The intracellular distribution of sterols in *Eurycotis floridana* and its possible relation to subcellular membrane structures

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ABSTRACT The roach *Eurycotis floridana* was reared on a semisynthetic diet containing minimal cholesterol-4-^{14}C (0.005%) supplemented with cholestanol-7α-^{3}H (0.1%), and various tissues of the insect were separated into "nuclear," "mitochondrial," and "microsomal" fractions by differential centrifugation. Muscle and salivary gland were satisfactorily fractionated and results from these are reported together with some, as yet, incomplete results for nerve.

The major concentrations of unesterified sterol and lipid phosphorus were in the mitochondrial and microsomal fractions.

The unesterified sterols of the subcellular fractions were separated by alumina and gas–liquid chromatography. The emergent peaks corresponding to cholesterol, cholestanol, and Δ^7-cholestenol (a metabolite of cholestanol) were assayed for ^{3}H and ^{14}C. No sterol was confined to any one subcellular fraction. The proportions of the three sterols were closely similar in all membranous components of the same tissue, but were different in different tissues.

It is suggested that the different subcellular membranes of a given tissue contain similar repeating structural units in which the individual sterols occupy sterically characteristic spaces.

KEY WORDS roach · muscle · salivary gland · nerve · fractionation · sterols · subcellular distribution · membrane structure · repeating unit

THE COCKROACH *Eurycotis floridana*, in common with other insects that have been carefully studied (1), has a dietary requirement for sterol which can be satisfied entirely by cholesterol. Under conditions of aseptic rearing 95% of the cholesterol requirement can be "spared" by cholestanol, which is partly transformed in the insect's tissues to Δ^7-cholestenol (2, 3). The preceding paper (4) describes a study of the distribution and turnover of the sterols present in the tissues of these insects when they are reared on an artificial diet containing 0.1% cholestanol-7α-^{3}H and 0.005% cholesterol-4-^{14}C. The results of that study suggest that the bulk of the unesterified sterol becomes incorporated into the tissues of the insect in at least two compartments or "functional spaces" that are characterized by the structures of the sterols which they accommodate under the conditions of the experiment.

There is extensive evidence (5–12, 14) that unesterified sterols are associated with phospholipids as structural components of the subcellular membranes of mammalian tissues. An analysis of the intracellular distribution of sterols in the tissues of the roach, reared under the above conditions, has therefore been undertaken in the expectation that it would reveal something of the similarities or differences between the functional spaces accommodating the sterol molecules in the different membranous subcellular components. Such a study is potentially interesting in relation to the concept of continuity or common structural origin of the subcellular membranes that has been proposed on the basis of the findings of electron microscopy (13).

The functional differences between the membranes of the cell wall, the mitochondria, the endoplasmic reticulum, and the nucleus presumably account for the fact

Abbreviations: TCA, trichloroacetic acid; GLC, gas–liquid chromatography.

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that their total lipid contents, even within the same tissue, are appreciably different (14), but if the concept of their continuity or common origin is valid, it is to be expected that they should possess some essential structural component in common. This point has recently been emphasized by Vandenheuvel in discussing the intermolecular interactions of the components of membrane systems (15). If it is assumed that the sterols contribute to this common element of membrane structure by occupying structurally different functional spaces that are specific for each type of sterol molecule, then the distribution of sterols in all of the subcellular membrane systems of a tissue should be the same.

There are many alternative possibilities, but the following may be briefly considered. The sterols may not be exclusively concerned with the maintenance of the structural integrity of the membranes but may have some other special "metabolic" functions (e.g., in stabilizing the conformations of certain specific enzymes). Further, even if the sterol is exclusively a structural component, the membrane matrix to which it contributes may in fact be different in the different subcellular membrane systems. In either of these cases it might be expected that the distribution of different sterols in the various subcellular membranes would be different. Yet another alternative is that a random arrangement of sterols may lead to their appearance in similar proportions in all of the membranes of the cell on a purely statistical basis. This seems unlikely, in view of the great importance generally attached to highly ordered structures in living systems, and is rendered all the more improbable by the fact that each tissue of the insect's body has its own characteristic proportion of different sterols in the experiments described earlier (4).

With these various possibilities in mind, the subcellular distribution of sterols was studied in several different tissues of *E. floridana* reared under "cholesterol-sparing" conditions in which the diet contained minimal cholesterol-4-^{14}C supplemented by cholestanol-7α-^{3}H. Particular attention was paid to the unesterified sterols which, as expected, were found localized almost exclusively in the membranous subcellular fractions. Extraction of the sterols and their separation by chromatography and final assay by radioactivity measurements have given evidence that the different sterols are incorporated into all the membranous systems of the cell in a constant relationship to each other. A preliminary report of this work has been given (16).

EXPERIMENTAL PROCEDURE

Materials and Methods

The roaches *Eurycotis floridana* were of the same origin as those described in the preceding paper and were reared by the same aseptic technique on the same synthetic diet containing cholesterol-4-^{14}C (0.005%) and cholestanol-7α-^{3}H (0.1%). The source of sterols and method of preparation of cholestanol-7α-^{3}H were also as described in the preceding paper (4).

Preparation of Tissues and Fractionation Procedures

The insects were inactivated in ice water and dissected in a cold room at 0–5°C with a dissecting microscope, and the tissues were placed immediately in ice-cold sucrose medium (see below). In most experiments samples of tissue from several insects were pooled. Great care was taken to secure samples of individual tissues as free as possible from contamination by others, and particular attention was paid to the removal of fat that adhered to the nerve cord. The various collected tissues were weighed after brief blotting with filter paper.

The tissues were homogenized with 0.32 M sucrose–0.01 M phosphate buffer solution, pH 7.7 at a concentration of 50 mg of tissue per ml for muscle and fat body, and 15 mg/ml for other tissues studied. These concentrations of tissue, which are lower than those generally used in fractionation of mammalian tissues, were dictated by the small weights of tissues available and the necessity of avoiding work with inconveniently small volumes of homogenate. Homogenization was performed in the smallest convenient homogenizers with pestles of Teflon on stainless steel shafts and was carried out as gently as possible, since early experiments quickly revealed that the mitochondria were highly fragile. The final volume of the homogenate was measured in a graduated conical glass centrifuge tube and a small aliquot was removed for analysis. Homogenates of tissues other than muscle were centrifuged without further treatment, but that of muscle was filtered under slightly reduced pressure through a loose mesh of teased cotton in a Hirsch funnel or small Büchner funnel in order to remove fragments of unbroken tissue. The fractionation procedure, essentially similar for muscle, salivary gland, and nerve, is outlined in Fig. 1. The sedimented material was washed by resuspension in a volume of medium equal to about one-half the volume of the suspension from which the particulate material was isolated, and then recentrifuged. Resuspension was carried out with the aid of a Vibro-mixer fitted with a small-bore glass rod with a flattened end. Supernatant fluids were withdrawn from the centrifuge tubes and transferred for recentrifugation by means of disposable Pasteur pipettes which were washed with a few drops of medium; the washings were combined with the transferred fluid. The washed sediments were generally kept at 0°C until the whole fractionation was completed and they could be analyzed. For assay of succinic dehydrogenase, the sedimented materials were resuspended im-

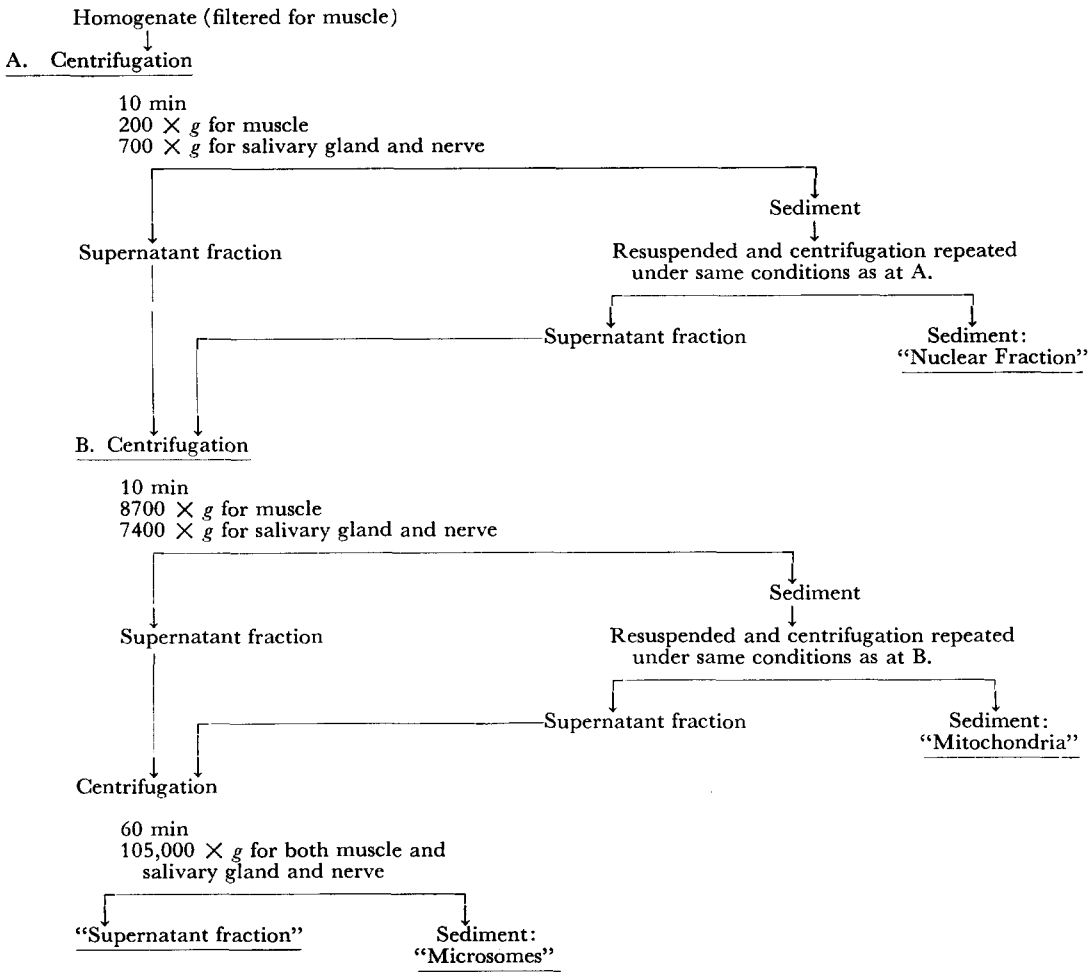

FIG. 1. Fractionation scheme for *E. floridana* (muscle, salivary gland, and nerve).

mediately after isolation in 0.1 M phosphate buffer, pH 7.7 containing 0.005 M disodium EDTA.

Evaluation of Tissue Fractionation Procedures

Since methods for the total fractionation of insect tissues as required for our purposes had not previously been described, it was necessary to show that the procedures adopted were adequate. For this purpose many trial fractionations were carried out with various modified methods until conditions were arrived at which consistently gave an acceptable degree of segregation of the various subcellular fractions. The fractions were analyzed for DNA, RNA and succinic dehydrogenase as indicators for the major concentrations of nuclei, microsomes, and mitochondria respectively. All fractions were also analyzed for protein.

Analysis for Protein. Protein was measured by the microbiuret method of Westley and Lamberth (17) on trichloroacetic acid (TCA) precipitates of aliquots of the whole homogenates and of the various subcellular fractions. The TCA precipitates were dissolved by allowing them to stand with N NaOH for not more than 30 min at room temperature. When necessary, the rate of solution was increased by heating in boiling water for less than 5 min, but this was found to decrease color formation in the assay. Measurements were made in a Klett-Summerson colorimeter (A. H. Thomas, Philadelphia, Pa.) with a No. 44 filter. The uric acid stored by *Eurycotis* makes the method of Lowry (18) inapplicable.

Nucleic Acids. After tissue samples had been extracted according to the procedure of Schmidt and Thannhauser (19) the precipitates were hydrolyzed in 5% TCA for 30 min in a boiling water bath. Aliquots of the hydrolysate were then taken for measurement of RNA by the method of Webb (20) and of DNA by the method of Webb and Levy (21). For the RNA assay, xylene was treated with concentrated sulfuric acid, then distilled prior to use.

Authentic samples of bacterial DNA and RNA of tobacco mosaic virus were obtained from Dr. P. Doty, Department of Chemistry, Harvard University, and the results of the assay of these were used for the determination of correction factors that must be applied when

commercially available DNA and RNA are used as standards.

Color measurements were made in a Klett-Summerson colorimeter, with a No. 44 filter for RNA assay and No. 54 filter for the DNA assays.

Lipid Phosphorus. Extracts prepared with ethanol and ether according to Schmidt and Thannhauser were pooled, the solvent was evaporated, and the lipid sample obtained was analyzed for phosphorus by the method of Fiske and Subbarow as modified by Allen (22). Our only modifications were that the final total volume was 10 ml and that *p*-(*N*-methyl)aminophenol (Elon, Eastman, Rochester, N. Y.) was the organic reducing agent. One milliliter of perchloric acid was added to the dry sample and the tube heated to 140°C until the contents became clear. Readings were made in a Klett-Summerson colorimeter with a No. 66 filter.

Succinic Dehydrogenase. The assay of succinic dehydrogenase in subcellular fractions was based on the colorimetric assay of Nachlas, Margulies, and Seligman (23). It was found difficult to account for all the enzyme activity of the homogenate when adding together activities in the various fractions. A great deal less activity was lost, however, if the washed mitochondrial pellet was immediately resuspended in phosphate buffer, 0.1 M, pH 7.7, containing 5 mM disodium EDTA. In addition, activity was enhanced at the addition of EDTA to the assay medium (but not by addition of bovine serum albumin). Accordingly, the final assay medium contained the following:

Substance	Volume
	ml
Succinate, 0.2 M	0.5
Phosphate buffer, 0.1 M, pH 7.7	1.5
Gelatin 0.1%	0.5
EDTA, 0.02 M, pH 7.7	0.5
2-*p*-Iodophenyl *p*-nitrophenyl 5-Phenyltetrazolium chloride (INT) (2 mg/ml)	1.0
Phenazine metho-sulfate (8 mg/ml)	0.5
Enzyme in phosphate buffer	0.5

The first five components could be mixed and kept frozen until ready for use; the last two were added just prior to assay. The assay was stopped by placing the tubes in ice and the readings were made in a Klett-Summerson colorimeter with a No. 54 filter.

Since it was found that some samples, especially those from microsomal fractions, gave rise to a nonspecific reduction of tetrazolium dye, a control tube was run for each sample. This tube contained enzyme, but 0.1 M fumarate was substituted for succinate. Blanks contained no enzyme. The color production was found to vary linearly with the amount of a suspension of mitochondria. For these experiments:

one enzyme unit =

$$\mu g \text{ INT reduced per min} = \text{Klett units} \times 0.08$$

This formula was determined by performing an assay with a constant enzyme concentration but with various amounts of INT which were completely reduced under the assay of conditions. The slope of the resulting curve indicated 0.08 Klett unit/μg of dye reduced, and the assay was routinely carried out in duplicate for 15 min.

Disodium fumarate and succinate were obtained from Calbiochem, Los Angeles, Calif., INT from Dajac Laboratories, Borden Chemical Co., Philadelphia, Pa.

Analysis of the Fractions for Sterols

The washed pellets obtained from the fractionation procedure (Fig. 1) were extracted, as described for the whole tissues (4), with ethanol, acetone, and ethyl acetate. The extractions were carried out in centrifuge tubes and the supernatant solutions were removed after centrifugation at 5000 × *g* for 10 min. The soluble supernatant fraction remaining after separation of the microsomal fraction was extracted with ether and alcohol as described for serum (24). After the final extract had been partitioned between water and ether, the ether phase was washed three times with water to remove sucrose. All lipid extracts were evaporated under a stream of pure nitrogen in a water bath at about 50°C. The residues were stored, when necessary, at −5°C under pure nitrogen.

The esterified sterols, unesterified sterols, and more polar sterol metabolites were separated either by chromatography on alumina as previously described (4), or by thin-layer chromatography on Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) with benzene–ethyl acetate 4:1 as the solvent (25). The areas of silica gel containing the sterol ester and free sterol respectively were transferred to small sintered glass filters and eluted with 20 ml of ether. The area of the plate containing the polar metabolites was eluted with 20 ml of methanol–ether 1:4.

The free sterol fraction was analyzed further by GLC after conversion of the sterols to their methyl ethers, under conditions previously described (26). The methyl ethers of cholestanol, cholesterol, and Δ^7-cholestenol were trapped on emergence from the detector by leading the effluent argon stream through 3 mm glass tubing into a small test tube immersed in liquid nitrogen in a larger test tube, which in turn was placed in a small Dewar flask containing liquid nitrogen. When the collection of a fraction was completed, the conducting tube was detached from the outlet of the gas chromatograph and the larger test tube, containing the smaller tube and conducting tube, was allowed to stand at room temperature. In this way the evaporation of the argon that had condensed in the smaller tube was allowed to take place

TABLE 1 EFFICIENCY OF SEPARATION OF SUBCELLULAR FRACTIONS OF MUSCLE, SALIVARY GLAND AND NERVE IN *E. floridana*

Fraction	Muscle				Salivary Gland				Nerve	
	DNA	RNA	Succinic Dehydrogenase	Protein	DNA	RNA	Succinic Dehydrogenase	Protein	Succinic Dehydrogenase	Protein
Nuclei	92	0	28	25	67	9	27	17	24	6
Mitochondria	0	10	72	5	12	8	56	12	57	6
Microsomes	0	51	0	6	19	65	17	15	19	4
Supernatant fraction	7	39	0	64	1	18	0	56	0	84
% Recovery	61	100	67	83	100	100	58	100	71	100

Values are given as percentage distribution in various fractions and percentage recovery based on assay of an aliquot of the whole homogenate.

slowly and loss of radioactive material by explosive boiling was prevented. The radioactivity of the recovered material was assayed for ^{3}H and ^{14}C by means of a dual channel liquid scintillation counting system as previously described (4). In the present experiments the conducting tube and collecting test tube were rinsed with a total of 15 ml of toluene scintillation fluid, which was then transferred to vials for counting.

RESULTS AND DISCUSSION

The fractionation of five tissues of *E. floridana* has been attempted: muscle, salivary gland, nerve, fat body, and mid-gut. Complete results which clearly indicate acceptable fractionation have been obtained from the first two. These results are therefore reported together with data from a partial analysis of nerve, which are in general agreement with the findings for salivary gland and muscle. The fat body, an abundant and readily isolable tissue, has unfortunately so far proved difficult to study, partly on account of the high concentration of free fat, which interferes with the segregation of the different particulate fractions, but also because of the presence of excretory microcrystals of urate (27) and intracellular symbionts or bacteroids (28). These cell inclusions contaminate the nuclear and mitochondrial fractions. The unsatisfactory results obtained with the mid-gut tissue were probably due, in part, to autolytic action of digestive enzymes secreted by this part of the intestine. Moreover, this section of the gut comprises many different cell types with widely varying secretory and absorptive functions, besides layers of circular and longitudinal muscle cells. This cellular heterogeneity, together with individual variations in metabolic state, almost certainly contributes to the difficulty of obtaining consistent results with this tissue.

From Table 1 it can be seen that the fractionation method finally adopted gave the major concentration of DNA in the "nuclear" fractions from muscle and salivary gland but that, in keeping with the simplicity of this fractionation procedure, there was an overlap of succinic dehydrogenase, characterizing the mitochondria, into the nuclear fraction. In the case of the muscle somewhat more than one-fourth of the mitochondrial fraction could have contaminated the nuclear fraction, while in the preparations from salivary gland and nerve, the overlap was closer to one-third. The contamination of the nuclear fraction by mitochondrial and microsomal material was shown by microscopical examination to be due partly to the adhesion of mitochondria to the nuclei and partly to the presence of some unbroken cells.

A more rigorous purification of the nuclei of the salivary gland was therefore undertaken, with a sucrose density gradient. The nuclear fraction from the preparation was devoid of succinic dehydrogenase activity and now contained only 5% of the total free sterol of the homogenate, an amount that was inadequate for analysis by GLC, but it was possible to show that the ratio of ^{3}H:^{14}C sterols in this preparation was identical with that of the mitochondrial and microsomal fractions.

As was to be expected, the results with respect to the mitochondrial and microsomal fractions were more satisfactory, since in the muscle preparation the microsomes contained about five times as much RNA as the mitochondria and in the salivary gland about eight times as much. On the other hand, although no succinic dehydrogenase was measurable in the microsomal fraction of muscle, that of the salivary gland contained 30% of the amount of succinic dehydrogenase found in the mitochondria. A similar overlap of succinic dehydrogenase into the microsomal fraction was found in the preparation from nerve.

Electron micrographs prepared in the Department of Biology, Harvard University, through the courtesy of Dr. Keith Porter, qualitatively confirmed the results of the above analyses.

Several previous studies of mammalian tissues have shown that the unesterified sterol and phospholipids (5–12, 14) of the cell are predominantly associated with the mitochondrial and microsomal fractions, and similar results are reported here for the roach (Table 2). Convincing proof that the unesterified sterols are essentially

TABLE 2 CONTENT OF STEROL AND PHOSPHOLIPID IN SUBCELLULAR FRACTIONS OF MUSCLE, SALIVARY GLAND, AND NERVE

Fraction	Muscle			Salivary Gland			Nerve		
	Phospho-lipid	Sterol Ester	Free Sterol	Phospho-lipid	Sterol Ester	Free Sterol	Phospho-lipid	Sterol Ester	Free Sterol
		μg/mg protein			*μg/mg protein*			*μg/mg protein*	
Nuclei	79	0.91	1.45	68	0.15	8.66	90	0.17	8.77
Mitochondria	400	2.10	8.75	115	0.27	11.06	237	1.58	10.18
Microsomes	520	0.34	14.42	223	0.12	12.01	252	1.0	13.61
Supernatant	9.5	0.18	0.34	10.4	0.04	0.36	0	0.28	0.33

Results for "phospholipid" are derived from lipid phosphorus assays by multiplying the lipid phosphorus value by 25. The values for sterols are for ^{14}C and ^{3}H sterols combined.

TABLE 3 MOLAR PROPORTIONS OF CHOLESTEROL-4-^{14}C, CHOLESTANOL-7α-^{3}H AND Δ^7-CHOLESTENOL-7-^{3}H IN SUBCELLULAR FRACTIONS FROM MUSCLE, SALIVARY GLAND, AND NERVE

Fraction	Muscle		Salivary Gland		Nerve
	Molar Ratios	Mean	Molar Ratios	Mean	Single Analysis
Nuclei	1:2.0:1.2		1:3.4:0.9		1:0.70:0.07
		1:2.3:1.2		1:3.8:1.0	
	1:2.5:1.2		1:4.2:1.1		
Mitochondria	1:1.9:0.8		1:3.7:1.0		1:0.52:0.05
		1:2.3:0.9		1:3.8:1.1	
	1:2.6:0.9		1:3.8:1.0		
Microsomes	1:1.9:0.8		1:4.2:1.2		1:0.65:0.06
		1:2.0:0.9		1:4.3:1.3	
	1:2.0:0.9		1:4.4:1.4		
Supernatant fraction	1:3.5:1.5		1:5.6:0.4		1:0.82:0.12
		1:5.5:1.5		1:16.4:2.3	
	1:7.4:1.5		1:27.1:4.1		

For the muscle and salivary gland, values from analyses of two insects are presented separately, together with mean values. Results of a single fractionation of nerve are given (with cholesterol arbitrarily = 1 in each case).

bound to the membranous elements of these subcellular components calls for a more detailed analysis which is in progress. In a preliminary study, treatment of the microsomal fraction of the salivary gland with ribonuclease, followed by recentrifugation for 60 min at 105,000 × *g* resulted in no loss of unesterified sterol from the sedimented material and the ^{14}C:^{3}H ratio of this fraction was unchanged. The esterified (^{3}H) sterol, however, was solubilized to the extent of 25% by this treatment. The results shown in Table 2 indicate that the ratios of sterol to phospholipid in the mitochondrial and microsomal fractions are of the same order as those of mammalian tissues, though they are somewhat lower. The characterization of the phospholipids of these insects' tissues has not yet been completed, but results so far obtained indicate the presence of phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, and phosphatidyl inositol as major components.[1] It seems probable, therefore, that the structural role of sterols in the insect is analogous to that in the mammal and that it involves essentially similar intermolecular association with the phospholipids in the membrane structure. Presumptive models of these associations have frequently been discussed (15, 29–32).

Table 3 shows the distribution of the three unesterified sterols cholesterol, cholestanol, and Δ^7-cholestenol in the various cell fractions obtained from muscle, salivary gland, and nerve. It is clear that no individual sterol is incorporated selectively into any one fraction in any of the tissues.[2] On the contrary, the proportions of the three sterols are clearly very similar in all subcellular fractions from the same tissue, but these proportions are markedly different for the different tissues. It is tempting to speculate that the values obtained may reflect a constant whole number relationship: for muscle 1:2:1, and for salivary gland 1:4:1. In the case of the nerve, the closest whole number ratio would be 17:10:1, a relationship that is strikingly different from the other two. In seeking an explanation for this difference it might be important to recall that the nervous tissue of the insect, as of mammals, consists of two major categories of cells, the glial and the neurones. Conceivably Δ^7-cholestenol is excluded

[1] Wientjes, C. M., and R. B. Clayton, unpublished work.

[2] This conclusion is consistent with the results of an extensive autoradiographic histological study of the distribution of labeled sterols in the various tissues of the insect (Zanon, C., N. L. Lasser, and R. B. Clayton, unpublished work).

entirely from the glial cells while being present in higher proportions in the neurones.

It has been pointed out in the foregoing discussion that the various subcellular fractions obtained by our procedures show some degree of cross-contamination (Table 1) but it seems unlikely that if the true ratios of sterols were appreciably different among the different fractions this degree of mixing could account for the similar proportions of sterols found in this study. The significance of the results is increased by the fact that the proportions of sterols in the supernatant fraction differ considerably from those in the particulate fractions obtained from the same homogenate (Table 3). This makes it unlikely that the uniformity of distribution is a result of nonspecific absorption of the sterols from the supernatant fluid. Moreover, although the results obtained with the two other tissues which have been studied cannot be considered entirely acceptable at the present time (for the various reasons outlined above), they all point to a similar constant pattern of distribution of the three sterols among the various subcellular fractions of the same tissue.

It is clear from the results reported in the preceding paper (4) that under the dietary conditions used (cholesterol present in an amount minimal for growth, together with 0.1% cholestanol) cholesterol is selectively incorporated into the unesterified sterol fraction of each tissue in preference to cholestanol (and Δ^7-cholestenol). The extent of this selective incorporation is characteristic of each tissue and since cholesterol is available in minimal amounts it is assumed that its distribution characterizes the distribution of a functional space that is specific for cholesterol.

The question of whether cholestanol and Δ^7-cholestenol are also structurally specific for the spaces which they occupy in the membranes is at present more difficult to answer and is being studied further. It is not clear, for example, whether all of the insects' tissues can effect the desaturation of cholestanol to Δ^7-cholestenol. Earlier work (2) suggests that it is more likely that certain tissues of the gut are more active in this respect than other tissues such as fat body and muscle. If this is so, then the availability of the two sterols for incorporation into various organs and tissues should be dependent both upon the relative efficiency of mechanisms for their transport and upon their relative affinity for receptor binding sites. Alternatively, the activity of an intracellular Δ^7-dehydrogenase system might control the relative intracellular concentrations of the two sterols and in some tissues both transport and dehydrogenase activity might be controlling factors.

In any event, nonspecific incorporation of cholestanol and Δ^7-cholestenol into the membrane structure would most probably lead to a pattern of distribution in which the ratios of unesterified sterols in the supernatant fraction were identical with those found in the membranes. This is clearly not the case (Table 3) and some form of selectivity in the binding of the different unesterified sterols into the membranes seems at present to be the most reasonable assumption. Such selectivity might be due to the presence of either a single type of structural space with a different affinity for each of the two sterols, or two topographically different spaces, each with its own selective affinity for a particular sterol. There might also be a combination of these circumstances (i.e. different, but not completely structurally specific, spaces that would allow some overlap of incorporated sterol types). The closely similar proportions of the three sterols in all fractions, except the supernatant, suggest the same average frequency of occurrence of at least two and possibly three topographically different functional spaces for unesterified sterol molecules in all the subcellular membrane systems of the same tissue (4). The marked differences between the proportions of the three sterols in the various tissues indicate corresponding differences in the average frequency of occurrence of these different types of spaces.

In view of the uncertainties, previously discussed (2), concerning the relative specific activities of cholestanol-7α-^{3}H and the Δ^7-cholestenol-7-^{3}H derived from it, the molar values derived from the radioactivity measurements in the present experiments can only be regarded as approximate. Attempts to relate the structures of sterols and associated membrane lipids in a rational spatial arrangement, which we aim to undertake, will obviously require much further work and must await the results of more precise analyses in terms of mass-distribution of the sterols. However, preliminary work along these lines, using a gas chromatograph equipped with a hydrogen flame detector, indicates that the distribution of the different sterols according to mass also shows a constant relationship in the various subcellular fractions.

It is also obvious that the analytical approach used here can only provide statistical information concerning the distribution of membrane lipids and cannot by itself determine whether these compounds are present in any regularly recurring spatial pattern. However, the concept of membrane continuity, with the sterols serving as components in a common repeating structural unit, as outlined in the Introduction, is consistent with the results so far obtained. The alternative possibility that the sterols are taken up in a spatially random, yet statistically equivalent relationship in all the different subcellular fractions of the same tissue, but enter different tissues in distinctly different proportions, is more difficult to rationalize. The differences between the proportions of sterols in the membrane fractions of the different tissues suggest corresponding structural differences between the

repeating units of membrane structure, which may reflect tissue specificity of structural proteins of the membranes. Such structural differences might reasonably be expected to underlie the great functional dissimilarities between the tissues.

The concept of repeating subunits of membrane structure that may be similar in both the mitochondrion and the endoplasmic reticulum has been discussed from a variety of viewpoints, notably by Fernandez-Moran and Green and their collaborators (33 and other references therein). The concept has been developed to the point at which consideration of hypothetical mechanisms of assembly of such subunits has been deemed appropriate (34). Where these discussions have centered upon the chemical components of the membrane systems, the cholesterol, which is a constant though quantitatively minor constituent, has consistently been neglected and attention has been paid only to the phospholipids as the major (and, supposedly, more important) lipid constituents (35, 36). In the present experiments sterols, which are indispensable in the diet of this insect, are mainly utilized by incorporation into the mitochondria and endoplasmic reticulum, together with phospholipids, in concentrations which are of the same order as those found in mammals, and their slow rate of turnover (4, 37) can be readily rationalized only by the assumption that they perform a function which is essentially structural rather than metabolic. It seems questionable, therefore, whether adequate models of the membrane structures in the mammal, no less than in the insect, can be derived without taking their sterol component into account.

It will be clear from this discussion that the interpretation of our results in terms of repeating structural units accommodating sterol molecules in the subcellular membrane system is entirely tentative. It is offered as an indication of what promises to be a useful new approach to the problem of the structural role of sterols and as a stimulus to further investigations in this area. In this connection, however, it is important to emphasize that we are considering the possible existence of one particular type of repeating structural unit, common to all subcellular fractions, that accommodates most, if not all, of the unesterified sterol. On the evidence of many published data, such a structural unit cannot accommodate all of the phospholipids, though presumably it accommodates some of them. From this point of view, studies such as that of Ashworth and Green (38), in which it was noted that analyses of the total lipid constituents of various subcellular fractions offered no support for the existence of common structural units, are irrelevant.

The authors are grateful to Dr. Konrad Bloch for his continued interest in this work.

These studies are supported by Grants-in-Aid from the American Heart Association and the Santa Clara Heart Association and by Grant GM-12493 from the National Institutes of Health. This work will form part of a thesis to be presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Harvard University, by N. L. Lasser.

Manuscript received 29 October 1965; accepted 11 February 1966.

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