Behavior of sterols of insect muscle during homogenization and differential centrifugation

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ABSTRACT Labeled sterol introduced in vitro into a homogenate of insect muscle became distributed in the various subcellular fractions in a manner similar to that obtained by fractionation of muscle of insects to which labeled sterol had been administered in vivo. If two differently labeled sterols, cholesterol and cholestanol, were added to the in vitro preparation, the ratio of concentrations of the two compounds remained the same for the supernatant as for the particulate fractions. This was true for widely different total concentrations of the sterols. This result differs from that previously reported for the in vivo incorporation of these sterols and is viewed as further support for the proposal that some form of structural selectivity regulates the in vivo incorporation of the sterols into the membranous parts of the cell.

Mitochondria into which labeled sterols had been incorporated under various conditions, both in vitro and in vivo, were combined with a second, unlabeled homogenate which was then fractionated in the usual way and assayed for radioactivity to assess the extent of redistribution of labeled sterol to other fractions. The results indicate that in the usual homogenization and fractionation procedure there is little redistribution of mitochondrial sterol that has been incorporated under long-term in vivo conditions. Sterol taken up by the mitochondria in vitro was redistributed to a significantly greater extent and somewhat similar results were obtained with sterol incorporated under short-term in vivo conditions. No differences between cholesterol and cholestanol with respect to their tendency to redistribute to other subcellular fractions could be detected in any of these experiments.

KEY WORDS sterols · muscle cell fractionation · roach · distribution in vitro · stability of binding

AN EARLIER REPORT FROM THIS LABORATORY described experiments in which the distribution of sterols in the subcellular membrane components of insect tissues was studied by means of differential centrifugation of whole homogenates of the tissues (1). The insects, which lack the enzymes for sterol synthesis, were reared on diets containing a mixture of sterols and under conditions that were assumed to permit the incorporation of different sterols into the membrane matrix in spaces which had different structural specificities. The results of this preliminary study showed a similar distribution of the different sterols within the various subcellular membrane systems of a particular tissue, but the patterns of distribution in different tissues were greatly different from one another. On the basis of these findings it was proposed that there might be a structural element of subcellular membranes, of which the sterols were an essential component, which was common to the various membrane systems of a given cell type, but which was different for cells of different types.

In attempting to extend this work, we found that further investigations of the fractionation technique were necessary. As pointed out previously (1), the results of the fractionation studies could be open to various interpretations. Some of these would depend upon the behavior of the biological material in the course of homogenization and fractionation. Accordingly, a series of experiments has been carried out in an attempt to gain further information on the following points: (*a*) the affinity of the particulate cell fractions for sterols that might conceivably be present in the cell sap at the outset of homogenization; (*b*) the selective affinity, if any, of the particulate fractions for sterols of different structures that might be available for exchange or uptake from the supernatant fraction during homogenization; (*c*) possible redistribution of sterols initially present in a specific particulate fraction into other cellular fractions during homogenization and centrifugation; and (*d*) the firmness of binding of sterols to subcellular membranes at short and long time periods after incorporation

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

into the tissues. Information in this last area might help in establishing criteria for structurally significant binding as distinct from fortuitous adsorption.

Although the experiments to be described still leave many unanswered questions concerning the utility of the tissue fractionation technique for the purposes envisaged in our earlier work, they have supplied several points of necessary information concerning the behavior of sterols associated with subcellular fractions derived from tissue homogenates. Since this information seems likely to be of general interest, it is presented in this communication.

EXPERIMENTAL

Insects

Stock colonies of the roach, *Eurycotis floridana*, descended from insects kindly furnished by Dr. Louis Roth of the U.S. Army Quartermaster Corps, provided experimental animals for much of this work. In recent work we used stock colonies descended from insects kindly furnished by Mr. Bart Jones. Stock colonies were reared in glass aquaria on a diet of Kasco dog food with the following supplements[1]: vitamin mixture (2) 0.22%, choline hydrochloride 0.40%, corn oil 2.00%, salt mixture USP IV 4.00%, and cholesterol 0.10%. The insects were supplied with water from cotton-stoppered 150 × 25 mm test tubes and reared under 70–80% relative humidity at 27°C.

Experimental Diets and Injections

Radioactive sterols were fed as additives to a sterol-free, semi-synthetic diet (2) as in previously reported experiments (3). Samples of sterols for injections were prepared by dissolving the sterol in hot (70–90°C) 1,2-propanediol at concentrations of 52.4 mg/100 ml for cholesterol and 49.9 mg/100 ml for cholestanol. These solutions were stored at room temperature.

10 μl of the sterol solution was injected by means of a 10 μl Hamilton Syringe into the hemocoel through the soft part of the dorsal abdominal exoskeleton of an insect that had been inactivated in ice. The injection was made laterally to the midline to avoid damage to the heart and care was taken to avoid penetration of any visceral structures. The insect was then kept in isolation, given free access to water, and usually was mobile and active within an hour.

Cholesterol-4-^{14}C, cholesterol-7α-^{3}H, cholesterol-1,2-^{3}H, and cholestanol-5,6-^{3}H were obtained from New England Nuclear Corp., Boston, Mass. Sterols were purified by TLC on silica gel in benzene–ethyl acetate 1:1. The purity of the sterol was checked by dilution of an aliquot of the radioactive material with the unlabeled compound followed by GLC of the methyl ether with radioassay of the effluent material (4). Cholestanol-4-^{14}C was prepared from cholesterol-4-^{14}C as described (3) for cholestanol-7α-^{3}H. All samples of labeled cholestanol, including cholestanol-5,6-^{3}H, were treated with permonophthalic acid and then lithium aluminum hydride, and finally chromatographically purified as previously described (3).

Homogenization

The following procedures were always carried out on ice or in the cold room. After an insect was inactivated in ice water, the coxae were isolated. These were blotted, weighed, and cut into small pieces, and then homogenized for 1.75 min (12 passes) in 0.16 M KCl–phosphate buffer, at a concentration of 50 mg of muscle tissue per ml of buffer, with the smallest convenient pestle and homogenizer tube. In preliminary experiments it was determined that the muscle comprised approximately 82% of the total wet weight of the coxae, and this factor was used to determine the volume of buffer. The whole homogenate was filtered through teased cotton to remove the chitin as described previously (1).

Fractionation

The usual procedure followed the outline of Lasser and Clayton (1), with modification. The "nuclear fraction" was obtained by centrifugation at 650 *g* for 10 min, the "mitochondrial fraction" by centrifugation at 25,000 *g* for 15 min, and the "microsomal fraction" by centrifugation at 100,000 *g* for 90 min.[2] Each fraction was washed by resuspension in approximately one-half the volume of the fraction from which it was isolated, followed by recentrifugation under the original conditions.

The Experiment Testing for Redistribution of Mitochondrial Sterol to Microsomal and Supernatant Fractions

The usual experiment testing for redistribution is schematized in Fig. 1. A mitochondrial fraction, M_L, was obtained from a homogenate (B, Fig. 1) of muscle of an insect to which labeled sterols had been administered. M_L was resuspended in the supernatant fraction obtained from a homogenate (A, Fig. 1) of unlabeled insect muscle. After some purification, the resuspended mitochondrial fraction M* was mixed with a whole homogenate (C, Fig. 1) of unlabeled insect muscle which was then fractionated and analyzed for redistribu-

[1] Kasco dog food, which formerly has served as a complete diet for the growth and reproduction of *E. floridana*, is not adequate as available commercially at the present time. The deficiency has not been identified, but the addition of the supplements listed here renders the dog food entirely satisfactory.

[2] These figures are based on extensive preliminary studies carried out in these laboratories by Miss Kathryn E. Lord.

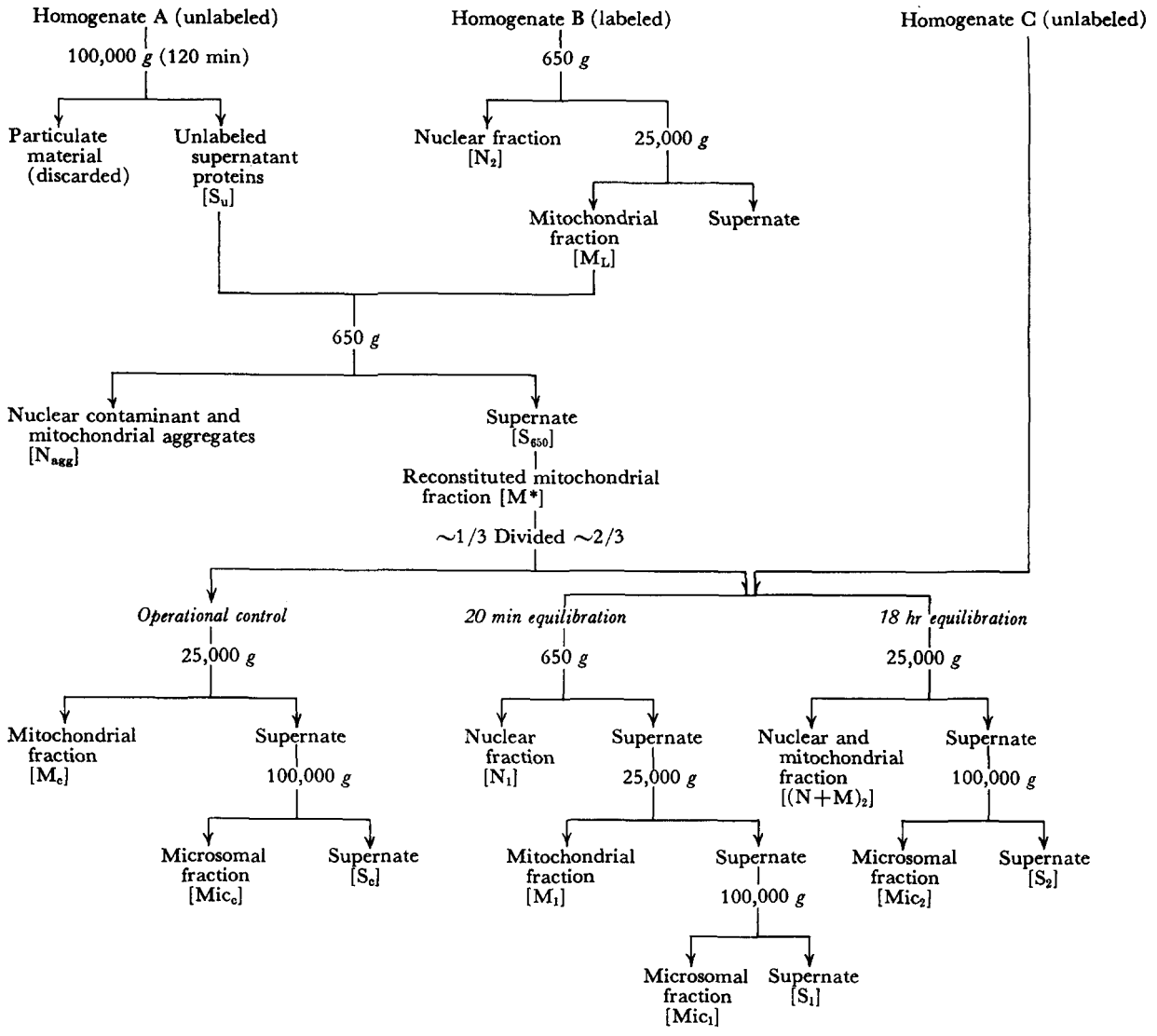

FIG. 1. The figure shows the origin and designation (in brackets) of various fractions referred to in Tables 2–5. Centrifugation at 650 *g* was always carried out for 10 min, at 25,000 *g* for 15 min, and at 100,000 *g* for 90 min, except in the preparation of S_u which was obtained by centrifugation at 100,000 *g* for 120 min. In all cases except for the preparation of S_u the particulate fractions were washed and the washings were combined with the corresponding supernatant fraction. These washings are not shown in the figure. The reconstituted mitochondrial fraction M* contained approximately $^3/_4$ of the material of fraction M_L.

tion of the label of M* to other fractions. Suitable control experiments were performed and all volumes and concentrations were adjusted so that the total concentration of mitochondrial fraction in the final system was close to that in the original homogenate B from which M_L was obtained.[3]

The unlabeled supernate (S_u), used for resuspension of the labeled mitochondria, was obtained by centrifugation of homogenate A at 100,000 *g* for 120 min (30 min longer than usual). No washings were combined in this procedure.

The labeled homogenate B, having a volume of 2.5–3 ml, was fractionated in the standard way to yield mitochondrial fraction M_L. We assayed the supernatant and nuclear fractions from this fractionation for radioactivity in order to establish that the sterol had distributed itself in the homogenate in the normal way.

2 ml of the supernate (S_u) from homogenate A was divided between the tubes in which M_L was sedimented. A Vibro-mixer fitted with a glass stirring rod as described in (1), was used to partially resuspend M_L by agitation for 30 sec. Then the combined materials were transferred to a 10 ml centrifuge tube, subjected to further agitation until

[3] In calculating these volume and concentration adjustments we ignored the amount of material lost as N_{agg}, i.e. nuclear fragments and mitochondrial aggregates, since this introduced an error of less than 10% in the concentration of the mitochondrial fraction in the final equilibration mixture.

the sediment appeared dispersed (max 1 min), and spun at 650 *g* for 10 min, a procedure designed to remove nuclear contaminations and mitochondrial aggregates that had failed to disperse. The tubes in which M_L was sedimented were rinsed with a further volume of S_u and these rinsings were used in turn to wash the 650 *g* sediment obtained above. The volume of S_u used in the rinsing procedure (and subsequent washing procedure) was 2 ml less than the volume of homogenate B. Hence, the S_{650} fraction was combined with the washings of the sediment (N_{agg}) to yield the reconstituted mitochondrial fraction, M*, in a volume similar to that of homogenate B.

Homogenate C was obtained in the usual way. A measured volume, approximately 2/3 of M*, was added to the homogenate which was then thoroughly mixed with a Vortex mixer for 2 min. The homogenate was divided into two measured quantities of approximately equal volume. One part was fractionated in the usual way, beginning 20 min after the addition of M* to the undivided homogenate C, to give nuclear fraction N_1, mitochondrial fraction M_1, microsomal fraction Mic_1, and supernatant S_1. The second part was stored at 0–4°C for 18–24 hr before being fractionated similarly to give corresponding fractions N_2, M_2, Mic_2, and S_2.

The remaining 1/3 of M* was used as a control. The control was spun in the same centrifugation runs used to prepare the fractions, M_1, Mic_1, and S_1. It thus corresponded most closely to the conditions of the 20 min equilibration experiment.

Sterol Analysis of the Fractions

All particulate fractions were extracted with chloroform–methanol 2:1 in the quartz or polyallomer centrifuge tubes in which the pellets were sedimented. The pellet together with the solvents was transferred into a 12 ml glass centrifuge tube in which it was dispersed. The supernatant solution was removed after centrifugation at 2000 rpm for 10 min and the original tubes and pellet sediments were washed twice with chloroform–methanol 2:1. The combined supernatant solutions and washings were evaporated to dryness under a stream of pure nitrogen in scintillation vials in a heating block at about 70°C. The residues were extracted three times with benzene; usually, more than 90% of the activity was recovered in the benzene extract. The residue after evaporation of the benzene extract under nitrogen was found to be suitable for direct counting in a Packard scintillation counter with a scintillation fluid containing 5 g of PPO and 0.3 g of POPOP per liter of toluene. All samples were counted until the total counts above background exceeded 5000, $P = 0.01$, percentage error < 3.0. Occasionally this criterion necessitated counting for 500 min. The benzene extraction yielded a sample for counting that was free from substances soluble in water or, in chloroform–methanol 2:1, e.g. inorganic salts, simple sugars, contaminating protein, and phospholipid. The amounts of these substances varied from sample to sample but would frequently cause excessive quenching as well as contamination of the samples with ^{40}K, which occasionally leads to serious errors in counting samples with low activity.

In Vitro Labeling Experiments

A homogenate containing labeled sterol was prepared in vitro as follows. The labeled sterol was suspended by a standardized technique in the supernatant proteins of an unlabeled insect, and muscle tissue of a second insect was then homogenized in the supernatant protein solution of the sterol.

Supernatant proteins were obtained from homogenized muscle of an unlabeled insect (or insects) as described above. We prepared the protein–sterol suspension in a centrifuge tube by first adding a measured volume of insect supernatant protein solution to the tube, then adding, drop by drop, one-tenth of this volume of sterol solution in 1,2-propanediol. During this addition the tube was agitated on a Vortex mixer at low speed and mixing was continued vigorously for 30 sec after the last drop was added. A volume of insect protein solution equal to the initial volume was now added drop by drop with continued mixing on the Vortex. To ensure removal of insoluble sterol, we centrifuged the tubes at 130,000 *g* for 120 min (both greater force and longer time than in the usual preparation of the supernatant fraction). Any traces of floating materials that occasionally appeared at the surface after this operation were drawn off by means of a Pasteur pipette. The clear supernatant solution was in turn removed by means of a Pasteur pipette into a volumetric flask of convenient size, and made up to volume with KCl–buffer solution. Aliquots were assayed for protein and for sterol.

The coxae of a second unlabeled insect were isolated, blotted, and weighed. Assuming that 82% of the gross weight represented muscle, we homogenized the coxae in the solution of sterol–supernatant protein at a concentration of 50 mg of muscle per ml.

Fractionations and studies of the redistribution of the labeled sterol were carried out as described above.

Statistical Methods

Whenever possible, the data were subjected to appropriate statistical treatment. The usual formulae were employed. Differences between experiments presented in Tables 2–5 (below) were analyzed either by the "*t*" test for unpaired variates:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{\frac{\sum_i (\bar{x}_1 - x_{1i})^2 + \sum_i (\bar{x}_2 - \bar{x}_{2i})^2}{N_1 + N_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

where df = $N_1 - 1 + N_2 - 1$, or by the "t" test for paired variates:

$$t = \frac{\bar{d}}{\sqrt{\frac{\Sigma_i (d_i - \bar{d})}{N(N-1)}}}$$

where df = N − 1. The F test was also applied using the s^2 estimates of the variance.

RESULTS

In an attempt to simulate the free equilibration of sterol between particulate and supernatant fractions, homogenates were labeled in vitro with sterols, as described above, and then subjected to differential centrifugation. Experiments using muscle tissue from adult male insects from the stock colony were performed as described, except that the homogenizations were carried out for 2 min instead of for 1.75 min, and the nuclear fraction was sedimented by centrifugation at 1500 g for 10 min. The results are summarized in Table 1, which shows the percentage distributions of label among the different subcellular fractions with two widely different concentrations of cholesterol and with mixtures of cholestanol-^{3}H and cholesterol-^{14}C, also at widely different total concentrations.

We performed four groups of experiments to determine whether the labeled sterol contained in a previously isolated mitochondrial fraction would migrate to other fractions when the labeled mitochondria were combined with a second homogenate. The results of these experiments represent the tendency for redistribution of sterols that were incorporated into the mitochondria under three different sets of conditions in vivo (Tables 2, 3, 5) as well as in the case where the mitochondria had been labeled in vitro (Table 4).

Table 2 presents the results of experiments testing for

TABLE 1 DISTRIBUTION OF LABEL IN HOMOGENATES INTO WHICH LABELED STEROL WAS INTRODUCED BY THE IN VITRO TECHNIQUE

Experiment	Total Radioactivity Recovered*	Concentration of Radioactive Sterol†	Percentage Distribution‡ of Recovered Radioactivity			
			N	M	Mic	S
	dpm	*μg/100 mg muscle*				
Cholesterol (higher concentration, average of 3 experiments)	2.71×10^6	6.78	12.5	25.4	30.0	32.1
s^2		0.09	3.9	0.7	1.5	5.9
SEM		0.18	1.1	0.5	0.7	1.4
Cholesterol (lower concentration)	3.32×10^5	0.0100	12.8	39.8	15.9	31.7
Cholestanol/cholesterol (higher concentration, ratio = 22.1)	3.24×10^5	10.46	7.9	41.2	24.3	26.6
	1.18×10^5	0.472	7.9	41.7	26.0	24.4
Cholestanol/cholesterol (lower concentration, ratio = 19.6)	5.85×10^5	0.2564	8.1	36.4	18.4	37.1
	4712	.01306	7.4	40.5	20.0	32.2

* The activity recovered from the individual fractions by the method described in the text always exceeded 90% of the activity added to the homogenates.

† The amount of radioactive sterol was calculated from the total radioactivity recovered, using the appropriate specific activities. The range of muscle mass for the six experiments above was 122–156 mg.

‡ N = nuclear fraction, M = mitochondrial fraction, Mic = microsomal fraction, S = supernatant fraction, prepared as described under *Fractionation*.

TABLE 2 REDISTRIBUTION OF LABEL TO OTHER FRACTIONS FROM MITOCHONDRIA OF INSECTS FED CHOLESTEROL-4-^{14}C

Time on Diet Containing Labeled Sterol*	Distribution of Label after 20 Min Equilibration†					Total Radioactivity Recovered‡	Distribution of Label after 18 Hr Equilibration†			Total Radioactivity Recovered‡	Distribution of Label in Experimental Control†			Total Radioactivity Recovered‡
	N_1	M_1	$(N+M)_1$	Mic_1	S_1		$(N+M)_2$	Mic_2	S_2		M_c	Mic_c	S_c	
days						*dpm*				*dpm*				*dpm*
45	18.0	67.5	85.5	11.1	3.9	3,859	84.4	12.6	3.0	3,912	85.2	9.0	5.8	4,493
60	10.9	74.7	85.6	11.3	3.0	7,535	79.4	11.8	8.8	6,087	80.4	13.7	6.0	11,291
64	18.1	64.1	82.2	13.0	4.8	6,836	76.2	16.2	7.7	6,405	76.6	15.3	8.1	8,316
Averages	15.6	68.8	84.4	11.8	3.9		79.9	13.5	6.5		80.7	12.7	6.6	
s^2	17.1	29.3	3.8	1.0	0.8		17.1	5.5	9.3		19.2	10.7	1.7	
SEM	2.4	3.1	1.1	0.6	0.5		2.4	1.4	1.8		2.5	1.9	.8	

* Insects in their final instar were selected from the stock colonies and placed upon a diet containing 0.1% cholesterol-7α-^{3}H of specific activity 10,120 dpm/μg. The muscle tissue of three of these insects that had become adults during feeding on the labeled diet was used for experiments as described in the text and outlined in Fig. 1.

† As percentage of total recovered. See Fig. 1 for designation of fractions.

‡ See footnote* to Table 1.

the redistribution of label from the mitochondrial fractions isolated from insects fed a diet containing labeled cholesterol. The column headings refer to fractions originating as outlined in Fig. 1, and the values are for the percentage distribution of label initially present in the mitochondrial fraction.

The experiment had three parts. In one experiment, designed to simulate the normal fractionation procedure, the combined homogenate and labeled mitochondria were allowed to stand for 20 min at 4°C. In a second experiment, the mixture was allowed to stand for 18 hr at 4°C to permit complete equilibration. In this experiment, the values for the nuclear fraction N_2 and the mitochondrial fraction M_2 are not presented separately since these fractions proved difficult to separate reproducibly after 18 hr. The control experiment yielded information about the reconstituted labeled mitochondrial fraction M* in the absence of the unlabeled homogenate. It is clear that this control has only an operational significance and may reflect a variety of forms of "redistribution" of the label. It could represent the extent of contamination of M* by nuclear fragments, mitochondrial aggregates, microsomal fragments, and supernatant proteins, but may also reflect the disintegration of the mitochondria during the resuspension procedure as well as extraction of sterol–protein complexes by the buffer. Since the control value of M_c represents that fraction of M* which sediments at less than 25,000 *g* (15 min), it is properly compared with the sum of $(N + M)_1$ rather than with M_1 alone.

Table 3 summarizes the data from tests for redistribution of labeled cholestanol from the mitochondrial fractions of insects 30 or more days after they have been injected with a solution of cholestanol-4-^{14}C. These data might reasonably be compared with the experiments in which we used cholesterol, summarized in Table 2. The 18 hr experiment was not performed, since the data of Table 2 had indicated that there was no significant difference between the results of equilibration for 20 min and for 18–24 hr. With that exception, the experiments were performed in a manner similar to those of Table 2.

Table 4 presents the results of experiments testing for the redistribution of label from the mitochondrial fraction isolated from a homogenate which had been labeled in vitro with either cholesterol-^{14}C or a mixture of cholesterol-^{14}C and cholestanol-^{3}H by the method described above. It can be assumed that none of the labeled sterol incorporated under these conditions has any structural significance and hence may be expected to show maximal tendency to redistribution.

Experiments involving equilibration for 20 min and 20–22 hr and experimental controls were carried out as in the experiments shown in Table 2. Only one control experiment using equilibration for 20 min was successfully performed. A second control experiment (see **, Table 4) in which the mitochondrial suspension was allowed to stand for 20 hr at 4°C showed a higher percentage of label in the supernate.

The experiment involving mixed cholestanol-^{3}H and cholesterol-^{14}C was carried out to determine whether these compounds would behave differently in this test system.

Table 5 summarizes the results of experiments testing for the redistribution of labeled sterol from mitochondrial fractions isolated from the muscle of insects at various times after injection of a solution of cholesterol-7α-^{3}H. The results are expressed as percentage distributions of label within the fractions of the homogenate in which the labeled mitochondria were resuspended. Experiments involving equilibration for 20 min and 18–24 hr, as well as experimental controls, were performed in a manner similar to those summarized in Table 2, except that the microsomal and supernatant fractions were not separated. These data therefore reflect the relationship between the time during which the sterol has been present in the tissue and its stability of binding to the mitochondria.

DISCUSSION

The results obtained in these studies give some further insight into the problems stemming from our earlier work and enumerated in the introduction to this paper.

Table 1 shows the distribution of labeled sterol that results from its introduction into the homogenate in the form of a previously prepared solution in the supernatant protein fraction. The sterols in these supernatant protein preparations are considered to be soluble inasmuch as they remain in the supernatant fraction, even when this is subjected to a stronger centrifugal field, and for longer times, than are usually used for the isolation of the supernatant fraction from a homogenate. It seems reasonable to assume that these preparations consist of sterol–lipoprotein complexes. It is, of course, not clear what relationship they may bear to the physiologically normal state of association between sterols and supernatant proteins.

The results shown in Table 1 clearly demonstrate the marked affinity of the particulate fractions of the homogenate for sterols introduced in an in vitro solubilized form. Over the 700-fold difference in concentration used in these experiments, the percentage of sterol retained by the supernatant fraction after homogenization and fractionation was essentially the same, with an average of 30.8% (SEM = 1.3, n = 3). We chose amounts of labeled sterol comparable to, or very much less than, the known concentrations of unesterified sterol in this insect's muscle (~28 μg/100 mg wet weight) (3). Thus, it might seem reasonable that a quantity of labeled sterol of the order

TABLE 3 REDISTRIBUTION OF LABEL TO OTHER FRACTIONS FROM MITOCHONDRIA OF INSECTS INJECTED WITH CHOLESTANOL-4-^{14}C

Time After Injection*	Distribution of Label after 20 Min Equilibration†					Total Radioactivity Recovered‡	Distribution of Label in Experimental Control†			Total Radioactivity Recovered‡
	N_1	M_1	$(N + M)_1$	Mic_1	S_1		M_c	Mic_c	S_c	
days						*dpm*				*dpm*
30	11.5	72.5	84.0	14.2	1.8	6831				
36	12.1	66.5	78.6	17.4	4.0	6889	84.9	13.1	2.0	6197
37	7.6	81.4	89.0	8.7	2.3	13147	87.4	11.3	1.3	5944
Averages	10.4	73.5	83.9	13.4	2.7		86.2	12.2	1.6	
s^2	6.0	56.2	27.1	19.4	1.3		3.1	1.6	0.3	
SEM	1.4	4.3	3.0	2.5	0.7		1.2	0.9	0.4	

* Insects in their final instar were selected from the stock colonies and were injected with 4.99 μg cholestanol-4-^{14}C, specific activity 2.84 $\times$ 10^5 dpm per μg, as described in the text. The insects were killed after they had attained adulthood and mitochondria isolated from their muscles were tested for redistribution of sterol as described.

† As percentage of total recovered. See Fig. 1 for designation of fractions.

‡ See footnote* to Table 1.

TABLE 4 REDISTRIBUTION OF LABEL TO OTHER FRACTIONS FROM MITOCHONDRIA THAT HAD BEEN ALLOWED TO INCORPORATE LABELED STEROLS IN VITRO

Experiment*	Distribution of Label after 20 Min Equilibration†					Total Radioactivity Recovered‡	Distribution of Label after 20–22 Hr Equilibration†					Total Radioactivity Recovered‡	Distribution of Label in Experimental Control†			Total Radioactivity Recovered‡
	N_1	M_1	$(N + M)_1$	Mic_1	S_1		N_2	M_2	$(N + M)_2$	Mic_2	S_2		M_c	Mic_c	S_c	
						dpm						*dpm*				*dpm*
Cholestanol-^{3}H§	13.9	54.5	68.4	22.6	8.9	10,392	11.2	51.4	62.6	24.6	12.8	12,473				
Cholesterol-^{14}C‖	12.5	52.8	65.3	23.4	11.3	4,131	11.0	49.1	60.1	24.5	15.4	5,128				
Cholesterol-^{14}C¶	12.7	44.0	56.7	31.5	11.8	122,899	48.2	17.8	66.0	21.7	12.4	78,736	53.4	29.7	16.9	130,647
Cholesterol-^{14}C¶	16.1	43.7	59.8	28.9	11.3	71,921	53.0	16.5	69.5	19.9	10.6	57,057	54.6**	14.4**	31.0**	77,290
Average††	13.8	46.8	60.6	27.9	11.5				65.2	22.0	12.8					
s^2	4.1	26.7	19.0	17.1	0.1				22.6	5.4	7.9					
SEM	1.2	3.0	2.5	2.4	0.2				2.7	1.3	1.6					

* The equilibrations for 20 min and 20–22 hr as well as the operational control were carried out as in the experiments shown in Table 2. The mitochondrial fraction used in the preparation of M* was isolated from an in vitro labeled homogenate similar to the mitochondrial fractions of the experiments shown in Table 1. The overall distribution of label in the homogenates used for these mitochondrial preparations was within the range represented in Table 1.

† As percentage of total recovered. See Fig. 1 for designation of fractions.

‡ See footnote* to Table 1.

§ Specific activity, 2.54 $\times$ 10^4 dpm/μg.

‖ Specific activity, 2.05 $\times$ 10^5 dpm/μg.

¶ Specific activity, 2.84 $\times$ 10^5 dpm/μg.

** Equilibrated for 20 hr.

†† For the purpose of calculation of s^2 and the standard error, the figures for the two experiments utilizing cholesterol were pooled with the cholesterol-^{14}C data of the experiment utilizing the cholestanol-^{3}H to cholesterol-^{14}C ratio of 19.9.

TABLE 5 REDISTRIBUTION OF LABEL TO OTHER FRACTIONS FROM MITOCHONDRIA ISOLATED FROM INSECTS AT INCREASING TIMES AFTER INJECTION OF CHOLESTEROL-7α-^{3}H

Time after Injection*	Experiment	Total Radioactivity Recovered†	Distribution of Label‡			
			N	M	N + M	Mic + S
		dpm				
80 Min	Control	391.7			63.2	36.8
	20 min	1499.4	20.1	53.7	73.8	26.2
	24 hr	1636.1	20.3	44.1	64.4	35.6
25 Hr	Control	380.2			63.5	36.5
	20 min	533.9	15.6	52.4	68.0	32.0
	22 hr	635.4	16.6	48.7	65.3	34.7
10 Days	Control	728.7			74.1	25.9
	20 min	798.7	14.1	65.9	80.0	20.0
	18 hr	1094.0	18.3	61.1	79.4	20.6

* These insects were injected with 5.24 μg of cholesterol-7α-^{3}H with specific activity 2.22 $\times$ 10^5 dpm/μg. Muscle mitochondria were isolated at the times indicated and tested for redistribution of label as described in the text and in Fig. 1.

† See footnote* to Table 1.

‡ As percentage of total recovered. See Table 1 for abbreviations.

of 0.01 μg could be taken up from the supernatant and accommodated in the particulate fractions, but the finding that approximately 10 μg of sterol will also partition in a similar manner is perhaps more surprising.[4] It is clear that if sterols were initially present in the cell sap or in the extracellular fluid in some form which precluded their attachment to membranes in vivo, they might nevertheless become attached to the particulate fractions during homogenization. In the absence of other information, these sterols would then be indistinguishable

[4] The higher concentrations of sterol introduced into the supernatant fraction in the experiments recorded in Table 1 are close to the maximum that can be achieved with these techniques. The results do not allow conclusions as to whether the particulate fractions are saturated with sterol even at the highest concentrations. Furthermore, the information at present available is insufficient to establish the significance of the changes in percentage distribution of labeled sterol between mitochondrial and microsomal fractions that appear to accompany the changes in total concentration of sterol in the system.

from sterols that are intrinsically a part of the subcellular membrane structures.

The overall percentage distribution of sterols in the in vitro system described here is similar to that of unesterified sterols in a homogenate of muscle tissue from an insect reared throughout its life on a diet containing labeled sterols. A typical distribution pattern in the latter case was: nuclear fraction 13%, mitochondrial fraction 33%, microsomal fraction 43%, supernatant fraction 11% (1,5). Possible differences between these values and those shown in Table 1 cannot at present be considered significant because of differences between the conditions of fractionation. Thus, the figures for the overall in vitro distribution of sterol do not bear significantly on the question of whether the in vivo subcellular distribution of sterols reflects a specific structural involvement in membrane formation.

In this connection, however, the data relating to the distribution of mixtures of cholestanol and cholesterol are significant, for in our earlier experiments (1) we noted that the ratio of the concentrations of unesterified cholestanol and cholesterol, in the supernatant fraction of the in vivo labeled muscle homogenate, was twice that found in the particulate fractions, whereas in the present in vitro labeling experiments, the ratios of these two components were the same in particulate and supernatant fractions. This contrast between the outcome of in vitro and in vivo processes of distribution and incorporation supports the proposal that the in vivo distribution of the sterols in the particulate subcellular fractions reflects certain structurally specific roles for the sterols in their membranes. As we have pointed out previously (1), a process of random, nonfunctional incorporation in vivo would seem more likely to lead to the result obtained in the present in vitro experiments.

Similar arguments may be offered as evidence that there is little extraction and redistribution of sterols from the particulate fractions via the supernate during the usual homogenization and centrifugation procedures, since such processes, if they occurred on a significant scale, would also be expected to lead to equalization of the ratios of different sterols in all fractions. More definitive evidence on this point is presented in Tables 2–5, which summarize the results of experiments carried out under various conditions to assess directly the extent of migration of sterols from the mitochondrial fraction to other fractions. The mitochondria were chosen for this study since they are a relatively well-defined subcellular fraction and can be obtained in good yield and in a satisfactorily intact state from the muscle homogenate.

When cholesterol-4-^{14}C was fed to insects for more than 45 days and their muscle mitochondria were isolated and then equilibrated with a second homogenate (Table 2), the mitochondrial fraction, after 20 min equilibration, retained 68.8% (SEM = 3.1, n = 3) of the label. Average values for the sterol migrating to the supernatant and microsomal fractions were 3.9% and 11.8%, respectively; 15.6% was associated with the nuclear fractions but seems likely to represent aggregated mitochondria rather than nuclear material per se. No significant differences ($P = 0.05$) could be found between corresponding fractions in experiments involving equilibration for 20 min or 18 hr, or between the corresponding values in either of these experiments and the experimental controls, except ($S_e - S_1$). If the control is assumed to represent mainly the breakdown of the mitochondria, this result is further evidence that the sterols, once associated with the mitochondria through long term in vivo processes of incorporation, do not migrate to other fractions during homogenization.

Earlier studies in our laboratory have shown that the turnover of cholestanol in the tissues of the cockroach is markedly more rapid than that of cholesterol (3). While it was clear from Table 2 that cholesterol did not migrate from the mitochondria to other fractions to any significant extent, it remained possible that cholestanol, incorporated into mitochondria in vivo, might show a greater tendency to redistribute. To test this possibility, we carried out the experiments recorded in Table 3. The sterol (cholestanol-4-^{14}C) was injected into the insects whose muscle mitochondria were isolated 30–37 days later and used for the tests of redistribution of the label in the usual way. There were no statistically significant differences between the results obtained in these experiments and those shown in Table 2 for the redistribution of cholesterol after 45–64 days ingestion of the sterol.[5] Obviously the difference between the half-lives of nonesterified cholesterol and cholestanol in vivo cannot be explained in terms of a simple difference in the firmness of binding of the sterols such as might be detected in this type of experiment.

The evidently stable incorporation of sterols into mitochondria under long-term in vivo conditions raised the question whether sterols incorporated into mitochondria in vitro, under conditions similar to those used for the experiments shown in Table 1, might have a demonstrably greater freedom to redistribute to other fractions. A possibility that seemed to us to be of practical interest for future work was that the standard redistribution experiment described here could be used diagnostically to distinguish between sterols that were structurally incor-

[5] The identity of the labeled sterols isolated in these fractions has not been examined in detail. However, preliminary thin-layer chromatographic analyses of the labeled material isolated from the mitochondrial fraction and from each fraction of the 20 min equilibration experiment have been carried out. These showed no differences in the pattern of distribution of label between the free and esterified sterols and the more polar steroids that could suggest selective redistribution of any of these fractions.

porated into mitochondria and sterols that were nonstructurally, superficially attached.

The data of Table 4 represent the results of experiments directed to these questions. A comparison of these results with those shown in Tables 2 and 3 confirms the expectation that the in vitro bound sterol is more freely redistributed than the in vivo incorporated material. All differences for corresponding fractions were significant at the $P = 0.01$ level. As might be expected from the results discussed above, there was no significant difference in this test between the behavior of in vitro bound cholesterol and cholestanol.

Although these results were consistent with our expectation of looser binding of sterols to mitochondria in vitro, they discouraged attempts to apply this type of test diagnostically in its present form, since the redistribution of the sterols that had become attached to mitochondria, even under in vitro conditions, was far from complete (i.e. did not approach the distribution shown in Table 1) and the pattern after 24 hr was not significantly different from that at 20 min. Nevertheless, some attempts to demonstrate a transition of sterols in vivo, from a "loosely bound" state such as might be comparable to the in vitro situation just discussed to the more firmly bound state typical of long term in vivo incorporation, have been moderately successful.

The results of these experiments are shown in Table 5. Cholesterol-7α-^{3}H was injected into insects which were killed 80 min, 25 hr, and 10 days later. Their muscle mitochondria were tested for redistribution of labeled sterol in the usual way. The percentage of the label found to redistribute to fractions sedimenting at higher centrifugal forces fell by about 10% of the total between the 1st and 10th days. The values shown in Table 2 for redistribution after 45–64 days feeding on a diet containing labeled sterol show a still smaller percentage of label migrating to the microsomal and supernatant fractions. These results, therefore, are consistent with the expectation that sterols administered to these insects by injection require an appreciable time (probably some days at least) to attain a state of functional incorporation which is characterized by maximal firmness of binding as revealed by these techniques. These experiments do not, of course, bear on the question of structurally specific binding of sterols in the mitochondria.

The broad purpose of the experiments described here was to determine whether the techniques of tissue fractionation and homogenization, as used in our earlier work, could be expected to give more precise data concerning the intracellular distribution of sterols and other lipids, which might help to elucidate their mode of intermolecular association. On the one hand, the results are encouraging in that they point to a very limited extent of randomization of mitochondrial sterols between different fractions in the course of homogenization and fractionation. On the other hand, there are only small differences in behavior during homogenization and centrifugation between sterols that are presumed to be structurally bound to the mitochondria and sterols that almost certainly are not. It is clear, therefore, that the isolation of sterols attached to a particulate fraction is not proof that the sterols are significant structural components in the fraction, nor even that they originated in it, and firm conclusions cannot be drawn in the absence of other evidence.

There are few studies in the literature that deal with the specific problems of uptake and release of sterols by mitochondria. Two which have appeared recently (6, 7) demonstrate a high affinity of mitochondria of various mammalian tissues in vitro for exogenous sterols. These observations are similar to our own, though the experimental conditions are somewhat different. Our results are quite dissimilar from those obtained in the study of Graham and Green (7), in which it was reported that 85–91% of the mitochondrial cholesterol of several tissues exchanged with cholesterol supplied in a lipoprotein-bound form in the medium. A major difference between this study and our own was the choice of conditions under which exchange was allowed to take place, for whereas our most strenuous conditions involved equilibration for 24 hr at 4°C, the above authors incubated their preparations for the same length of time but at 37°C. It seems doubtful whether physiological significance can be attached to results obtained under the latter incubation conditions, which might be expected to lead to extensive autolysis.

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