THE QUANTITATIVE RETENTION OF CHOLESTEROL IN MOUSE LIVER PREPARED FOR ELECTRON MICROSCOPY BY FIXATION IN A DIGITONIN-CONTAINING ALDEHYDE SOLUTION

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ABSTRACT

A major methodological problem in the intracellular localization of cholesterol is the nearly complete extraction of sterols during routine dehydration and embedding procedures for electron microscopy. Cholesterol digitonide (a sterol complex with digitonin), however, is qualitatively insoluble in these solvents. Mouse liver has been prepared as follows: (*a*) Flickinger's aldehyde fixative, 20 hr; (*b*) Flickinger's fixative containing 0.2% digitonin, 24 hr; (*c*) cacodylate wash, 24 hr; (*d*) 1% OsO_4, 2 hr; (*e*) acetone dehydration; and (*f*) Epon 812 infiltration under vacuum, 28 hr. After the last step, an analysis of the tissue for sterol content under optimal analytical conditions demonstrates a retention of 99% of the unesterified cholesterol present in unfixed mouse liver. Liver prepared in an identical manner except for omission of digitonin is essentially devoid of sterols. Cholesterol isolated chromatographically from liver processed as outlined above has been identified unequivocally by mass spectrometry. Liver from step (*f*) also has been polymerized, thin-sectioned, and examined in the electron microscope. A remarkable quality of fine-structural preservation is obtained. The major alteration encountered is the presence of small cylindrical "spicules," often occurring as tightly packed concentric lamellae, at membrane surfaces.

INTRODUCTION

Although much as been learned concerning the biosynthesis of cholesterol (4), little is known about the structural localization of cholesterol within the cell. A crucial technical difficulty is that tissues routinely prepared for electron microscopy are nearly devoid of sterols as the result of extraction during dehydration and embedding procedures (2, 6, 18).

We have solved this problem by the employment of Flickinger's fixative (2% formaldehyde and 2.5% glutaraldehyde; see reference 9) containing 0.2% digitonin. Whereas cholesterol is freely soluble in dehydration solvents such as ethanol and acetone, cholesterol digitonide is qualitatively insoluble in these solvents. Analysis of liver after treatment with (*a*) Flickinger's fixative containing digitonin, (*b*) osmium tetroxide, (*c*) a dehydration solvent, and (*d*) Epon 812 reveals a retention of 99% of the unesterified cholesterol present in the unfixed control. Liver handled in an identical

manner except for omission of digitonin is essentially devoid of sterol. Unequivocal identification of the digitonin-retained sterol as cholesterol has been obtained with high resolution mass spectrometry. Fine-structural documentation is included to demonstrate that utilization of this digitonin-containing aldehyde fixative is capable of achieving excellent preservation.

MATERIALS AND METHODS

Reagents

Trioxymethylene (USP, paraformaldehyde) and glutaraldehyde (G-151, 50% w/w, Biological Grade) were purchased from Fisher Scientific Company, Fair Lawn, N. J.; sodium cacodylate, from K & K Laboratories, Inc., Plainview, N. Y.; digitonin, from Nutritional Biochemical Corporation, Cleveland, Ohio; calcium chloride (No. 4140), from Mallinckrodt Chemical Works, St. Louis, Mo.; osmium tetroxide, from Engelhard Industries, Inc. (Chemical Division), Newark, N. J.; Unisil silicic acid (100–200 mesh), from Clarkson Chemical Co., Inc., Williamsport, Pa.; Supercel, from Johns-Manville, N. Y. Cholesterol used as a reference compound was purified through the dibromide (8). All other reagents were of Analytical Reagent quality.

Preparation of Cacodylate/HCl Buffer

Mix equal parts of a 0.4 M sodium cacodylate solution and a 0.022 M HCl solution. The resulting solution is the 0.2 M cacodylate/HCl buffer used below; pH is 7.2 (19).

Preparation of Flickinger's Fixative

SOLUTION I: Place 1 g of paraformaldehyde in a flask. Add 22.5 ml of distilled water. Heat to nearly boiling while stirring (solution clears partially). Add 2–3 drops of 1 N sodium hydroxide, and the solution becomes clear. Cool to room temperature (9).

SOLUTION II: Add 2.5 ml of 50% aqueous glutaraldehyde to 2.5 ml of 0.2 M cacodylate/HCl buffer.

Add 5 ml of Solution II to Solution I. Then add 22.5 ml of 0.2 M cacodylate/HCl buffer to give a final volume of 50 ml. Dissolve 0.025 g of calcium chloride. The final solution contains 2% formaldehyde, 2.5% glutaraldehyde, and 0.05% calcium chloride in 0.1 M cacodylate/HCl buffer (pH 7.2).

Preparation of Flickinger's Fixative Containing Digitonin

SOLUTION III: Dissolve 0.1 g of digitonin in 22.5 ml of 0.2 M cacodylate/HCl buffer (pH 7.2) by careful boiling (avoid foaming) until solution clears. Cool to room temperature.

Combine Solutions I, II, and III. Then dissolve 0.025 g of calcium chloride. The final solution may possess a faint opalescence which can be disregarded. The final solution contains 2% formaldehyde, 2.5% glutaraldehyde, 0.05% calcium chloride, and 0.2% digitonin in 0.1 M cacodylate/HCl buffer (pH 7.2). This fixative is stored at room temperature and used within 3 days.

Preparation of Liver

PRELIMINARY: Adult male C3H mice were decapitated, and after a brief period to permit the drainage of blood, their livers were rapidly excised. Liver designated as the unfixed control was diced uniformly into 1–2 mm cubes under 0.9% saline at room temperature. Following three changes of 0.9% saline the liver cubes were separated from the saline by filtration through cheese cloth. Liver wet weight was then determined (1–2 g). In Experiment 3 (see below) 7 g of liver were used to facilitate isolation of cholesterol. The unfixed control was processed chemically as detailed below. Liver designated as experimental was diced uniformly into 1–2 mm cubes under Flickinger's fixative at room temperature. After two changes of fixative, the liver cubes were fixed for 20 hr and separated from the fixative by cheese cloth filtration. Liver wet weights were determined for two approximately equal portions (1–2 g) which were designated: (*a*) Flickinger's fixative and (*b*) Flickinger's fixative containing digitonin. Subsequent processing of the two experimental portions was performed as outlined in Table I.

CONCLUSION: Both experimentally handled portions of liver were processed chemically as detailed below. Liver handled as specified in Experiment 3 also was polymerized stepwise at 37°C, 45°C, and 60°C over a 4-day period. Thin sections cut with a diamond knife on a Huxley ultramicrotome were stained in alcoholic uranyl acetate (10) followed by lead citrate (20) and photographed in a Hitachi HU-11 C electron microscope. This material served as the source for the hepatocytes shown in Figs. 1, 2, and 3.

Chemical Methods

EXTRACTION: The unfixed control was extracted directly with 2:1 chloroform:methanol as described below, and the cholesterol content was determined by Method A (Experiments 1 and 3) and Method B (Experiment 4). In the case of the liver cubes processed for electron microscopy, Epon 812 was removed by diethyl ether (Experiments 1 and 2) or by acetone (Experiments 3 and 4). The sterols were extracted with a solution of 2:1 chloroform:methanol (40 ml/g liver, wet weight) for 20

hr at room temperature in a stoppered Erlenmeyer flask provided with magnetic stirring. The resulting black suspension was filtered through a scintered glass funnel, and the dark brown filtrate was reduced to dryness at room temperature under a stream of nitrogen. After the addition of a known volume of 2:1 chloroform:methanol (10 ml/g liver, wet weight), aliquots were transferred to tubes with Teflon-lined screw caps. The aliquot was reduced to dryness under a stream of nitrogen. The residue was then treated as described in either Method A or Method B.

TABLE I

Experimental Protocol for Experiments 1–4

Fixation—room temperature	Flickinger's fixative	Flickinger's fixative containing digitonin
	hr	*hr*
(*a*) Flickinger's fixative—before wet weight	20	20
(*b*) Flickinger's fixative—after wet weight	24	0
(*c*) Flickinger's fixative containing 0.2% digitonin	0	24
(*d*) Wash: 0.05 M cacodylate/HCl buffer + 0.05% $CaCl_2$ (pH 7.2)—X 3	24	24
(*e*) 1% OsO_4 in 0.1 M cacodylate/HCl buffer + 0.05% $CaCl_2$ (pH 7.2)	2	2

Experiments 1 and 2

Procedure	Flickinger's fixative	Flickinger's fixative containing digitonin
	min	*min*
Dehydration—ethanol		
50%—X 2	3	3
70%	3	3
90%	3	3
95%	3	3
100%—X 3	5	5
Embedment—Epon 812—with rotation		
2:1 ethanol:Epon	30	30
1:1 ethanol:Epon	30	30
1:2 ethanol:Epon	30	30
Epon	60	60
Epon removal		
100% diethyl ether—X 4	10	10
Liver analyzed for sterol content (see Chemical Methods)		

TABLE—*continued*

Experiments 3 and 4

(As above steps *a* through *e*)

Procedure	Flickinger's fixative	Flickinger's fixative containing digitonin
Dehydration—acetone		
50%—X 2	3 min	3 min
70%	3 "	3 "
90%	3 "	3 "
100%—X 4	5 "	5 "
Embedment—Epon 812—under vacuum		
Epon—1st change	4 hr	4 hr
Epon—2nd change	24 "	24 "
Epon removal		
100% acetone—X 4	5 min	5 min
Liver analyzed for sterol content (see Chemical Methods)		

METHOD A: A 1:1 solution of ethanol:water (15 ml/g liver, wet weight) was added to the residue followed by an equal volume of petroleum ether. The contents were mixed by using a Vari-Whirl vortex agitator (Van Waters and Rogers, Albuquerque, N. M.). The two layers were separated at low speed in a PR-2 International Centrifuge (In-, ternational Equipment Co., Needham Heights Mass.). The upper layer (petroleum ether) was transferred to another flask with a Pasteur pipette. The extraction was repeated twice more. The combined petroleum ether extract was reduced to dryness under a stream of nitrogen at room temperature, a known volume of petroleum ether was added, and aliquots were taken for colorimetric determination of cholesterol by a modification of the Liebermann-Burchard reaction (1).[1] A correction was

[1] Extraction Method A did not yield a complete extraction of cholesterol from cholesterol digitonide. This was detected by the addition of known amounts of synthetic cholesterol digitonide to aliquots of the extract from tissue fixed in Flickinger's fixative. Recoveries were approximately 30–35%. This analytical difficulty was solved by Extraction Method B which completely extracts cholesterol from cholesterol digitonide.

made for yellow material which absorbed 1½ min after addition of the reagent. Cholesterol has no absorbance at this time, but develops maximal absorbance 30 min after addition of the reagent.[2] The remainder of the petroleum ether extract was subjected to silicic acid chromatography (Experiment 3, see below).

METHOD B: A modification of the method of Issidorides et al., (13) was used to break the cholesterol digitonide complex. Dimethyl sulfoxide (5 ml) was added to the residue, and the sample was placed in a mechanical shaker at room temperature for 20 min. Petroleum ether (15 ml) was added, and the contents were mixed by using a Vari-Whirl vortex agitator. The two layers were separated by centrifugation, as described in Method A, and the upper layer (petroleum ether) was transferred to another flask. The extraction was repeated three more times with 15 ml portions of petroleum ether. Aliquots were assayed for cholesterol content (1). Little or no correction was needed for nonspecific absorbance at 1½ min, in contrast to Method A.

CHROMATOGRAPHY: In order to identify cholesterol in an unequivocal manner from liver fixed in Flickinger's fixative containing digitonin, cholesterol was isolated by chromatography so that a high-resolution mass spectrum could be obtained. The residue from the petroleum ether extract was dissolved in 9:1 carbon tetrachloride:benzene and applied to a 1.2 × 7 cm, 5:1 Unisil silicic acid: Supercel column. Elution was continued with the same solvent and the first fraction (Fraction 1, 30 ml) was collected. The eluting solvent was then changed to benzene (redistilled immediately before use), and a second fraction (Fraction 2, 60 ml) was collected, followed by a third fraction (Fraction 3) of 30 ml. Fraction 2 contained cholesterol. In an aliquot, the amount of sterol present was measured for Experiment 3 by the Liebermann-Burchard reaction (3a in Table II). The remaining material in Fraction 2 from Experiment 3 was dissolved in anhydrous methanol and filtered through a scintered glass funnel. After removal of solvents, a portion of this material was subjected to mass spectrometry.

MASS SPECTROMETRY: Mass spectral studies were performed with a CEC (Consolidated Electrodynamics Corporation, Pasadena, Calif.) 21-110B mass spectrometer.[3] The samples were introduced into the ion source by using the direct introduction probe technique. The reference compound was cholesterol purified through the dibromide (8). The ion source was held at 135°C, and the mass spectra were recorded on photographic plates at a probe temperature of 120°C.

DETERMINATION OF ESTERIFIED CHOLESTEROL: An aliquot of the 2:1 chloroform:methanol extract from the unfixed control was reduced to dryness under a stream of nitrogen, dissolved in 3:1 carbon tetrachloride:benzene, and applied to a 5:1 Unisil silicic acid:Supercel column (1.2 × 7 cm). Fraction 1 (60 ml) was collected, followed by three fractions of 10 ml each (Fractions 2–4); the solvent was then changed to benzene and fractions of 60 ml (Fraction 5) and 30 ml (Fraction 6) were collected. Esterified cholesterol was present in Fraction 1 and unesterified cholesterol in Fraction 5 as measured colorimetrically (1).

SOLUBILITY DETERMINATIONS: Cholesterol digitonide was prepared as previously described (23). 40-mg samples were placed in test tubes with Teflon-lined screw caps, and 10 ml of the indicated solvent were added (Table III). The contents were mixed thoroughly with a Vari-Whirl vortex agitator, and allowed to stand for 1 hr at room temperature. The tubes were then centrifuged, and aliquots were taken from the supernatants for colorimetric determination (1).

RESULTS

Chemical

Table II reveals the sterol content of mouse liver under the comparative conditions studied in this report: (*a*) the unfixed control; (*b*) treatment with Flickinger's fixative; and (*c*) treatment with Flickinger's fixative containing 0.2% digitonin. Experiments 1 and 2 utilized ethanol dehydration. Experiments 3 and 4 utilized acetone dehydration. In each experiment a marked retention of cholesterol occurs in mouse liver which has been treated with Flickinger's fixative containing digitonin. Experiments 1, 2, and 3 show a retention of 26–44% of the unesterified cholesterol present in the unfixed control (see Table II). In Experiment 4 we utilized an improved method for recovery of cholesterol from cholesterol digitonide in the liver specimens (Extraction Method B). This Experiment is the best measure of the true capability of the digitonin fixative; 99% retention of cholesterol is obtained. The identity of this retained material, presumed to be cholesterol on the basis of its characteristic Liebermann-Burchard color reaction, has been verified by chromatographic isolation and subjection of the material to high-resolution mass spectrometry (see Chemical Methods).

[2] The absorbance at 1½ min (620 mμ) was subtracted from the absorbance obtained at 30 min (620 mμ).

[3] We gratefully acknowledge the assistance of Dr. E. D. Loughran of Los Alamos Scientific Laboratory for the mass spectral studies.

TABLE II

Cholesterol Content of Mouse Liver Prepared for Electron Microscopy

Experiment*	Unesterified cholesterol expressed as mg/g of liver, wet weight‡			Retention of unesterified cholesterol for Flickinger's fixative containing digitonin
	Unfixed control	Flickinger's fixative §	Flickinger's fixative containing digitonin	
				%
1	1.36	⩽0.02	0.60	44
2	1.38‖	⩽0.06	0.36	26
3	1.40	⩽0.06	0.49	35
3a¶		⩽0.01	0.32	
4**	1.59	⩽0.04	1.58	99

* See Methods for experimental details. Cholesterol was extracted from the liver cube specimens in Experiments 1, 2, and 3 by Extraction Method A.

‡ Unesterified cholesterol accounted for 90% and esterified cholesterol accounted for 10% of the total cholesterol in the unfixed control (Experiment 3); e.g., total cholesterol = 1.56; unesterified cholesterol = 1.40; esterified cholesterol = 0.16.

§ The results shown in this column are maximal figures; more precise measurement was not possible because of the extremely small amounts of cholesterol present in these samples.

‖ Calculated on the basis of the average of unfixed controls 1 and 3.

¶ The petroleum ether extract in Experiment 3 was further purified by silicic acid chromatography (see Methods). No attempt was made to correct for any losses involved in chromatography.

** Cholesterol was extracted from the liver cube specimens by Extraction Method B.

The calculated mass of the cholesterol parent ion is 386.47826. Identical values of 386.47770 were measured for authentic cholesterol and the presumptive cholesterol isolated from the digitonin-treated liver in Experiment 3. This represents a difference of only 0.56 millimass units and established the empirical formula for the retained material as $C_{27}H_{46}O$. The high-resolution mass spectrum of this material is essentially identical to the spectrum of authentic cholesterol. Such data provide unequivocal documentation that the substance retained in mouse liver which has been exposed to digitonin is cholesterol.

For the quantitative data depicted in Table II to be reliable, a complete removal of all liver sterols must occur during extraction. Saponification of the unfixed control residue (after chloroform:methanol extraction), followed by a second extraction of this saponified material as described previously (21), has been performed to evaluate sterol removal during the initial extraction. A colorimetric estimation of the sterol content (1) of this second extract reveals it to be essentially sterol-free.

As a control for the colorimetric determinations, 5 ml of a 2% solution of digitonin in 50% ethanol (an amount equal to the total amount of digitonin used in the fixative) is extracted with petroleum ether. One-fifth of this extract has been tested by means of the Liebermann-Burchard reaction (1). No color development has ever occurred under these conditions.

Table III depicts the solubility of synthetic cholesterol digitonide in several organic solvents. Of the solvents examined, cholesterol digitonide appears to be least soluble in acetone (1.2% dissolved, Table III). Accordingly acetone has been adopted as the dehydrant of choice for optimal cholesterol preservation under the conditions of this report. It should be noted that cholesterol digitonide is completely soluble at a 0.4% concentration in 2:1 chloroform:methanol. This latter mixture is employed for the extraction of sterols from all liver specimens under analysis. Furthermore, cholesterol is also 100% soluble at a 0.4% concentration in each of the solvents listed in Table III.

TABLE III

*Solubility of Cholesterol Digitonide in Several Common Organic Solvents**

Solvent	mg/ml of cholesterol digitonide dissolved	Dissolved
		%
2:1 Chloroform:methanol	4.000	100
Acetone	0.048	1.2
Ethanol	0.112	2.8
Diethyl ether	0.108	2.7
Propylene oxide	0.244	6.1

* See Methods for experimental details.

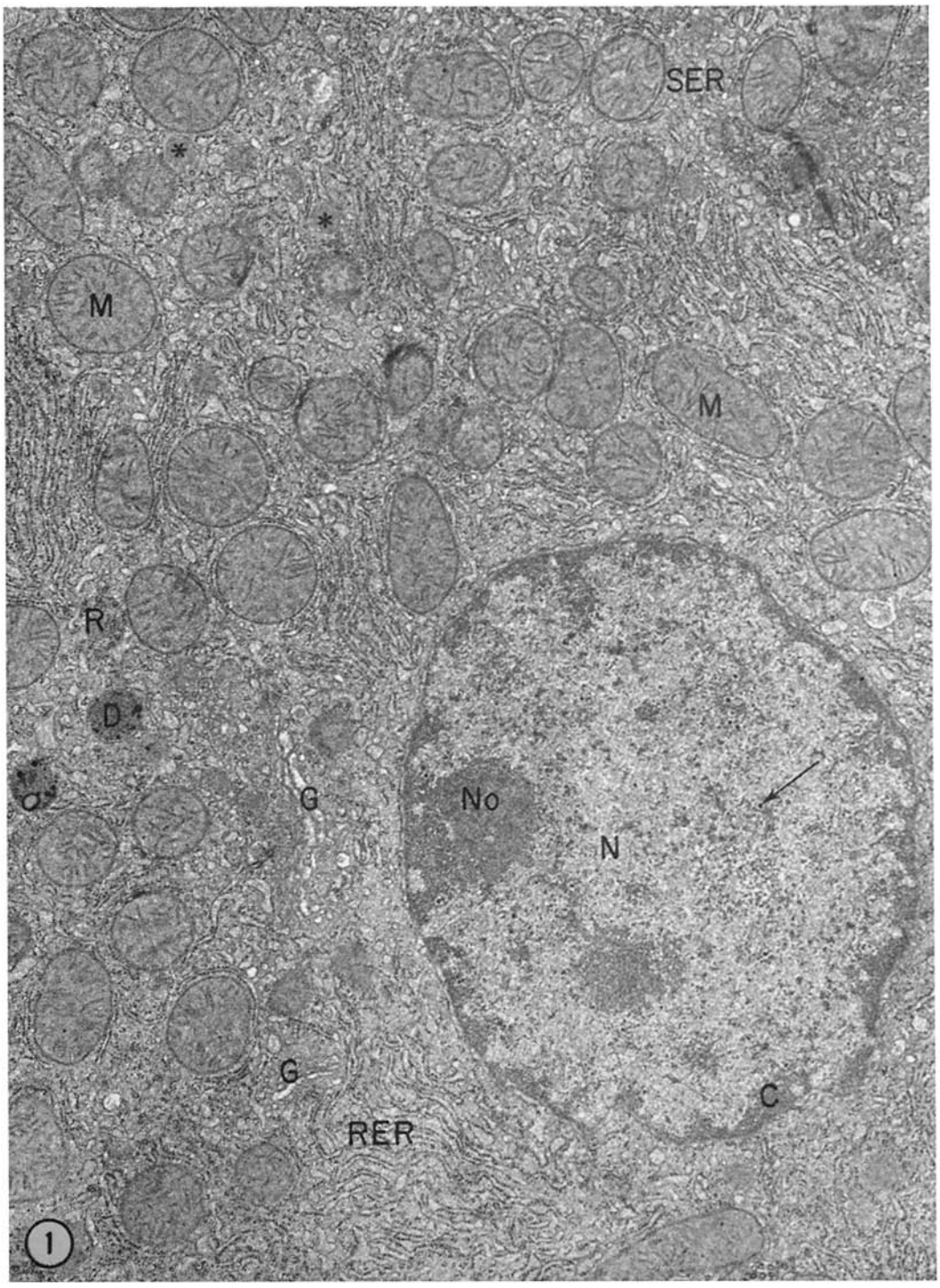

FIGURE 1 Mouse hepatocyte fixed in 2% formaldehyde-2.5% glutaraldehyde containing 0.2% digitonin; acetone dehydration; omission of propylene oxide. A high quality of fine-structural detail is evident in the: nucleus (*N*) with nucleolus (*No*), perichromatin granules (arrow), and clumped chromatin (*C*); Golgi zone (*G*); mitochondria (*M*); rough-surfaced (*RER*) and smooth-surfaced (*SER*) endoplasmic reticulum; free ribosomes (*R*); dense bodies (*D*); and presumptive microbodies (*). × 16,000.

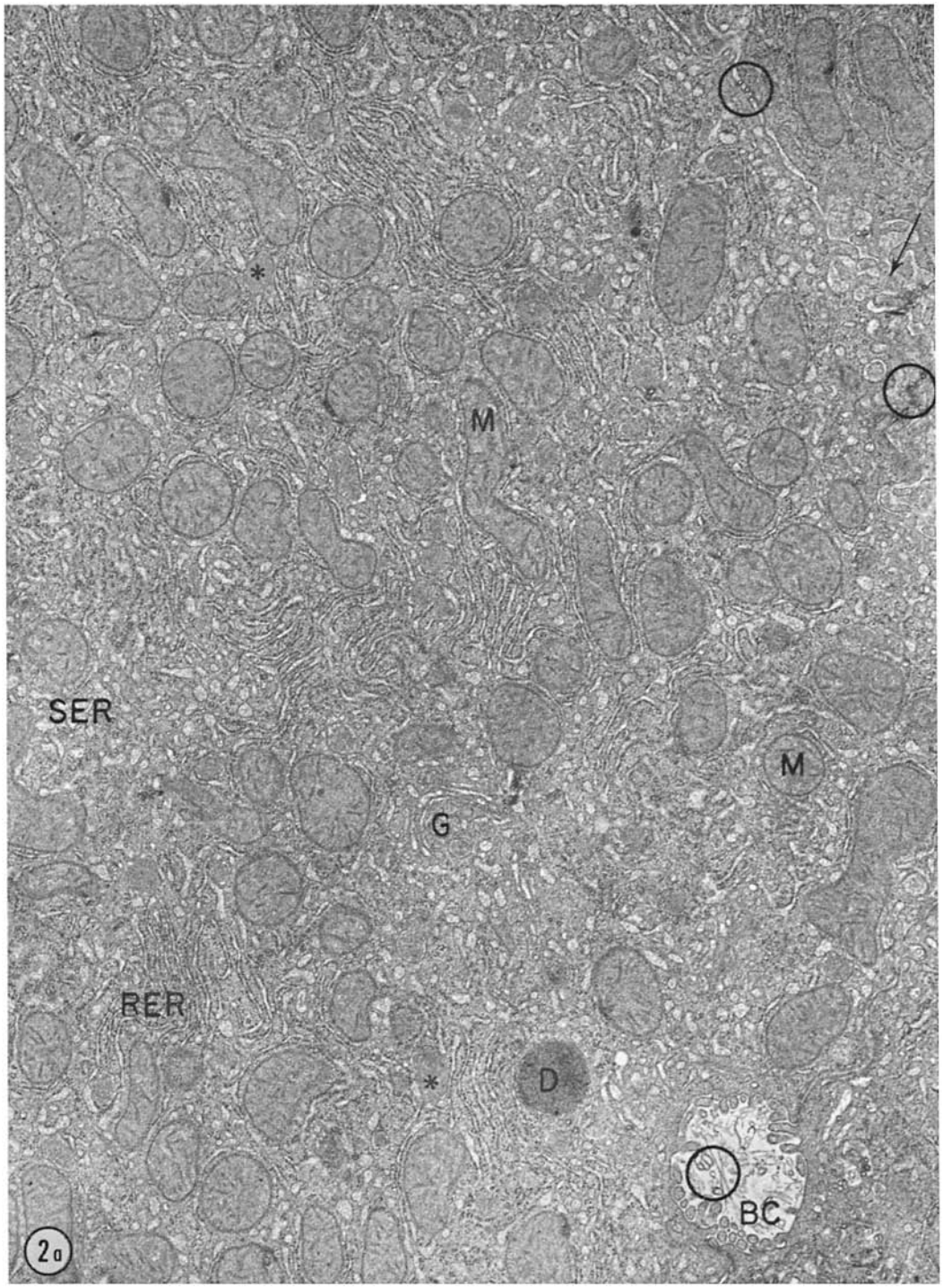

FIGURE 2 *a* Another hepatocyte prepared as in Fig. 1. This cell similarly demonstrates good preservation of the: Golgi zone (*G*); mitochondria (*M*); rough-surfaced (*RER*) and smooth-surfaced (*SER*) reticulum; dense bodies (*D*); and presumptive microbodies (*). The space of Disse (arrow) with an intercellular extension and a biliary canaliculus (*BC*) are indented by microvilli. Each space contains digitonin-induced spicules (encircled) in various planes of section. The presence of such spicules in these three locations is verified at higher magnification in Figs. 2 *b*, 2 *c*, and 2 *d*. × 16,000.

Fine Structure

We are utilizing currently the hypertrophic effect of various barbiturates upon liver smooth-surfaced endoplasmic reticulum to facilitate the investigation of cellular mechanisms of cholesterol biosynthesis (14). The hepatocytes illustrated in Figs. 1, 2, and 3 are derived from the liver of a mouse which has received, by intraperitoneal injection, three daily doses of Nembutal (7 mg/100 g body weight). These cells can be considered essentially normal for the purpose of this report. They differ morphologically from normal murine hepatocytes (7, 12) and rat hepatocytes (5) by the virtual absence of cytoplasmic glycogen (15). Hypertrophy of the smooth endoplasmic reticulum, however, is not a prominent feature.

The incorporation of 0.2% digitonin into a formaldehyde-glutaraldehyde fixative is capable of preserving 99% of the unesterified cholesterol present in mouse liver after routine preparatory techniques for electron microscopy. A remarkable quality of fine-structural detail can be achieved with this fixative. Figs. 1 and 2 *a* represent survey electron micrographs of typical hepatocytes present in mouse liver when handled as outlined above (see Materials and Methods). The nucleus is round to oval in shape, possesses a prominent nucleolus, contains easily visible perichromatin granules, and displays a clumped chromatin pattern (Fig. 1). A single cell usually demonstrates at least one well-developed Golgi zone, with normal appearing vesicular, vacuolar, and parallel-lamellar components (Figs. 1 and 2 *a*). Numerous mitochondria having round, oval, or elongate profiles occur throughout the cytoplasm. There is no evidence of swelling or distortion of cristae suggesting poor fixation (Figs. 1 and 2 *a*). Rough- and smooth-surfaced elements of the endoplasmic reticulum are abundant. The rough-surfaced variety predominates and is organized commonly into parallel stacks of 4–6 lamellae. Cisternae of the endoplasmic reticulum are generally narrow and do not demonstrate prominent vesiculation (Figs. 1 and 2 *a*). Clusters of unattached (free) ribosomes are

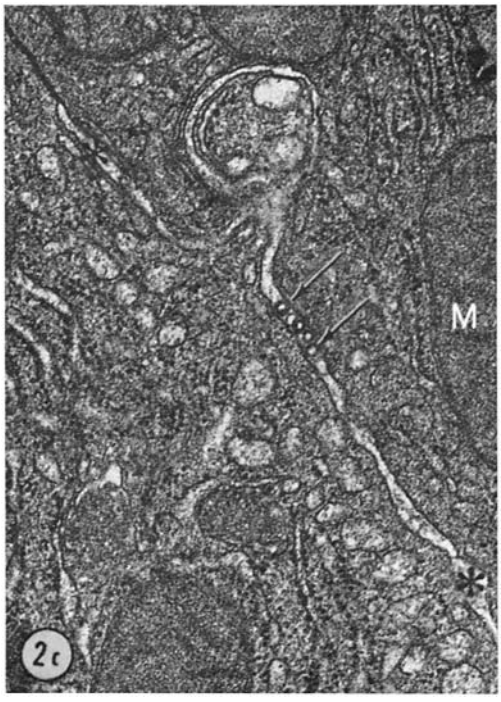

FIGURE 2 *b* Higher magnification of the space of Disse illustrated in Fig. 2 *a*. The space of Disse (*SD*) contains numerous digitonin-induced spicules (arrows) in various planes of section. × 37,000.

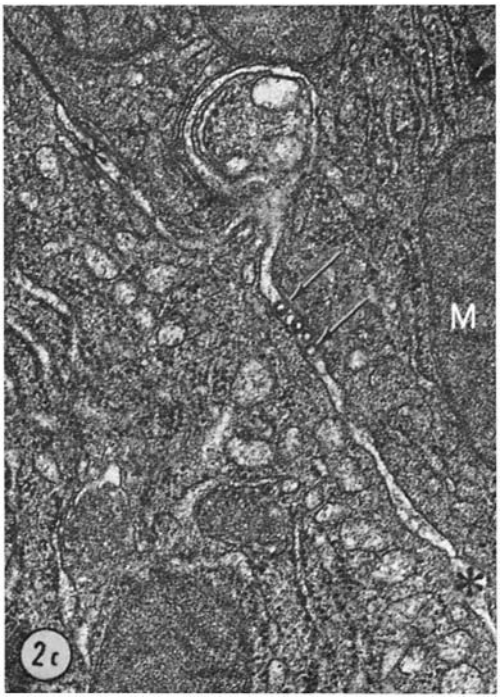

FIGURE 2 *c* Higher magnification of the intercellular extension of the space of Disse present in Fig. 2 *a*. The intercellular space (*) contains several cross-sectional profiles of digitonin-induced spicules (arrows). *M*, mitochondrion. × 37,000.

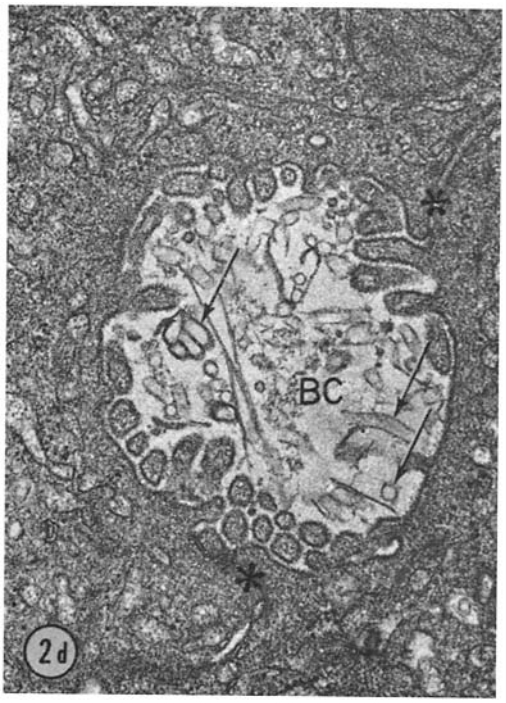

FIGURE 2 *d* Higher magnification of the biliary canaliculus depicted in Fig. 2 *a*. The canaliculus (*BC*) contains numerous digitonin-induced spicules (arrows) in various planes of section. Junctional complex (*). × 37,000.

distributed widely within the cytoplasm (Figs. 1 and 2 *a*). The background cytoplasmic matrix is uniformly dense with an even distribution of organelles (Figs. 1 and 2 *a*). Membrane-limited cytoplasmic inclusions which possess pleomorphic electron-opaque material (presumptive lysosomes) or homogeneous finely granular contents (presumptive microbodies) represent another prominent component of the liver cells under study (Figs. 1 and 2 *a*). The space of Disse and a biliary canaliculus are visible in Fig. 2 *a*. Both of these extracellular compartments are indented by protruding microvilli from the adjacent hepatocytes. The cytologic features outlined above are identical with the fine-structural characteristics of normal liver cells described in earlier reports (5, 7, 12). Inclusion of this brief account of murine liver cell ultrastructure serves to document that incorporation of 0.2% digitonin into Flickinger's fixative (9) and omission of propylene oxide prior to Epon infiltration do not compromise the quality of cell preservation.

At least two characteristic alterations occur, however, in association with the presence of digitonin in the aldehyde fixative. The first and most striking is the development of small, elongate, cylindrical spicules often in proximity to membrane surfaces such as: (*a*) the plasma membrane of the cell (they are prominent in the space of Disse and within the biliary canaliculus; see Figs. 2 *b*, 2 *c*, and 2 *d*; (*b*) the Golgi lamellae; (*c*) the outer and inner mitochondrial membranes (Fig. 3); and (*d*) the endoplasmic reticulum membranes (Fig. 3). These spicules assume the form of tightly ordered concentric lamellae surrounding an electron-transparent central core (Fig. 3). The approximate dimensions of a typical spicule are: (*a*) length, 2000 A; (*b*) outside diameter, 800 A; (*c*) diameter of the electron-transparent central core, 250 A; (*d*) thickness of a single electron-opaque lamella, 35 A; and (*e*) thickness of a single electron-transparent lamella, 25 A. The appearance of these digitonin-induced spicules and their approximate dimensions are identical essentially to those of the digitonin-induced "needles" observed independently by Ökrös in rat liver, testis, and adrenal cortex (17). A second alteration associated with digitonin is the development of scattered trilaminar unit membranes which possess greater relative contrast between adjacent electron-opaque and electron-transparent lamellae than is usually encountered in preparations lacking digitonin (Fig. 3). The approximate thickness of such unit membranes is 90–95 A, with the inner and outer opaque lamellae each measuring 35 A and the intermediate transparent lamella measuring 25 A.

DISCUSSION

Windaus (24) discovered the unique property of certain saponins to precipitate cholesterol and other 3β-hydroxy sterols. This has led to the use of this reaction as a quantitative method for the determination of unesterified cholesterol (23). In an extensive light microscopic investigation of cat adrenal cortex, Bennett (3) reports the zonal localization of sterol-digitonin birefringent crystals by polarizing optics. To minimize sterol extraction Bennett has used aqueous digitonin following or during formalin fixation. The distribution of these sterol-digitonin crystals in frozen sections is interpreted as an index of cholesterol localization (3). Levy et al. (16) depict in an electron micrograph (their Fig. 9) the fine structural appearance of a

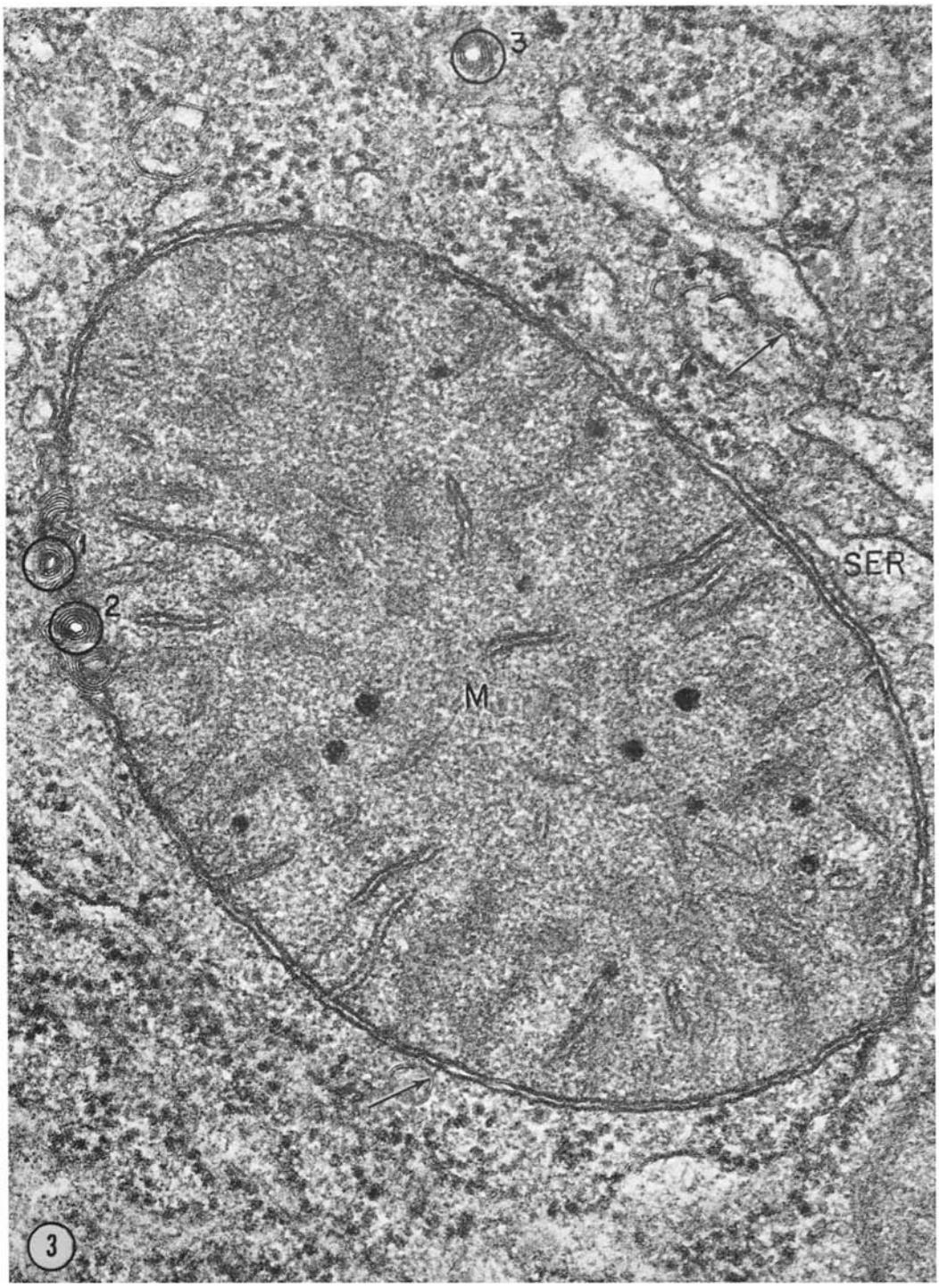

FIGURE 3 A mitochondrion (*M*) from a mouse hepatocyte prepared as in Fig. 1. Cross-sections of digitonin-induced cylindrical spicules (encircled) are visible in association with: the inner and outer mitochondrial membranes (encircled 1, 2) and a probable endoplasmic reticulum membrane (encircled 3). Arrows indicate portions of endoplasmic reticulum unit-membranes which possess a high degree of contrast between the trilaminar lamellae. *SER*, smooth-surfaced reticulum. × 83,000.

"cholesterol-digitonin complex obtained *in vitro* from commercial cholesterol." The approximate thicknesses of the periodic electron-opaque and electron-transparent lamellae present in this complex are 31 A and 25 A respectively (our measurements). Similar dimensions have been observeo fdr the concentric opaque and transparent lamellae which occur in the spicules described in this report, i.e. 35 A and 25 A, respectively. In a study of the effects of surface-active agents upon leukocytes, Graham et al. (11) report the development of "cylindrical projections" from the plasma membrane of cells incubated in various media containing digitonin. These cylindrical projections have a diameter of 450–700 A, which is similar to the 800 A outside diameter of the spicules in the present study. Those authors (11) discuss this digitonin-induced phenomenon in relation to the ability of digitonin to complex with cholesterol. While our studies concerning digitonin were in progress, Ökrös (17) independently employed the digitonin reaction after glutaraldehyde fixation as a histochemical approach to the problem of the fine-structural localization of cholesterol. He reports the presence of "digitonin-cholesterol needles" at the periphery of adrenal lipid droplets, within and adjacent to adrenal capillaries, around peribiliary hepatic mitochondria, in hepatic sinusoids, and in the vicinity of testicular Sertoli cell-germ cell plasma membrane contacts (17). The procedure of Ökrös differs from the one presented here by employing two digitonin solutions in succession after glutaraldehyde fixation and a buffer wash: (*a*) 1.2% digitonin in 0.1 M phosphate buffer at 4°C and (*b*) 0.5% digitonin in 35% ethanol at 4°C.

To the best of our knowledge, the data presented here represent the first quantitative demonstration of a method capable of retaining significant amounts of unesterified cholesterol in tissues prepared for electron microscopy. In fact, our method is capable of achieving a 99% retention of unesterified cholesterol in mouse liver prepared for electron microscopy (Experiment 4). The sterol present in mouse liver after treatment with Flickinger's fixative containing 0.2% digitonin has been purified by column chromatography and identified unequivocally as cholesterol by high-resolution mass spectrometry. This is important because it dispels any doubts that the isolated compound might be an artifact related to aldehyde or osmium tetroxide fixation or even digitonin itself.

The methods used for the isolation of sterols from liver prepared for electron microscopy (see Chemical Methods) are quite important. Many techniques employ saponification in refluxing alcoholic-KOH before extraction into petroleum ether (21). Although this procedure is satisfactory for unfixed tissue, serious problems arise during saponification of tissue fixed in osmium tetroxide. Significant amounts of cholesterol are destroyed when osmium tetroxide-fixed tissue is saponified in alcoholic-KOH. As a result, techniques such as solvent extraction at room temperature and silicic acid chromatography have been devised in order to minimize sterol degradation. It is especially important to realize that the retention of cholesterol observed in Experiments 1, 2, and 3 (26–44%) does not accurately reflect the total amount of cholesterol retained in the liver cubes. This is because of an analytical difficulty in Method A.[1] Significant losses occurred with Method A, which resulted in an apparent lowering of the amount of cholesterol retained. This analytical difficulty was solved by Method B which effectively breaks the cholesterol digitonide complex and allows extraction of cholesterol into the petroleum ether phase. Thus Experiment 4 demonstrates the true capability of the digitonin fixative to retain unesterified cholesterol.

It is important to emphasize that digitonin reacts with 3β-hydroxy sterols, such as cholesterol, and forms an insoluble complex (23, 24). If the 3β-hydroxy group is esterified with a fatty acid, the resulting esterified cholesterol is unreactive with digitonin. As a result, the present method is incapable of preserving esterified cholesterol.

Our data are in contrast to the results of Casley-Smith (6) who reports the inability of digitonin to preserve cholesterol in tissues prepared for electron microscopy. This failure is probably related to the fact that Casley-Smith employed 0.5% digitonin in absolute alcohol during dehydration (6) rather than in the fixative as outlined in this report.

The methods outlined in this report are capable of achieving not only a quantitative retention of unesterified cholesterol but also a high quality of fine-structural preservation and detail (see Figs. 1, 2 *a*, and 3). It is our subjective impression that tissues prepared and polymerized as described above tend to be somewhat brittle. Thin-sectioning may result in scattered small tears or cracks in areas of the section which contain tissue. This technical problem, however, has not become a

serious handicap in spite of the routine use of grids which lack a support film.

We have described a distinctive morphological alteration which appears in tissues treated with 0.2% digitonin in Flickinger's fixative. This alteration is the appearance of elongate, cylindrical spicules, which frequently possess a tightly stacked, concentrically lamellated wall enclosing a central core (Figs. 2 *b*, 2 *c*, 2 *d*, and 3). These spicules occur in the vicinity of intra- and intercellular membrane surfaces and appear identical with the digitonin-cholesterol crystalline needles of Ökrös (17). Our current interpretation of this spicule formation differs to some degree from that of Okros (17). We agree that the phenomenon depends upon the simultaneous presence of digitonin and sterols in the tissue under examination. It has not been proven, however, that Ökrös's needles are composed only of cholesterol digitonide. Substances such as protein and lipoprotein must be excluded as participating components. The dimensions of the longitudinal periodic lamellae present in the in vitro cholesterol-digitonin complex depicted by Levy et al. (16) are similar to our measurements and those of Ökrös. However, the information present in Fig. 9 of Levy et al. is insufficient to conclude that our spicules and Ökrös' needles are identical with a pure cholesterol digitonide complex. The complete retention of unesterified cholesterol when compared with the relatively low prevalence of spicule formation suggests to the authors that the retained cholesterol has a distribution far greater than the loci of the spicules.

In a recent report Scallen and Napolitano (22) have described a second prominent alteration associated with fixation in digitonin-containing glutaraldehyde, i.e., the consistent preservation of a double minor period in rat sciatic nerve myelin.

It is apparent that the method described here should make possible for the first time radioautographic localization of cholesterol by electron microscopy.

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