free sterols with 60–75 ml of 50% benzene in Skellysolve, and more polar materials with 25–50 ml of 20% methanol in ether. When lipid extracts from animals grown on diets containing labeled sterols were chromatographed in this way, essentially all the radioactivity was recovered from the column.

Radioactivity measurements were made with a dual channel Packard liquid scintillation spectrometer and were converted to disintegrations per minute by corrections for background and counting efficiency. The simultaneous assay of ^{14}C and ^{3}H was carried out with window settings adjusted to give 0.3% overlap of ^{3}H counts into the ^{14}C channel and counts were accumulated to give an error of 5% or less in the counts for ^{14}C. For most samples this procedure allowed calculation of ^{14}C and ^{3}H activities by neglecting the overlap of ^{3}H. In some cases, however, (^{14}C-ester, Table 2) the ratio ^{14}C/^{3}H was so low that this procedure led to results for ^{14}C that were too high. In these cases the results are expressed as maximum values which also incorporate a standard deviation calculated on the basis of the spurious ^{14}C counts.

RESULTS

The results of all experiments were derived in a similar way. The insects were reared aseptically from the time of hatching on a semisynthetic diet (14) containing labeled sterols of known activity. In some experiments the insects received the same diet throughout the experimental period; in others they were given a diet containing a different sterol or combination of sterols during a later phase of the experiment. The final measurement in all cases was the level of radioactivity found in whole lipid extracts of the insects' tissues or in fractions obtained from the total extracts by chromatography. The radioactivity measurements were then used to calculate concentrations of sterols, the assumption being that the specific activity of the tissue sterols was identical with that of the dietary sterols. Direct determinations of the specific activity of tissue sterols have shown that this assumption is valid, with the reservation noted below in connection with the desaturation of cholestanol to Δ^{7}-cholestenol.

The further assumption has been made that, except in the case of the "polar" materials, all the radioactivity was present either in unchanged cholesterol or in sparing sterol. As a test for this assumption, the free sterols from roaches grown on minimal cholesterol-^{14}C and cholestanol-^{3}H were obtained by saponification of the total lipid extracts followed by chromatography of the unsaponifiable material on alumina. Further analysis of these sterols by GLC showed that all the ^{14}C was con-

tained in cholesterol, but that the ^3H appeared in both cholestanol and Δ^7-cholestenol (19). A loss of up to 15% of the tritium occurs in the desaturation of cholestanol, most probably as a result of the presence of some tritium in either the 7β- or 8β-positions, or both (19). If all of the ^3H-sterol were present as Δ^7-cholestanol, this change in specific activity would mean that the actual ^3H-sterol concentration would be greater by approximately 18% than values calculated on the assumption that no loss of ^3H had occurred. In most cases rather more than 50% of the tritium sterol is present as Δ^7-cholestenol and the remainder as cholestanol. The values reported in the tables for the concentrations of ^3H-sterols may therefore be as much as 10% low, but no correction factor has been introduced since it is not certain that it should be exactly the same for all experiments and this inaccuracy does not invalidate any conclusions that will be drawn from the results.

Distribution and Concentration of Sterols

The concentrations of cholesterol and sparing sterols in the different tissues of *Eurycotis floridana* nymphs and adults, reared on various diets, are shown in Table 1. The table shows mean values derived from analyses of several individual insects and obtained from radioactivity measurements on total lipids. These values therefore indicate the level of total steroid, i.e., free and esterified sterols plus a small amount of polar steroid, in the tissues.

In the "mixed sterol" diet, having 0.1% cholestanol plus 0.005% cholesterol, the concentration of cholesterol is minimal; 0.1% cholesterol, as supplied in the second diet, is fully adequate for normal growth.

When the insect was reared on the mixed sterol diet, the cholesterol became concentrated in most tissues in preference to cholestanol. The extent of this preferential incorporation of cholesterol varies from tissue to tissue, but is most marked in the nerve, where the two sterols are present in approximately equal amounts. The sum of the concentration of ^{14}C- and ^3H-labeled sterols found in almost all the tissues of animals fed the mixed sterol diet is remarkably close to that found in animals reared on 0.1% cholesterol alone. Exceptions are the nerve (less than $^2/_3$ the concentration in the animals reared on 0.1% cholesterol) and the hind intestine (almost three times that in animals reared on 0.1% cholesterol).

The data of Table 1 as well as other data for nymphs of various ages, not given here, indicate that the growth of the insect is accompanied by only small changes in the sterol concentrations of most tissues and that in general these changes are in the direction of increased concentrations.

Distribution of Radioactivity in Different Steroid Fractions

The distribution of the total radioactivity in the tissues between fractions representing esterified and unesteri-

TABLE 1 CONCENTRATION OF STEROIDS IN TISSUES OF *E. floridana* REARED ON DIETS CONTAINING DIFFERENT CONCENTRATIONS OF CHOLESTEROL

Values shown indicate the concentration of total steroid, mean ±SD, as μg/100 mg fresh tissue. The numbers of insects analyzed in each group are given in parentheses. Specific activities used in the diets were as follows: for the "mixed sterol" diet, cholesterol-4-^{14}C, 947 dpm/μg, cholestanol-7α-^3H, 6000 dpm/μg; for 0.1% cholesterol-4-^{14}C only, 400 cpm/μg and 894 dpm/μg.

	0.1% Cholesterol-4-^{14}C in Diet	0.1% Cholestanol-7α-^3H + 0.005% Cholesterol-4-^{14}C in Diet					
	Adults (4)	Adults (8)			Nymphs (3)		
	Total ^{14}C-Steroid	^{14}C-Steroid	^3H-Steroid	Sum ^{14}C + ^3H	^{14}C-Steroid	^3H-Steroid	Sum ^{14}C + ^3H
Alimentary canal							
Crop	137.6 ± 58.5	11.9 ± 2.4	72.0 ± 23.7	83.9	15.1 ± 5.6	219.7 ± 151.8	234.8
Proventriculus	67.5 ± 5.2	10.1 ± 0.1	34.8 ± 7.0	44.8	10.0 ± 0.3	55.7 ± 14.6	65.7
Mid-intestine	106.7 ± 9.5	8.0 ± 1.5	111.6 ± 52.2	119.6	9.4 ± 1.7	343.7 ± 225.6	353.1
Gastric caeca	103.8 ± 14.4	6.3 ± 0.4	95.7 ± 60.8	102.0	5.5 ± 1.0	103.1 ± 46.2	108.6
Hind intestine	100.9 ± 6.1	18.0 ± 2.7	272.9 ± 84.5	290.9	13.6 ± 2.0	199.5 ± 35.1	213.0
Rectum	185.3 ± 27.4	32.0 ± 0.2	214.1 ± 15.6	246.1	25.6 ± 7.5	213.3 ± 108.4	238.9
Malpighian tubules	162.4 ± 19.9	10.4 ± 4.0	115.9 ± 32.4	126.3	9.8 ± 2.3	139.4 ± 20.8	149.1
Fat	121.7 ± 39.1	8.0 ± 3.1	117.3 ± 26.2	125.3	10.7 ± 0.9	118.2 ± 1.7	129.0
Salivary gland	298.0 ± 36.5	51.3 ± 14.7	271.8 ± 59.3	323.1	24.0 ± 4.1	196.0 ± 41.2	220.0
Muscle	32.8 ± 6.9	4.7 ± 1.0	35.3 ± 6.9	40.0	4.0 ± 0.8	24.2 ± 1.3	28.1
Nerve	325.1 ± 58.5	97.4 ± 12.5	99.6 ± 33.5	197.0	98.2 ± 8.5	86.1 ± 8.9	175.3
Cuticle	57.4 ± 22.8	9.5 ± 1.2	38.4 ± 7.1	47.9	6.4 ± 1.7	27.4 ± 2.2	33.8
Reproductive organs							
Male	58.2* —	17.7 2.5†	27.7 7.6†	45.4			
Female	177.4* —	34.8 12.6‡	106.5 6.1‡	141.3			

* One insect used for this tissue.
† Four insects used for this tissue.
‡ Three insects used for this tissue.

TABLE 2 CHROMATOGRAPHIC FRACTIONATION OF STEROIDS IN TISSUES OF *E. floridana* REARED ON DIETS CONTAINING DIFFERENT STEROLS

	0.005% Cholesterol-4-^{14}C + Cholestanol-7α-^{3}H (3 Nymphs)						0.1% Cholesterol-4-^{14}C (4 Adults)		
	^{14}C-Steroid			^{3}H-Steroid			^{14}C-Steroid		
	Ester	Free	Polar	Ester	Free	Polar	Ester	Free	Polar
Alimentary canal									
Crop	14.1 ± 11.6	80.9 ± 10.0	4.8 ± 2.0	42.9 ± 21.0	53.2 ± 19.5	3.6 ± 1.5	18.9 ± 17.0	77.9 ± 18.4	3.0 ± 1.7
Proventriculus	<3.0	91.3 ± 0.3	6.3 ± 1.1	14.0 ± 6.4	81.6 ± 4.9	4.1 ± 1.7	5.8 ± 4.6	87.9 ± 4.9	6.2 ± 4.7
Mid-intestine	8.5 ± 4.1	80.9 ± 8.9	10.4 ± 5.3	51.7 ± 18.6	45.7 ± 19.2	2.4 ± 0.5	1.4 ± 0.9	87.0 ± 5.7	11.4 ± 5.3
Gastric caeca	<10	86.5 ± 8.2	8.9 ± 4.5	34.7 ± 17.7	60.4 ± 15.3	4.7 ± 2.5	8.5 ± 4.8	79.4 ± 7.2	15.0 ± 4.6
Hind intestine	9.5 ± 5.0	85.3 ± 5.9	5.1 ± 1.1	32.7 ± 9.8	62.4 ± 8.7	4.8 ± 1.7	3.1 ± 1.7	93.5 ± 3.0	3.3 ± 1.5
Rectum	10.8 ± 5.4	82.2 ± 5.8	6.9 ± 1.0	31.4 ± 4.0	63.6 ± 5.4	4.8 ± 1.6	30.9 ± 5.3	66.7 ± 5.2	2.2 ± 0.6
Malpighian tubules	5.9 ± 7.5	84.6 ± 8.5	9.4 ± 2.4	9.5 ± 2.9	85.9 ± 1.8	4.3 ± 1.4	2.0 ± 0.7	86.3 ± 14.0	3.9 ± 2.1
Fat	<10.0	91.6 ± 2.1	3.5 ± 0.3	44.6 ± 8.1	53.8 ± 8.4	1.5 ± 1.0	47.4 ± 8.3	57.0 ± 12.6	1.3 ± 0.3
Salivary gland	<2.0	92.8 ± 2.7	6.5 ± 3.2	5.5 ± 2.1	88.9 ± 2.6	5.4 ± 3.0	1.2 ± 0.8	92.9 ± 5.2	2.7 ± 1.4
Muscle	<2.0	94.6 ± 1.4	3.9 ± 0.8	9.6 ± 8.9	87.0 ± 9.6	3.2 ± 0.9	7.7 ± 5.6	85.0 ± 7.3	3.1 ± 0.4
Nerve	<2.0	93.6 ± 1.3	5.4 ± 1.7	38.7 ± 5.5	58.1 ± 5.2	3.0 ± 0.7	32.9 ± 9.2	62.7 ± 7.3	4.1 ± 2.8
Cuticle	<3.0	94.4 ± 4.4	4.2 ± 3.2	18.6 ± 10.9	77.4 ± 11.7	3.9 ± 1.1	22.0 ± 6.2	73.0 ± 9.9	4.9 ± 3.6
Reproductive organs									
Male	—	—	—	—	—	—	1.3	98.2	0.6
Female	—	—	—	—	—	—	3.8	91.5	4.6

Values are percentages of labeled steroid recovered from column (deactivated alumina).

fied sterols and more polar steroids is shown in Table 2. The results are expressed as the percentage in each fraction of all the radioactive material recovered from chromatography of the total extracts of alumina, since recoveries were virtually quantitative. In the nymphs the cholesterol of the tissues other than the gut is almost exclusively in the unesterified form, while the percentage of esterified ^{3}H-sterol is often many times greater, e.g., in the nerve, <2.0%-cholesterol but 38.7% ^{3}H-sterol is in the esterified form. These results suggest that an essential role of the cholesterol in these animals involves its incorporation as the free sterol into certain sites for which the sparing sterol molecules are unsuited. Analyses (not shown) of the tissue sterols of adults which have continued to consume the same dietary sterol mixture indicate a closely similar pattern of distribution of the two types of sterols between the various fractions, though the cholesterol is now esterified to a somewhat greater extent than in the nymphs.[3] This observation probably reflects the fact that in the adult the demand for unesterified cholesterol to occupy specific functional spaces is much less than during growth, when the limited availability of cholesterol in the diet leads to a strict confinement of cholesterol to this space.

Polar Metabolites of Cholesterol

In most of the tissues of insects reared on the diet containing 0.1% cholesterol, substances more polar than cholesterol account for 1–6% of the radioactivity, but in the mid-intestine and gastric caeca 11–15% of the radioactivity is in the form of such materials (Table 2). These results are in good agreement with those of Ishii, Kaplanis, and Robbins (25), who found that 20 days after the injection of cholesterol-^{14}C into the roach *P. americana*, only 1–3% of the ^{14}C incorporated into most of the tissues of the insect was in polar steroids, but that in the mid-intestine and gastric caeca these materials accounted for 13% of the labeling. On the other hand, Ishii et al. found a similar high concentration of polar metabolites in the hind gut; this was not observed in our experiments with *E. floridana*.

Displacement of Cholesterol by Sparing Sterol

Table 3 shows the results of experiments to determine whether sparing sterol could replace cholesterol already incorporated into the insect's tissues. The insects were grown to adulthood on a diet containing 0.1% cholesterol-^{14}C; some were dissected and analyzed, and the remaining insects were transferred to a diet containing 5% cholestanol but no cholesterol. In an early series the cholestanol used in the later part of the experiment was nonradioactive and its incorporation into the tissues was inferred from the progressive fall in concentration of cholesterol-^{14}C. In a later study the diet in the second phase of the experiment contained 5% cholestanol-7α-^{3}H and the concentrations of both cholesterol-^{14}C and ^{3}H-labeled sparing sterol were followed for 8 months after transfer of the insects to the new diet. The results are shown as the sterol concentrations at the time of transfer to the new diet (zero time), 2 months later, and during the period from 3 to 8 months. Only an extremely slow exchange was observed in the last period.

Turnover of Sterols in Growing Insects

To obtain further information concerning the differences in distribution, metabolism, and dynamic state between cholesterol and the sparing sterol, we studied the turnover of sterols in growing insects. Individuals

[3] Clayton, R. B., and A. M. Edwards, unpublished results.

TABLE 3 DISPLACEMENT OF CHOLESTEROL FROM TISSUES OF *E. floridana* BY CHOLESTANOL

Insects were grown to adulthood on diets containing 0.1% cholesterol-4-^{14}C, 400 cpm/μg or 894 dpm/μg, then transferred to diets containing 5% cholestanol, with or without ^{3}H-labeling. Cholestanol-7α-^{3}H contained 1855 dpm/μg. Analytical values at "zero time" are for adults taken from the colony at the time of transfer. Other insects were analyzed at 2 months or between 3 and 8 months after transfer to the new diet. Figures in parentheses below column subheading indicate the number of insects analyzed.

	Zero Time	2 Months			3–8 Months		
	^{14}C-Steroid (4)	^{14}C-Steroid (3)	^{3}H-Steroid (2)	Sum ^{14}C + ^{3}H concns.	^{14}C-Steroid (5)	^{3}H-Steroid (3)	Sum ^{14}C + ^{3}H concns.
				μg sterol per 100 mg fresh tissue			
Malpighian tubules	162.4	114.4	85.0	199.4	80.5	123.0	203.5
Fat	121.7	88.5	37.8	126.3	44.4	67.5	111.9
Salivary gland	298.0	187.1	34.1	221.2	132.2	125.4	257.6
Muscle	32.8	25.8	4.5	30.3	21.5	26.2	47.7
Nerve	325.1	245.3	18.8	264.1	238.4	66.8	305.2
Cuticle	57.4	38.6	10.8	49.4	23.3	55.1	78.4

from a colony of nymphs grown for 7 months on 0.005% cholesterol-^{14}C and 0.1% cholestanol-^{3}H were dissected and the total concentrations of ^{14}C- and ^{3}H-labeled steroids and their distribution among the ester, free, and more polar fractions were determined for each tissue. The remainder of the colony was allowed to grow for a further 7 months on a diet containing the same concentrations of unlabeled sterols and its members were then analyzed as before in order that the concentrations of labeled steroids remaining in the various tissues could be ascertained. The results are expressed in Table 4 both as ratios of the concentrations of total labeled steroid in the tissues of the younger nymphs to those in the older nymphs, and as ratios of the corresponding labeling concentrations of individual steroid fractions obtained by chromatography on alumina. Appropriate correction for the increase in weight of the individual tissue is made in each case. Since the total steroid concentrations of most tissues tend to increase slightly during growth, or remain almost unchanged (see Table 1), these values give a direct measure of the extent to which labeled sterol incorporated in the earlier growth period is exchanged for sterol ingested during the later phase of the experiment. The data of Table 4 indicate that in the gut tissues (excluding the crop), the nerve ganglia, the muscle, and to a lesser extent the salivary gland, the displacement of cholesterol took place more slowly than that of the sparing sterol. This difference in behavior was particularly evident for the free sterol fraction. However, since cholesterol was present in the diet during the second part of the experiment in the limiting concentration of 0.005%, the slower turnover of labeled cholesterol could be due to the low availability of dietary cholesterol for exchange.

The experiment was therefore repeated under conditions designed to allow displacement of cholesterol at the maximal rate. The regimen during the first part of the experiment contained, as before, 0.1% cholestanol-^{3}H and 0.005% cholesterol-^{14}C, but the insects were given a diet containing 5% unlabeled cholesterol in the second phase of the experiment, which in this case lasted 2.8 months. Analyses for ^{14}C- and ^{3}H-labeled steroids in the various tissues were carried out as before, both before the change of diet and at the end of the experiment. The results are expressed in Table 5 as ratios calculated in the same manner as in the previous experiment, with correction for increase in weight of the individual tissues. (In the first experiment the average weight per whole insect increased by a factor of 3.3 and in the second experiment, by a factor of 2.0.) It is clear from these results that whether the diet in the second phase of these experiments contained the same (non-radioactive) sterol mixture as in the first stage (0.005% cholesterol and 0.1% cholestanol) or 1000-fold greater unlabeled cholesterol concentration (5.0% cholesterol), the rate of turnover of the cholesterol pool was generally less than that of the sparing sterol and that the unesterified fractions of the two types of sterol show the most striking differences in turnover rate. In both experiments and in every tissue except the fat, the free ^{3}H-sterol turns over more rapidly than the cholesterol.

DISCUSSION

Concentrations of Sterols in Various Tissues

The concentrations of sterols found in the tissues of *Eurycotis floridana* in this study (Table 1) are in general higher than those reported by Casida, Beck, and Cole for *Periplaneta americana* (10). They are somewhat lower than most values for corresponding tissues of vertebrates, but are generally of the same order and are consistent with the view that cholesterol fulfills similar functions in both mammalian and insect tissues. Thus, the concentration of sterol in the insect muscle is

close to that found in chicken muscle (20), and although the concentration of cholesterol in the nerve tissues of this insect is only about one-tenth as great as that of the adult mammalian brain (21), it is close to the values for fetal mammalian brain, as might be expected in view of the primitive state of myelination in insect nerve (22).

Other experiments in our laboratory[3] in which insects were reared on diets containing 1.0% cholesterol, rather than 0.1% cholesterol as in the experiments described, show that this 10-fold increase in dietary sterol concentration leads to relatively much smaller but significant increases in the sterol concentrations of most body tissues: salivary gland, 70%; nerve, 50%; muscle, 45%; and cuticle, less than 30%. An exception is the fat, which shows a 7-fold increase of sterol concentration under these conditions, suggesting a probable role for this tissue as a storage reservoir for sterol, which will be discussed more fully below.

The concentration of dietary cholesterol into the tissues in preference to cholestanol, together with the selective incorporation of cholesterol in the unesterified form, have been reported in a preliminary communication (8) and are fully confirmed by the more extensive experiments reported here. Since we have shown that cholestanol is absorbed from the insect's gut at least as readily as cholesterol (23), it seems unlikely that this aspect of sterol utilization can be responsible for the differential concentration of the two compounds into the tissues, particularly in view of the marked and characteristic differences between the concentrations of

TABLE 4 TURNOVER OF STEROIDS IN TISSUES OF *E. floridana* DURING GROWTH WHILE RECEIVING A DIET CONTAINING 0.005% CHOLESTEROL AND 0.1% CHOLESTANOL

The diet for the first 7 months of growth contained 0.005% cholesterol-4-^{14}C (1000 dpm/μg) and 0.1% cholestanol-7α-^{3}H (6000 dpm/μg). During the second 7 months of growth the diet contained the same concentrations of unlabeled sterols.

The results for total ^{3}H- or ^{14}C-steroid are calculated from the measured total specific activity of ^{3}H or ^{14}C in each tissue. Values for esterified, nonesterified and polar steroid are calculated from the measured ^{3}H or ^{14}C content of fractions obtained by alumina chromatography of total lipid extracts on deactivated alumina. Values are expressed as:

$$\frac{\mu\text{g labeled steroid per 100 mg tissue in younger nymphs}}{\mu\text{g labeled steroid per 100 mg tissue in older nymphs}} \times \frac{\text{weight of tissue in older nymph}}{\text{weight of tissue in younger nymph}}.$$

A value greater than 1 thus represents displacement while a value less than 1 indicates accumulation of labeled steroid in a given tissue.

Labeled material appearing in a fraction in older nymphs but absent from the corresponding fraction in younger nymphs is indicated as (+). The disappearance from a fraction in the older nymphs of labeled material that was present in the corresponding fraction in the younger nymphs is shown as (−). The absence of labeled material from a particular fraction in both younger and older nymphs is shown as (0).

	^{14}C-Steroid				^{3}H-Steroid			
	Total	Ester	Free	Polar	Total	Ester	Free	Polar
Alimentary canal								
Crop	3.1	—	2.8	—	3.3	0.9	5.2	1.3
Remainder	1.1	2.6	0.9	2.7	2.1	0.6	3.4	1.2
Salivary gland	1.9	0	1.9	0	2.3	0.3	4.5	0.3
Fat	0.5	+	0.7	0.3	0.3	0.01	0.5	0.8
Nerve (ganglia only)	1.5	0	1.3	—	2.7	0.7	5.8	0.9
Muscle	0.3	0	0.3	0	2.1	0.3	3.3	1.1
Cuticle	1.3	0	1.3	0	1.1	0.03	2.1	1.4

TABLE 5 TURNOVER OF STEROIDS IN TISSUES OF *E. floridana* DURING GROWTH WHILE RECEIVING A DIET CONTAINING 5% CHOLESTEROL

The diet during the first phase of the experiment (4 months) contained 0.005% cholesterol-4-^{14}C (1000 dpm/μg) and 0.1% cholestanol-7α-^{3}H (6000 dpm/μg). In the second phase of the experiment (3 months) the diet contained 5% unlabeled cholesterol. The results were obtained in the same manner as those presented in Table 4 and are similarly expressed.

	^{14}C-Steroid				^{3}H-Steroid			
	Total	Ester	Free	Polar	Total	Ester	Free	Polar
Alimentary canal								
Crop	2.6	5.3	2.5	1.2	14.0	33.4	9.3	8.0
Remainder	1.0	0.4	1.0	0.9	5.8	6.8	5.5	2.9
Salivary gland	0.5	0	0.5	0	1.6	0.8	1.7	5.5
Fat	0.1	0.02	0.3	+	0.2	0.1	0.4	0.2
Nerve	1.3	+	1.4	+	1.0	0.2	2.7	3.4
Muscle	1.0	+	1.0	+	2.5	0.8	3.2	4.6
Cuticle	1.5	0.2	1.9	+	2.1	1.1	3.6	1.5

the two sterols in individual organs and tissues. It should be noted that there is in general a close correspondence between the total concentrations of sterols in the tissues of insects reared on 0.1% cholesterol and of those reared on 0.1% cholestanol and 0.005% cholesterol. Thus, the results support the assumption that has been made in earlier publications (7, 24) that the cholesterol-sparing sterol substitutes for cholesterol in certain structural spaces that offer some degree of stereochemical flexibility. They also give further support to the suggestion (8) that the tissues of these insects contain at least one type of structural space which is sterically highly specific for unesterified cholesterol. The "cholesterol" space is evidently present in all tissues, but its quantitative importance differs greatly from one to another.

Polar Metabolites of Cholesterol

Our finding, and that of Ishii et al. (25), of appreciable concentrations of polar derivatives of cholesterol in the mid-gut and gastric caeca of two species of roaches, suggests that these regions of the intestinal tract may be concerned in the excretion of some polar metabolites of cholesterol. The identity of these materials remains to be elucidated, but preliminary results from thin-layer chromatography and autoradiography show that they consist of complex mixtures. Experiments[3] involving feeding of cholesterol doubly labeled with ^{3}H and ^{14}C indicate that the formation of these polar materials involves no measurable loss of 3α-H and a loss of only about 10% of C_{26}. A loss of 7α-H of the order of 30% is indicated, but presently available results do not show to what extent this loss is attributable to metabolism or to atmospheric oxidation, to which the 7-position of the cholesterol nucleus is particularly vulnerable. These preliminary findings together with the C_{27} steroid structure of the growth and moulting hormone, ecdysone (26, 27), suggest that the major pathways of sterol metabolism in insects are radically different from those of the vertebrates. Since the polar materials detected in our experiments and in those of Ishii et al. were found in adult insects, the likelihood that they represent metabolites of ecdysone seems remote, for with attainment of adulthood the prothoracic gland which produces this hormone degenerates (28).

Turnover of Sterols in Adult and Nymphal Insects

It was of interest to determine whether, in an adult insect whose tissues contained cholesterol as the sole sterol, this cholesterol could be exchanged readily with dietary cholestanol. This might not be the case if cholesterol were capable of much firmer binding at functional sites than either cholestanol or Δ^7-cholestenol. Experiments to test these possibilities indicated that cholestanol fed as 0.1% of the diet could displace cholesterol from the tissues, but so slowly that it was not practical to measure the rate of turnover. An accelerated rate of displacement could be achieved when cholestanol was fed as 5% of the diet (Table 3). The results show that cholestanol enters the body tissues, excluding the gut, with little increase in the total sterol concentration, as would be expected if the sparing sterol molecules occupied functional spaces initially filled by cholesterol. Even under these conditions, the rate of exchange of cholesterol for cholestanol is slow, requiring 3–6 months for approximately 50% displacement in most body tissues. This estimate of the half-life of the tissue cholesterol is to be compared with the estimate of 60 days for the half-life of total body cholesterol of *P. americana* derived by Vroman, Kaplanis, and Robbins (12) from experiments in which adult insects were fed a diet containing 0.1% cholesterol-^{14}C. It seems likely that the exchangeability of cholesterol in the tissues of these two species under comparable dietary conditions would be similar and the present results suggest that exogenous cholesterol is more suitable for exchanging than exogenous cholestanol, even for binding into sites in which cholesterol is nonessential. Since the two sterols are absorbed from the gut at similar rates (23), the slowness with which cholestanol displaces cholesterol is not due to poor absorption of cholestanol.

Approximate half-lives of the sterol pools in the growing insect can be calculated from the data of Tables 4 and 5 on the assumption that the decline in concentration of the labeled sterol takes place with first order kinetics and that the different isotopic labels characterized kinetically homogeneous sterol pools. The uncertainties inherent in such calculations based on concentration measurements at only two points in time are well known (29). Moreover, the possible differences between the rates of turnover of cholestanol-^{3}H and its known metabolite, Δ^7-cholestenol-^{3}H (19), have not so far been studied, yet they may well be of considerable interest. Nevertheless, such calculations leave no doubt that in many tissues the half-life of the free cholesterol pool is significantly longer than that of the free sparing sterol pool under both sets of experimental conditions used. This difference is most striking in the muscle and in the gut tissues other than the crop, where there is no measurable turnover of free cholesterol but the approximate half-life of the sparing sterol pool is only 1.6 and 1.1 months respectively. It is remarkable that in the crop, the principal site of absorption of cholesterol from the gut (23), despite the high flux of cholesterol when the insect consumes a diet containing 5% cholesterol, the half-life of the previously incorporated, unesterified cholesterol is about 2 months.

Storage of Sterols in the Fat of Growing Insects

The changes in the labeling of the ester fraction indicate that in the body tissues in both experiments depicted in Tables 4 and 5 (and also in the gut tissues in the experiment with minimal cholesterol) there is a shift of sterol incorporated in the earlier part of the experiment from the free to the esterified pool. The fat is exceptional in that it shows a marked accumulation of labeled sterol in both the free and esterified forms (mainly the latter) in both experiments. Analysis of the esterified sterols of the fat by GLC showed that they contained more than 80% Δ^7-cholestenol, a proportion much in excess of that found in any other tissue (30).

In the first experiment (Table 4) it was calculated that the gain in labeled sterol by the fat during the second phase was 0.18 μg of cholesterol and 14.3 μg of ^{3}H-sterol while the losses from the other tissues were 0.36 μg of cholesterol and 9.85 μg of ^{3}H-sterol. (The apparent gain by the fat of a larger amount of ^{3}H-sterol than was lost from the other tissues is due to the failure to include the remaining carcass in the analyses after dissecting out "all other tissues.") In the second experiment (Table 5) a similar calculation showed that approximately half of the labeled sterol displaced from other tissues was trapped by the fat. These calculations are necessarily approximate but serve to show that the fat effectively traps and stores sterols as their esters displaced from the other tissues during growth. Determination (as digitonides) of the concentrations of total sterols in the fat and other tissues at the end of the second experiment showed that storage of newly ingested sterol also occurred. A 20-fold increase occurred in the sterol concentration in the fat, but other tissues showed only a 2- to 3-fold rise, while some showed no measurable increase. Comparable results were obtained when insects were reared on a diet containing 1.0% cholesterol.

Storage of sterols is probably related to the needs of the insect for sterol to be incorporated into reproductive tissues during maturation. Monroe (31) has reported evidence for the utilization of sterol stored during larval development in the course of egg production in the housefly and similar evidence for sterol storage by *Dermestes vulpinus* during growth has been obtained in our laboratory.[1] The results of the present experiments indicate that the capacity for sterol storage on a significant scale is confined to the growing insect. The data of Table 3, and of other experiments[3] in which adult insects were fed for several months on a diet containing 5% labeled cholesterol, showed no marked accumulation of sterol in the fat. The results suggest that the storage of sterols in the fat body is under hormonal control. This question is being further investigated.

The mechanism by which newly ingested sterol displaces previously incorporated sterol molecules from the free to the esterified pool of the tissues and thence into the esterified (storage) pool in the fat is at present unknown. Cholesterol ingested as the free sterol is conveyed from the gut to the body tissues via the hemolymph, almost exclusively in the unesterified form (23). The observed results would be accounted for if this sterol were taken up more readily than esterified sterol, into the tissues other than the fat, while the fat had an equal or greater affinity for the ester. Specific sterol-transferring lipoproteins of the hemolymph may also be responsible, either entirely or in part, for the observed effect (32).

The results of this study all indicate that the sterols of the tissues of *E. floridana* reared with minimal dietary cholesterol, supplemented with cholestanol, are distributed in three major compartments in which they may be distinguished from each other by their structural specificity, their dynamic state, and their state of esterification. The cholesterol seems most likely to be required primarily to fill a functional space of high structural specificity.[4] It is unesterified and has a long half-life which, in some tissues of the growing animal, amounts to an absence of measurable turnover. Another functional space, requiring unesterified sterol, is apparently less structurally specific and the sterol in it has a distinctly greater mobility than cholesterol. Since, in these insects' tissues, the cholesterol-sparing "sterol" consists of a mixture of cholestanol and Δ^7-cholestenol, both free and esterified, it is further possible that these two sterols also do not function identically. Evidence for differences in the distribution of these sterols (19), together with more detailed studies described in the following paper (33), makes it seem likely that this is indeed the case. The third major sterol compartment comprises the sterol esters. There is good evidence for a role of esterification in relation to sterol storage in the fat of the cockroach, which may be analogous to the storage of cholesterol esters in the liver of the newly hatched chick (34); but the functional significance of the sterol esters in other tissues remains uncertain. It is not yet known, for example, whether the relative size of this compartment might be reduced under conditions of sterol deprivation.

The occurrence of cholesterol almost exclusively in the unesterified form suggests its structural involvement in the membranes of mitochondria and of the endoplasmic

[4] It is emphasized that the view presented here and in earlier studies with *Dermestes vulpinus* (24), that cholesterol is required to fill a structurally specific space in the subcellular membranes of these species, does not imply that this must be true of insect in general. There is evidence that this is not the case in the housefly (39) and recently a nutritional study was described in which, for the first time, it was shown that an insect (*Drosophila pachea*) that normally utilizes a phytosterol, Δ^7-stigmastenol, could not utilize cholesterol as its sole dietary sterol (40).

reticulum in a manner analogous to that of mammalian subcellular membrane systems (35–37). On the other hand, its extraordinarily low rate of turnover in the insect's tissues is more reminiscent of the behavior of cholesterol as a component of vertebrate myelin (5) and prompts the speculation that a stable membrane structure may permeate the tissues of the insect. This possibility is interesting in view of Wigglesworth's suggestion on quite different grounds (38) that an orienting "cyto-skeleton" is present in insect tissue.

In the following paper (33) evidence is presented for the occurrence of unesterified cholesterol, cholestanol, and Δ^7-cholestenol as components of all the subcellular membrane structures of the tissues of *Eurycotis floridana*.

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