AN ULTRASTRUCTURAL AND BIOCHEMICAL STUDY OF THE EFFECTS OF THREE INHIBITORS OF CHOLESTEROL BIOSYNTHESIS UPON MURINE ADRENAL GLAND AND TESTIS

Histochemical Evidence for a Lysosome Response

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ABSTRACT

Triparanol and 20,25-diazacholesterol inhibit cholesterol biosynthesis and result in the accumulation of desmosterol. AY-9944, another inhibitor, produces an accumulation of 7-dehydrocholesterol. Adult male C3H mice receive one of these drugs intraperitoneally. Livers, adrenal glands, and testes from each drug group are excised, and portions of each are analyzed by a modified Liebermann-Burchard reaction for quantitation of sterols. Adrenals and testes are examined also by electron microscopy. Fine-structural localization of acid phosphatase has been studied in triparanol-treated adrenal glands. Biochemical analysis reveals that 14–64% of the sterols occurs as desmosterol or 7-dehydrocholesterol. Fine-structural alterations in the adrenal glands and testes from each drug group are essentially identical. The predominant cytological feature is the occurrence of increased numbers of pleomorphic, unit-membrane–limited, electron-opaque, cytoplasmic inclusions. Hence, the cellular modifications following triparanol administration are not unique, as has been suggested. They represent a generalized phenomenon, probably related to inhibition of cholesterol biosynthesis, which is an effect common to each drug. Lead phosphate reaction product (indicating acid phosphatase activity) is demonstrable within these membrane-limited cytoplasmic bodies, identifying them as morphological lysosomes. The utilization of a lysosomal mechanism in sterol-synthesizing cells, which are accumulating cholesterol intermediates, is discussed.

INTRODUCTION

During the past ten years several chemical compounds have appeared which possess the ability to inhibit cholesterol[1] biosynthesis both in vitro and in vivo. Three structurally different drugs,[2] with an established enzymatic site for their inhibitory

[1] Names of sterols used in this paper: cholesterol (Δ^5-cholesten-3β-ol), desmosterol ($\Delta^{5,24}$-cholestadien-3β-ol), and 7-dehydrocholesterol ($\Delta^{5,7}$-cholestadien-3β-ol).

[2] Names of drugs used in this paper: triparanol (MER-29, 1-[*p*(β-diethylaminoethoxy) phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl) ethanol); 20,25-diazacholesterol (SC-12937, 20,25-diazacholest-5-en-3β-ol dihydrochloride); AY-9944 (trans-1,4-bis (2-

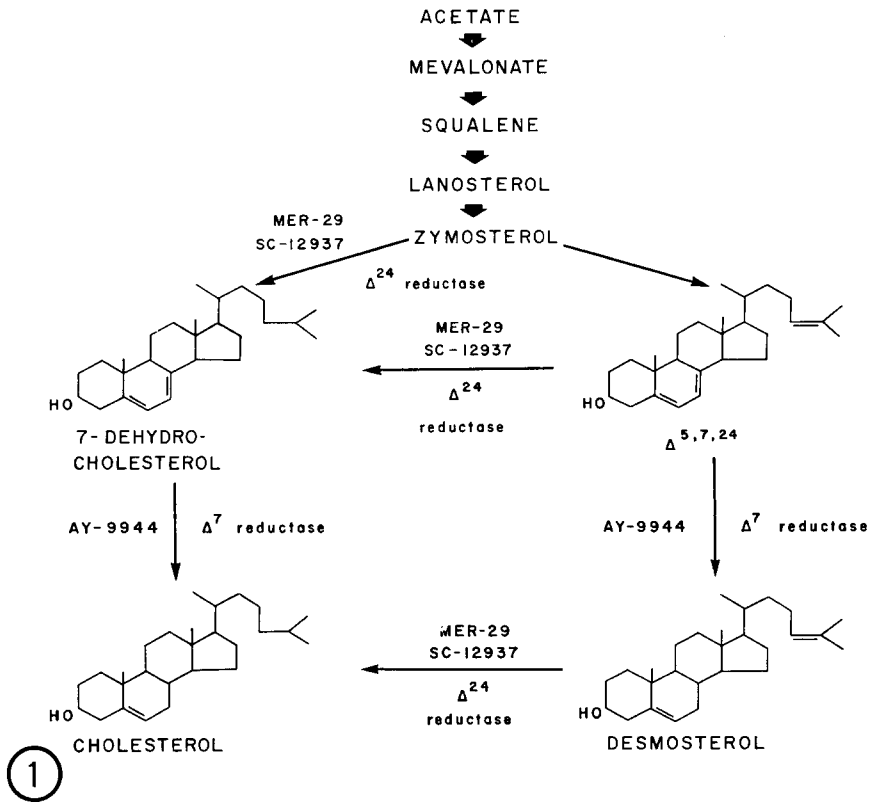


FIGURE 1 An abbreviated diagram of cholesterol biosynthesis. MER-29 (triparanol) and SC-12937 (20,25-diazacholesterol) are Δ^{24}-reductase inhibitors, which result in desmosterol accumulation. AY-9944 is a Δ^{7}-reductase inhibitor, which results in 7-dehydrocholesterol accumulation.

effects, have been utilized in the present investigation for a comparative study of the cellular mechanisms for handling accumulated cholesterol precursors. Triparanol (MER-29) is a Δ^{24}-reductase inhibitor, which results in the accumulation of desmosterol (Fig. 1). The identity of desmosterol as the principal precursor accumulating with administration of triparanol has been verified by ourselves (37) and by others (4, 19). 20,25-diazacholesterol (SC-12937) is another Δ^{24}-reductase inhibitor having a chemical structure distinct from triparanol. Desmosterol (Fig. 1) is also the principal precursor which accumulates with administration of 20,25-diazacholesterol (2, 33). AY-9944 is a Δ^{7}-reductase inhibitor, which results in the accumulation of 7-dehydrocholesterol (Fig. 1). The identity of 7-dehydrocholesterol as the principal precursor accumulating during AY-9944 administration has been documented previously (14).

Previous morphological investigations have dealt with the cellular effects of triparanol upon the adrenal gland, testis, ovary, liver, and pancreas. Light microscopic studies of triparanol-treated rat, monkey, and dog adrenal cortex (26, 15, 43) reveal a partial lipid depletion in frozen sections. Yates (44) reports that the dense cytoplasmic bodies present in the hamster zona fasciculata are histochemically positive for phospholipid. Ultrastructural studies of triparanol administration in a variety of cell types demonstrate a similarity of effects. In the rat liver and pancreas, Hruban et al. (24, 25) and Swift and Hruban (40) describe the presence of cytoplasmic, membranous whorls, which they refer to as "myeloid bodies." These "myeloid bodies" fail to react when incubated in the Gomori medium for acid phosphatase and have

chlorobenzylaminomethyl) cyclohexane dihydrochloride).

not been considered "typical" lysosomes. Acid phosphatase is demonstrable when "myeloid bodies" occur inside autophagic vacuoles. The reaction product is limited, however, to that portion of the autophagic vacuole containing non-myeloid cytoplasmic material. Hruban et al. conclude that the predominant effect of triparanol is "highly specific" and involves the accumulation of newly formed, abnormal, membranous material in the cytoplasm. Volk and Scarpelli (43) report, in the triparanol-treated rat adrenal gland, cellular alterations in the three zones of the cortex. The cortical cells contain hypertrophied, tubular, smooth endoplasmic reticulum and dense cytoplasmic bodies in the zona glomerulosa and reticularis. These authors interpret the primary effect of triparanol to be one of stimulation of the smooth endoplasmic reticulum. Yates and his coworkers (44, 45, 3, 8) have studied the response of the Syrian hamster adrenal cortex, corpus luteum, testis, and small intestine to triparanol administration. They substantiate the earlier reports of the appearance of opaque cytoplasmic bodies and hypertrophy of the smooth endoplasmic reticulum. The internal contents of these opaque cytoplasmic bodies appear as membranous whorls, as a crystalline lattice pattern, or as a "reticular" network of membranes. Hruban et al. (25) and Chen and Yates (8) believe that the membranous whorls ("myeloid bodies," myelin figures) appear first and transform, during continued triparanol administration, into opaque bodies with a "reticular" or crystalline lattice pattern. Yates et al. (45) and Arai et al. (3) have obtained an adrenal gland cell fraction containing the opaque cytoplasmic bodies. They report this fraction to be rich in desmosterol, triparanol, lipoprotein, and phospholipid. Yates et al. suggest that triparanol induces a cytoplasmic "lipodystrophy," characterized by a disorganization of membranous cell structures.

The present investigation was undertaken to clarify several problems relating to the effects of triparanol: (*a*) the specificity of triparanol in stimulating opaque cytoplasmic inclusions; (*b*) the nature of the drug response, i.e., is it the result of inhibition of cholesterol biosynthesis, or does it represent a response unrelated to this effect; and (*c*) the identity of the drug-induced inclusions as cellular organelles. The utilization of a sterol analysis of the treated organs permits a chemical demonstration of drug effect and provides an indication of treatment severity. Comparative studies with three different inhibitors allow conclusions to be formulated regarding triparanol specificity and the relationship of established drug actions to the stimulation of cytoplasmic inclusions. This study reports a similar effect of triparanol, 20,25-diazacholesterol, and AY-9944 in the production of increased numbers of pleomorphic, membrane-limited, cytoplasmic, dense bodies. Ultrastructural acid phosphatase activity is demonstrable within these membrane-limited inclusions, and hence they qualify as morphological lysosomes. Preliminary evidence is presented for a possible origin of these morphological lysosomes from the smooth endoplasmic reticulum. The utilization of lysosomal mechanisms by steroid-synthesizing cells for the sequestration of cholesterol precursors is discussed.

MATERIALS AND METHODS

Adult male C3H mice received one of the drugs in four evenly spaced intraperitoneal injections over an 8-day period. Control mice consisted of a group which received no injections, and another group which received four intraperitoneal injections of olive oil. On the 10th day following initiation of therapy, the mice from each drug and control group were anesthetized with Nembutal and the livers, adrenal glands, and testes were excised.

The drugs and the doses utilized in this study were: (*a*) triparanol (MER-29)[3]-300 mg/kg as an olive oil solution containing 10 mg/0.1 ml; (*b*) 20,25-diazacholesterol (SC-12937)[4]-30 mg/kg as an aqueous solution containing 1 mg/0.1 ml; and (*c*) AY-9944[5]-30 mg/kg as an aqueous solution containing 1 mg/0.1 ml.

Samples of the livers, adrenal glands, and testes representing each of the drug groups were analyzed quantitatively for sterol content. Colorimetric assay for sterols employed the method of Abell et al. (1) suitably modified for the estimation of desmosterol (4) and 7-dehydrocholesterol (30). In addition, infrared and ultraviolet spectroscopy were used to verify the identity of these sterols in a manner similar to that previously described (38).

[3] We wish to thank A. Wassermann, S.p.A. of Societa Italiana per Specialita Farmaco-Terapeutiche, Milan, for the gift of MER-29 used in our studies.

[4] We wish to thank Dr. R. E. Ranney of G.D. Searle and Co., Skokie, Ill., for the gift of SC-12937 used in our studies.

[5] We wish to thank Dr. D. Dvornik of Ayerst Laboratories, Montreal, for the gift of AY-9944 used in our studies.

TABLE I

Sterol Content of Mouse Liver, Adrenal, and Testis

Organ	Drug	Cholesterol		Desmosterol		7-Dehydrocholesterol		Other sterols§	
		*mg**	%‡	*mg*	%	*mg*	%	*mg*	%
Liver	None	0.156	100	0	0	0	0	0	0
	MER-29	0.057	36	0.102	64	0	0	0	0
	SC-12937	0.131	54	0.085	35	0	0	0.026	11
	AY-9944	0.165	71	0	0	0.068	29	0	0
Adrenal	None	0.830	95	0.040	5	0	0	0	0
	MER-29	0.566	65	0.227	26	0	0	0.078	9
	SC-12937	0.320	62	0.134	26	0	0	0.062	12
	AY-9944	1.28	75	0	0	0.427	25	0	0
Testis	None	0.273	100	0	0	0	0	0	0
	MER-29	0.159	60	0.106	40	0	0	0	0
	SC-12937	0.204	79	0.054	21	0	0	0	0
	AY-9944	0.207	86	0	0	0.033	14	0	0

* Expressed as mg of sterol per 100 mg tissue, wet weight.

‡ Expressed as % of total sterol.

§ Based upon rapid (1½-min) color development in the Liebermann-Burchard reaction.

Adrenal glands and testes, obtained simultaneously with the samples for chemical analysis, were prepared for routine electron microscopy. Each organ was fixed at room temperature by immersion in a solution of 2% formaldehyde-2.5% glutaraldehyde (17), which was buffered to pH 7.2 with 0.2 M sodium cacodylate (22) containing 0.05% $CaCl_2$. After 1 hr, the organs were diced and returned to the fixative for another hour. Following an overnight wash in 0.1 M sodium cacodylate (pH 7.2) containing 0.05% $CaCl_2$, the specimens were postfixed for 2 hr at room temperature in 1% osmium tetroxide buffered to pH 7.2 with 0.2 M sodium cacodylate. Dehydration was accomplished in a graded series of ethanols. The tissues were embedded in Epon 812 (29) and polymerized stepwise at 37°, 45°, and 60°C, over the next 4 days. Adrenals from mice treated with each drug were fixed also for 2 hr in ice-cold 1% osmium tetroxide without prior aldehyde exposure. These adrenal glands were then processed as outlined above.

Adrenal glands removed from the triparanol-treated group of mice were studied further by incubation in a modified Gomori medium (see reference 6 for details) for the fine-structural localization of acid phosphatase. These specimens were fixed for 2 hr in ice-cold 2% formaldehyde-2.5% glutaraldehyde, which lacked the $CaCl_2$ additive. Following a 5-hr wash in several changes of 0.1 M cacodylate buffer, the tissue blocks were sectioned at 50 μ on a Sorvall TC-2 Tissue Sectioner, and the sections were incubated for 20 min at 37°C in a medium containing sodium betaglycerophosphate, lead nitrate, and Tris-maleate buffer (pH 5) (6). The omission of sodium betaglycerophosphate and the addition of 0.01 M sodium fluoride to the routinely prepared medium served as separate controls for the histochemical reaction. All sections were washed in succession with distilled water, 2% acetic acid, and distilled water. Subsequent handling of the tissue was identical to the procedures outlined above, except for shorter time periods in osmium tetroxide and the ethanol series.

Thick sections stained with azure II-methylene blue (35) were examined by phase contrast microscopy, and the adrenal cortex and testicular interstitium were identified. Thin sections, cut with a diamond knife on a Huxley ultramicrotome, were stained in alcoholic uranyl acetate (21) followed by lead citrate (34) and photographed in an Hitachi HU-11A or HU-11C electron microscope.

RESULTS

Chemical

Table I summarizes our chemical findings. We are able to demonstrate, in the organs under study, an accumulation of the expected cholesterol precursors associated with drug administration. In mature animals, cholesterol normally accounts for virtually all of the sterol present (see untreated control organs, Table I). While desmosterol and 7-

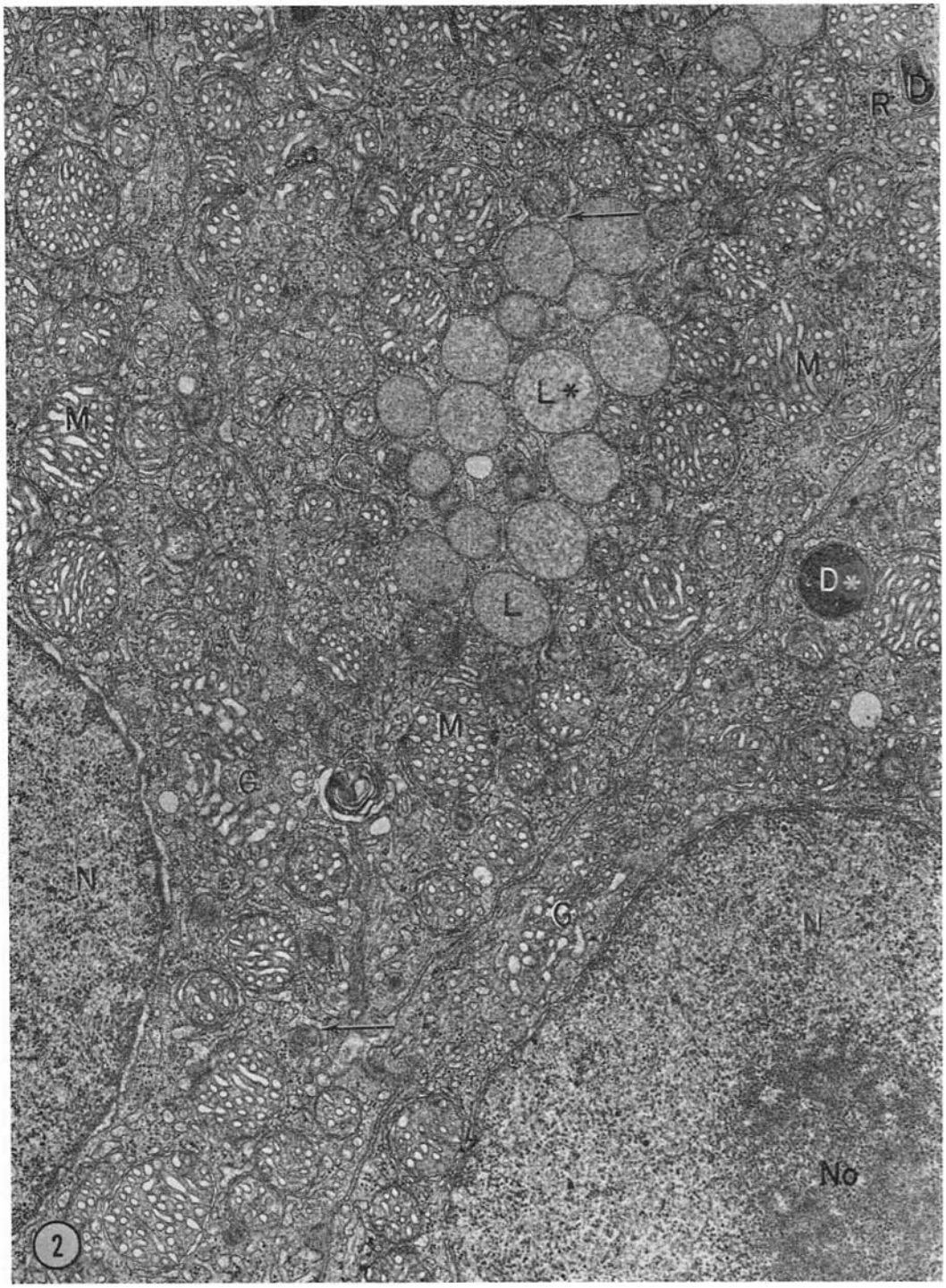


FIGURE 2 Portions of three cells in the normal zona fasciculata. Control adrenal from a mouse treated with olive oil. These cells possess several features in common with the zona glomerulosa: (*a*) a large, ovoid nucleus (*N*) containing a prominent nucleolus (*No*); (*b*) cytoplasmic lipid droplets (*L*); (*c*) a Golgi zone (*G*); (d) free ribosomes (*R*); (*e*) an occasional membrane-limited dense body (*D*); and (*f*) a smooth-surfaced endoplasmic reticulum (arrows). Round or oval mitochondrion with vesicular or flattened saccular cristae, *M*. *See Fig. 3 legend. × 18,000.

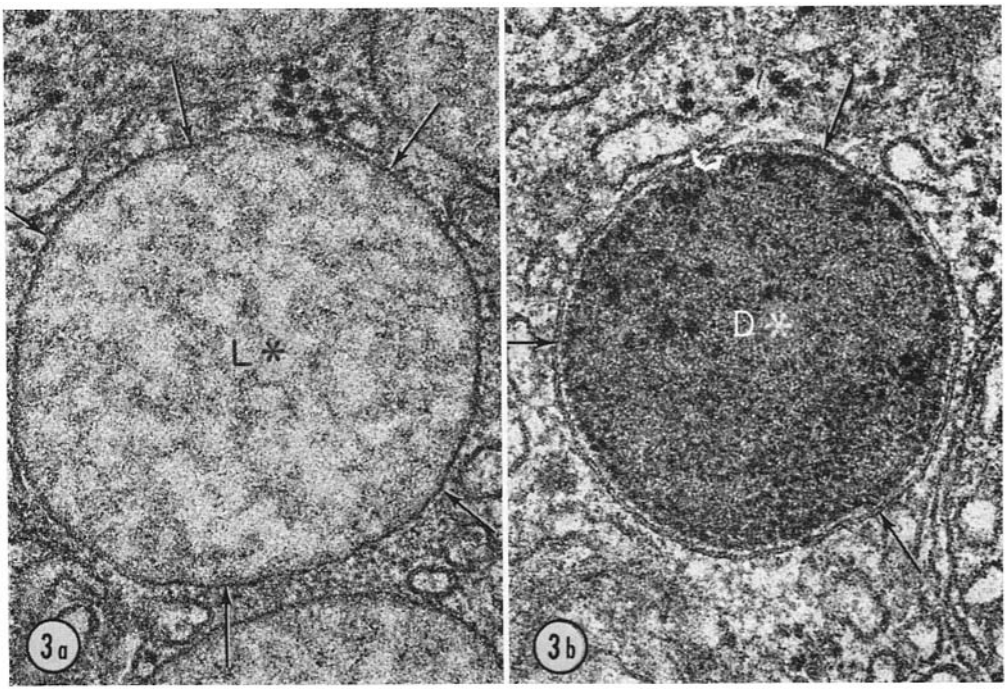


FIGURE 3 *a* An enlargement of the lipid droplet (*L**) from Fig. 2. Preservation of the contents is average for the conditions of this report. Although the plane of section passes close to the droplet center, there is no evidence of a trilaminar unit-membrane at any point (arrows) along the electron-opaque limiting interface. × 88,000.

FIGURE 3 *b* An enlargement of the dense body (*D**) from Fig. 2. A trilaminar unit membrane is demonstrable limiting the entire circumference (arrows) of this body. It is unlikely that fixation would permit adequate preservation of this limiting membrane and yet fail to preserve the limiting interface of a lipid droplet (*L**, Fig. 2) only 300 μ distant. × 88,000.

dehydrocholesterol are present in normal mature animals, these sterols occur in only trace quantities.

The percentage accumulation of desmosterol is greater in MER-29-treated mice (liver 64%, testis 40%, and adrenal 26%) than in mice receiving SC-12937 (liver 35%, testis 21%, and adrenal 26%). Small quantities of other sterol precursors, presumed by their color development to be Δ^7- and/or Δ^8-sterols, also occur in the liver and adrenal of mice receiving SC-12937 and in the adrenal of MER-29-treated mice. This observation is similar to the findings of Clayton, Nelson, and Frantz (11) in the skin of rats receiving MER-29. AY-9944 results in the accumulation of 7-dehydrocholesterol (liver 29%, testis 14%, and adrenal 25%).

Table I demonstrates further that the response of different organs to the same drug may vary more than twofold, i.e., 64% of the liver sterols, compared to only 26% of adrenal sterols, exists as desmosterol following MER-29 administration. It should be noted that substantial amounts of cholesterol occur in the liver, adrenal gland, and testis following treatment with all three drugs. Such data suggest that complete inhibition of cholesterol biosynthesis is not a necessary concomitant to drug administration.

Normal Fine Structure

ZONA FASCICULATA AND ZONA GLOMERULOSA: The reports of Zelander (47, 48) provide an extensive description of the electron microscopic appearance of normal mouse adrenal gland. His work should be consulted for the many details of the adrenal cortex peripheral to this study.

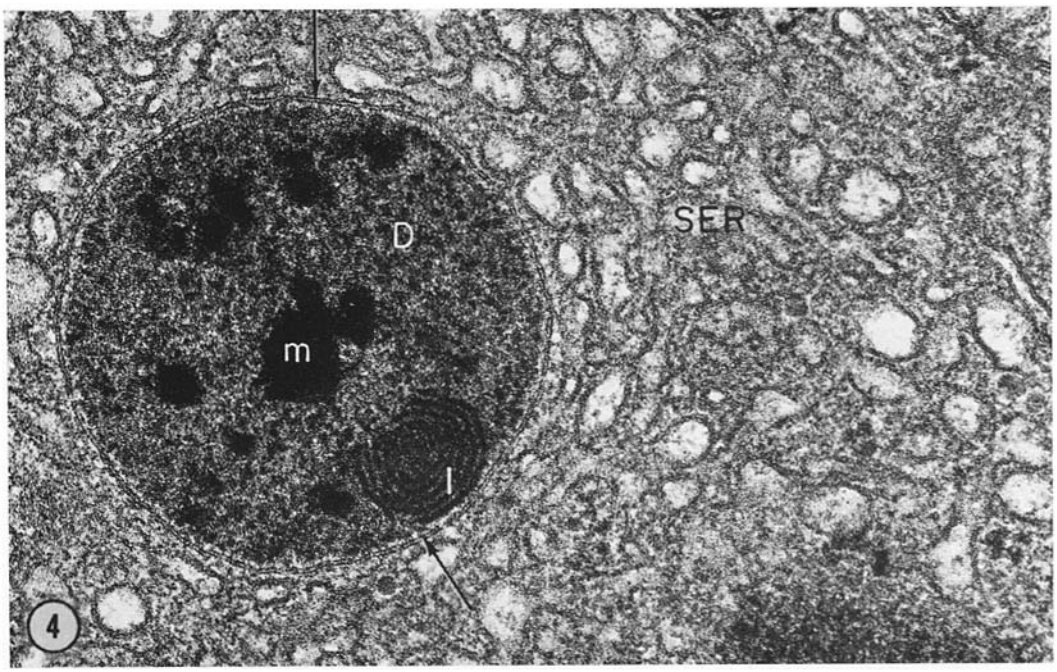


FIGURE 4 A portion of cytoplasm from a testicular Leydig cell. Control testis from a mouse treated with olive oil. Illustrated are two cellular features occurring in this cell: (*a*) an abundant smooth-surfaced endoplasmic reticulum (*SER*), and (*b*) an occasional unit-membrane-limited (arrows) dense body (*D*). These dense bodies (*D*) may contain amorphous masses of greater density (*m*) and electron-opaque concentric lamellae (*l*). × 88,000.

There are no significant differences in morphology between the adrenal cortices of untreated control mice and the adrenal cortices of control mice receiving intraperitoneal olive oil. We conclude that the olive oil vehicle for triparanol in the quantities used in this investigation is innocuous to mice. The adrenal cortical fine structure of C3H mice from both control groups substantiates most of Zelander's findings. Cells occurring in the zona fasciculata (Fig. 2) and zona glomerulosa share several characteristics in common: (*a*) a large, ovoid nucleus; (*b*) cytoplasmic lipid droplets; (*c*) a prominent Golgi zone; (*d*) occasional pleomorphic, unit-membrane–limited, cytoplasmic, dense bodies (Zelander's "globules"); and (*e*) a smooth-surfaced endoplasmic reticulum consisting of intertwining tubules, channels, vesicles, and small vacuoles. Adrenal cortical lipid droplets possess an electron-opaque interface delimiting their contents from the surrounding cytoplasm. We have been unable to resolve, in the many planes of section in which these lipid droplets are seen, a single example of a trilaminar, unit-membrane structure at this interface (Fig. 3 *a*). This observation is in agreement with the descriptions of Fawcett (18) and Long and Jones (27, 28) regarding lipid droplet fine structure in the liver and adrenal. A trilaminar unit-membrane is always demonstrable, in a favorable plane of section, delimiting the cytoplasmic dense bodies (Fig. 3 *b*). The presence of this trilaminar, limiting, unit-membrane serves as a reliable characteristic for distinguishing the two inclusions. These dense bodies may contain concentric lamellae of greater density as an accompanying component to the finely granular, moderately dense matrix. Similar dense bodies in the normal rat adrenal cortex possess fine-structural acid phosphatase and qualify as morphological lysosomes (32, 41).

TESTICULAR LEYDIG CELLS: Christensen and Fawcett (10) have published recently a detailed study of the ultrastructure of mouse testicular Leydig cells. The following description is confined to those Leydig cell features pertinent to the present study. No important morphological dif-

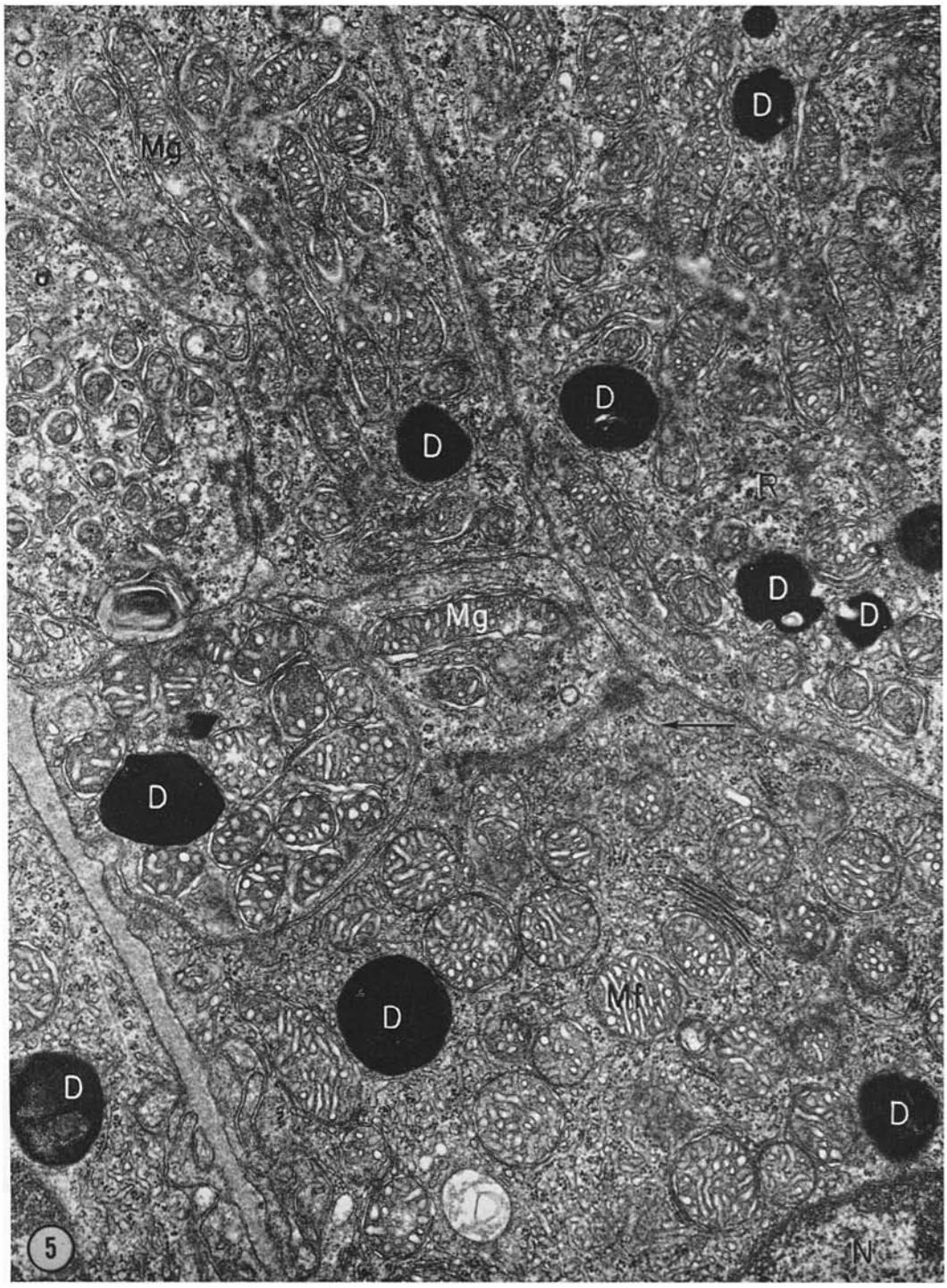


FIGURE 5 Portions of six adrenal cortical cells from a mouse treated with MER-29. The cellular effects of this drug are identical with the effects following SC-12937 and AY-9944. Administration of these drugs is associated with a marked increase in the number of pleomorphic, membrane-limited, cytoplasmic dense bodies (*D*). Compare the presence of nine dense bodies (*D*) in Fig. 5 with the occurrence of only two dense bodies (*D*) in control adrenal (Fig. 2). The predominant dense body shape is a slightly irregular, round-to-ovoid form. The figure allows a comparison of the elongate mitochondria in the zona glomerulosa (*Mg*) with the round or ovoid mitochondria in the zona fasciculata (*Mf*). Nucleus, *N*; ribosomes, *R*; smooth endoplasmic reticulum, arrow. × 18,000.

ferences occur between Leydig cells from untreated control mice and Leydig cells from control mice treated with intraperitoneal olive oil. The Leydig cell fine structure of both control groups agrees closely with earlier reports (10). Fig. 4 depicts a portion of a typical Leydig cell and illustrates certain morphological features which are present in these cells: (*a*) an abundant smooth-surfaced endoplasmic reticulum consisting of anastomosing tubules associated with scattered vesicles and small vacuoles, and (*b*) occasional pleomorphic, unit-membrane–limited, cytoplasmic, dense bodies. These bodies appear identical to their counterpart in the adrenal cortex and usually possess a finely granular matrix of moderate density. Such inclusions also may contain amorphous masses of greater density and electron-opaque concentric lamellae (Fig. 4).

Cytological Effects of Triparanol, 20,25-Diazacholesterol, and AY-9944

INTRODUCTION: Triparanol, 20,25-diazacholesterol, and AY-9944 produce identical cellular alterations in the zona glomerulosa, zona fasciculata, and testicular Leydig cells. Administration of these drugs is associated with the appearance of increased numbers of pleomorphic, membrane-limited, cytoplasmic, dense bodies. The effects of these drugs are readily apparent when the affected cells are examined in the electron microscope. Fig. 5 demonstrates the presence of nine dense bodies in a typical area from MER-29-treated adrenal cortex. This represents a significant increase over the two dense bodies visible in a typical area (at the same magnification) of control adrenal (Fig. 2). Similar fine-structural alterations have been reported in a variety of cell types by several workers (24, 25, 40, 43–45, 3, 8, 46). It is important to emphasize that the morphological alterations associated with drug administration are abnormal in relation to the increased quantity of cytoplasmic dense bodies. Similar membrane-limited dense bodies occur in the untreated control cells, but in greatly reduced numbers.

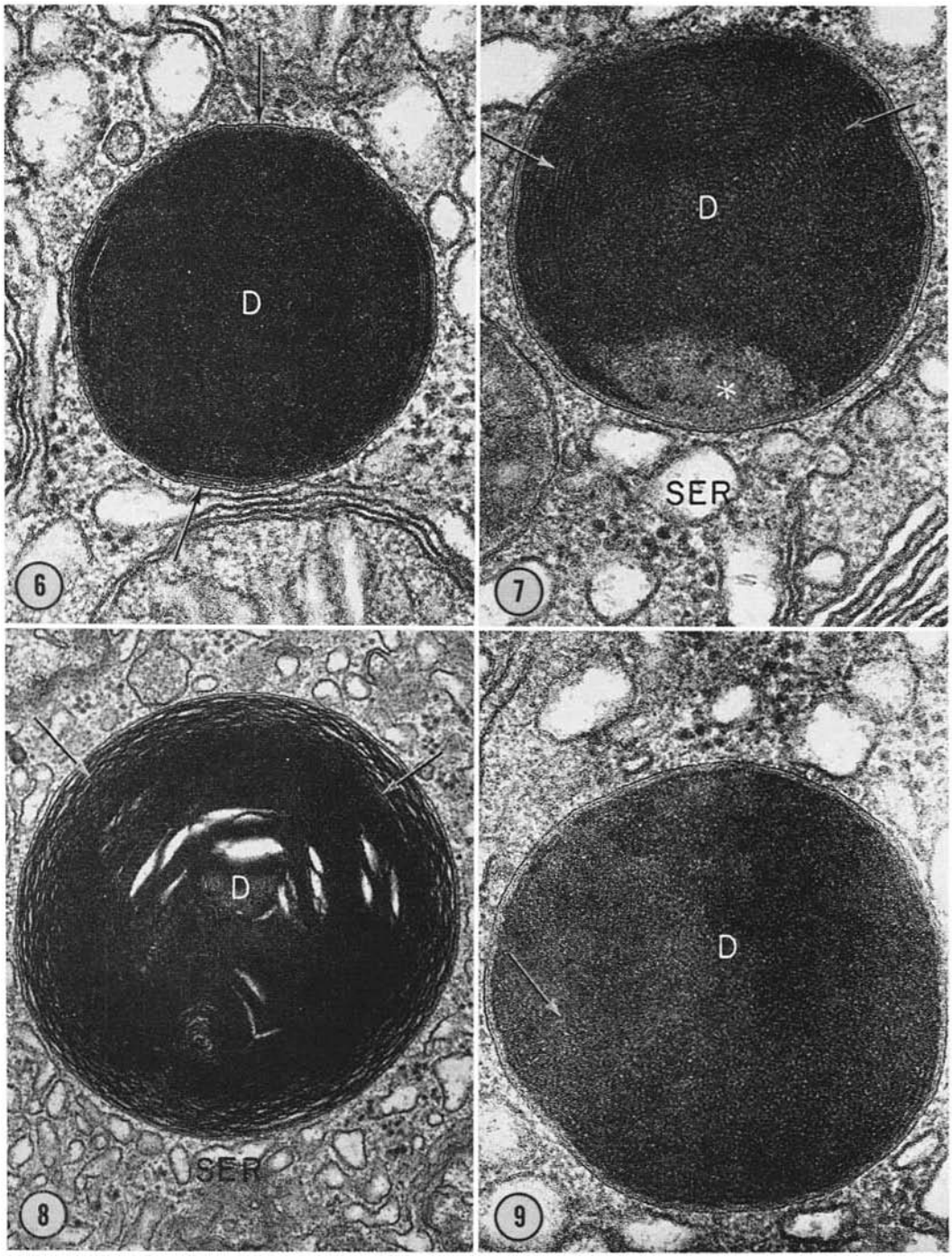
DENSE BODY FINE STRUCTURE: Drug-induced dense bodies occur exclusively in the cell cytoplasm (Fig. 5). Similar structures have not been encountered within the nuclei of cells exposed to the inhibitors. These bodies appear to be distributed randomly in the cytoplasm. Dense body diameters measure approximately 0.4–1.0 μ. Although the shape of these bodies is variable, a slightly irregular, round-to-ovoid form predominates (Fig. 5). At higher magnification, these dense bodies, which are identical to those depicted in Fig. 5, are delimited clearly by a trilaminar unit-membrane (Figs. 6, 7, and 9). In this respect, they are also identical to the dense bodies present in control preparations, as shown in Figs. 3 *b* and 4. The contents of these bodies are

FIGURE 6 A portion of cytoplasm in an adrenal cortical cell from a mouse treated with SC-12937. The dense body (*D*) is filled with a homogeneous, highly electron-opaque material and is delimited clearly by a trilaminar unit-membrane (arrows). A trilaminar unit-membrane is also visible limiting the dense bodies (*D*) in Figs. 7 and 9. OsO_4 fixed. × 83,000.

FIGURE 7 A portion of cytoplasm in an adrenal cortical cell from a mouse treated with SC-12937. The dense body (*D*) contains a finely granular matrix of moderate electron opacity (*) and concentric, regularly stacked lamellae (arrows) composed of electron-opaque material. Some vesiculation of the smooth reticulum (*SER*), which occurs inconsistently with OsO_4, is apparent. OsO_4 fixed. × 83,000.

FIGURE 8 A portion of cytoplasm in a Leydig cell from a mouse treated with MER-29. The dense body (*D*) contains a concentric, irregular lamination of membranes (arrows) forming a myelin figure (membranous whorl). Vesiculation of the smooth reticulum (*SER*) is essentially absent. OsO_4 fixed. × 44,000.

FIGURE 9 A portion of cytoplasm in an adrenal cortical cell from a mouse treated with SC-12937. The dense body (*D*) has a finely stranded, mesh-work arrangement of the electron-opaque material, which imparts a "reticular" pattern (arrow) to the contents (Chen and Yates' Type III body). OsO_4 fixed. × 83,000.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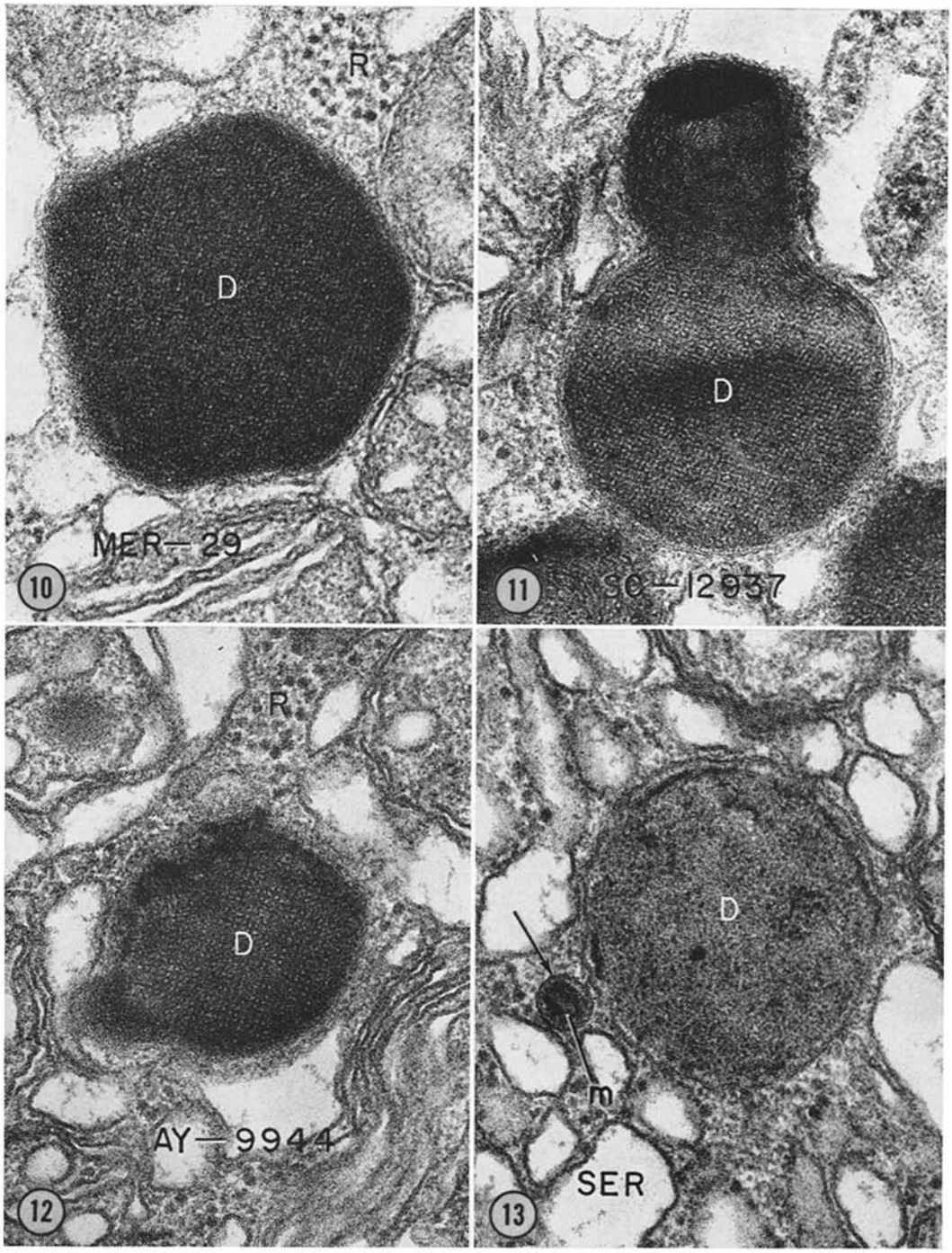


FIGURES 10–12 Portions of cytoplasm in three adrenal cortical cells, each from a mouse treated with a different drug. The dense bodies (*D*) possess a crystalline lattice arrangement of electron-opaque material, resulting in a rectangular or hexagonal repeating array (Chen and Yates' Type IV body). Fig. 10, treated with MER-29; Fig. 11, treated with SC-12937; and Fig. 12, treated with AY-9944. Ribosomes, *R*; smooth endoplasmic reticulum, *SER*. OsO_4 fixed. × 83,000.

FIGURE 13 A portion of cytoplasm in an adrenal cortical cell from a mouse treated with AY-9944. A smooth-surfaced, membrane-limited vesicle (arrow) occurs in the cytoplasm and appears similar to adjacent smooth reticulum (*SER*). This vesicle (arrow) is noteworthy in the presence of electron-opaque material (*m*) within the cisternal space. Compare the similar electron opacity of this material (*m*) with the opaque material present in many dense bodies (Fig. 6). Dense body, *D*. OsO_4 fixed. × 83,000.

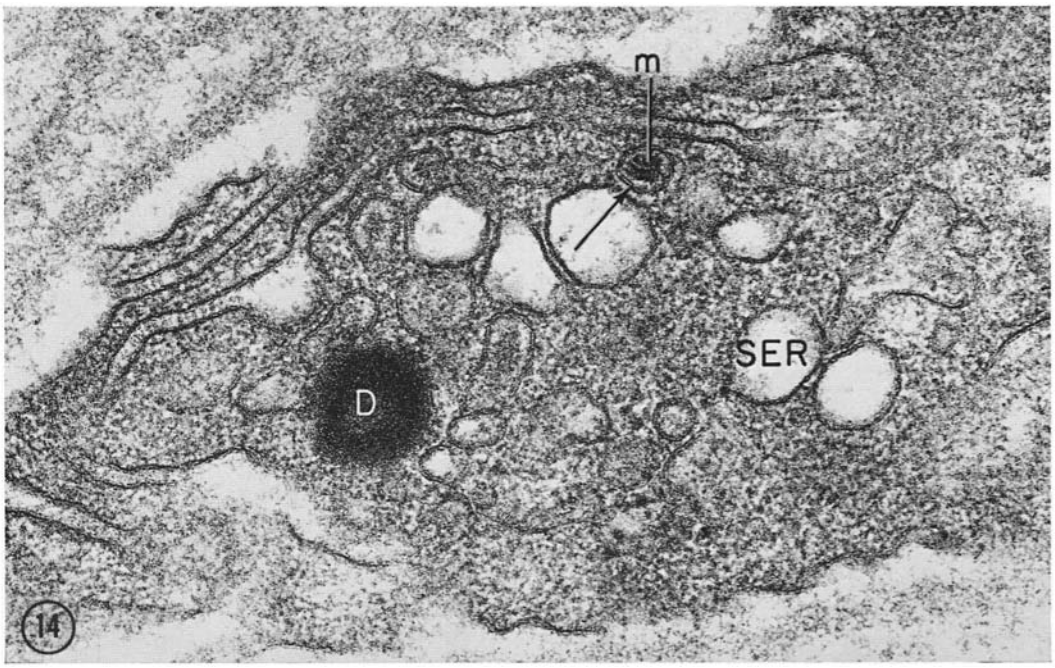


FIGURE 14 A portion of cytoplasm in an adrenal cortical cell from a mouse treated with SC-12937. A smooth-surfaced, membrane-limited vesicle (arrow) occurs in the cytoplasm, which appears similar to adjacent smooth reticulum (*SER*). This vesicle (arrow) contains electron-opaque material (*m*), which displays a lamellar organization, within the cisternal space. Compare the similar electron opacity and lamellar organization of this material (*m*) with the concentric lamellae visible inside some dense bodies (Fig. 7). Dense body, *D*. OsO_4 fixed. × 83,000.

pleomorphic and exist (often in combination) in several recognizable forms; (*a*) as a finely granular matrix of moderate electron opacity (Fig. 7); (*b*) as a homogeneous, amorphous, highly electron-opaque material, which may fill the body and obscure further detail (Fig. 6); (*c*) as concentric, regularly stacked lamellae composed of the electron-opaque material (Fig. 7); (*d*) as a concentric, irregular lamination of membranes forming a myelin figure or membranous whorl (Fig. 8), which appears identical to Chen and Yates' Type I body (8); (*e*) as a finely stranded, meshwork arrangement of the opaque material, which imparts a "reticular" pattern to the contents (Fig. 9), and appears identical to Chen and Yates' Type III "reticular" body (8); and (*f*) as a crystalline lattice arrangement of opaque material resulting in a rectangular or hexagonal repeating array (Figs. 10–12), which appears identical to Chen and Yates' Type IV body (8). Contrary to the data of Yates et al., reporting the presence of a crystalline lattice structure in hamster dense bodies only after triparanol administration (46), we have been able to demonstrate a crystalline lattice pattern in dense bodies of the murine adrenal cortex following treatment with triparanol (Fig. 10), 20,25-diazacholesterol (Fig. 11), and AY-9944 (Fig. 12). All of these parameters of fine structure are shared by the dense bodies, which accumulate in both adrenal cortex and Leydig cells, following administration of each of the drugs under study.

EFFECTS OF FIXATION: Two fundamental types of fixation have been employed in this study: (*a*) a mixture of formaldehyde-glutaraldehyde followed by osmium tetroxide (17), and (*b*) initial osmium tetroxide, omitting any aldehyde exposure. The former permits the most effective preservation of the numerous cell constituents, especially the smooth-surfaced endoplasmic reticulum, which often dilates and forms vesicles under less than optimal conditions (10). Osmium tetroxide appears to be an adequate fixative, although

some vesiculation of the smooth reticulum occurs as an inconsistent finding (compare Fig. 7 with Fig. 8). Dense body fine structure is identical, however, under both fixation procedures. An exception to this generalization is the presence of a crystalline lattice structure in the contents of certain dense bodies following drug administration (Figs. 10–12). The ease with which such a lattice pattern can be demonstrated is increased greatly by fixation in osmium tetroxide. We are not prepared to state, however, that lattice patterns occur exclusively in osmium tetroxide–fixed material.

PRESUMPTIVE SMOOTH-SURFACED ENDOPLASMIC RETICULUM. Earlier workers have reported that a hypertrophy of the smooth endoplasmic reticulum is associated with triparanol administration (43, 44). At the relatively low level of precursor accumulation achieved in the present study, we are unable to document convincingly an increase in the quantity of smooth endoplasmic reticulum in murine adrenal cortex and Leydig cells. Following treatment with each drug, however, smooth-surfaced, membrane-limited vesicles and tubules measuring approximately 50–90 mμ across the smallest diameter are encountered in the cytoplasm. These vesicles and tubules are similar to adjacent elements of the smooth-surfaced endoplasmic reticulum and are noteworthy in the presence of electron-opaque material within the cisternal space (Figs. 13 and 14). The electron opacity of this cisternal material is similar to the density of the opaque material present in many dense bodies (compare Figs. 13 and 14 with Fig. 6). Occasionally, the opaque cisternal contents display a lamellar organization suggestive of the concentric lamellae visible within dense bodies (compare Fig. 14 with Fig. 7). Morphologically, the cisternal contents of these vesicles are similar, if not identical, to the contents of the dense cytoplasmic bodies.

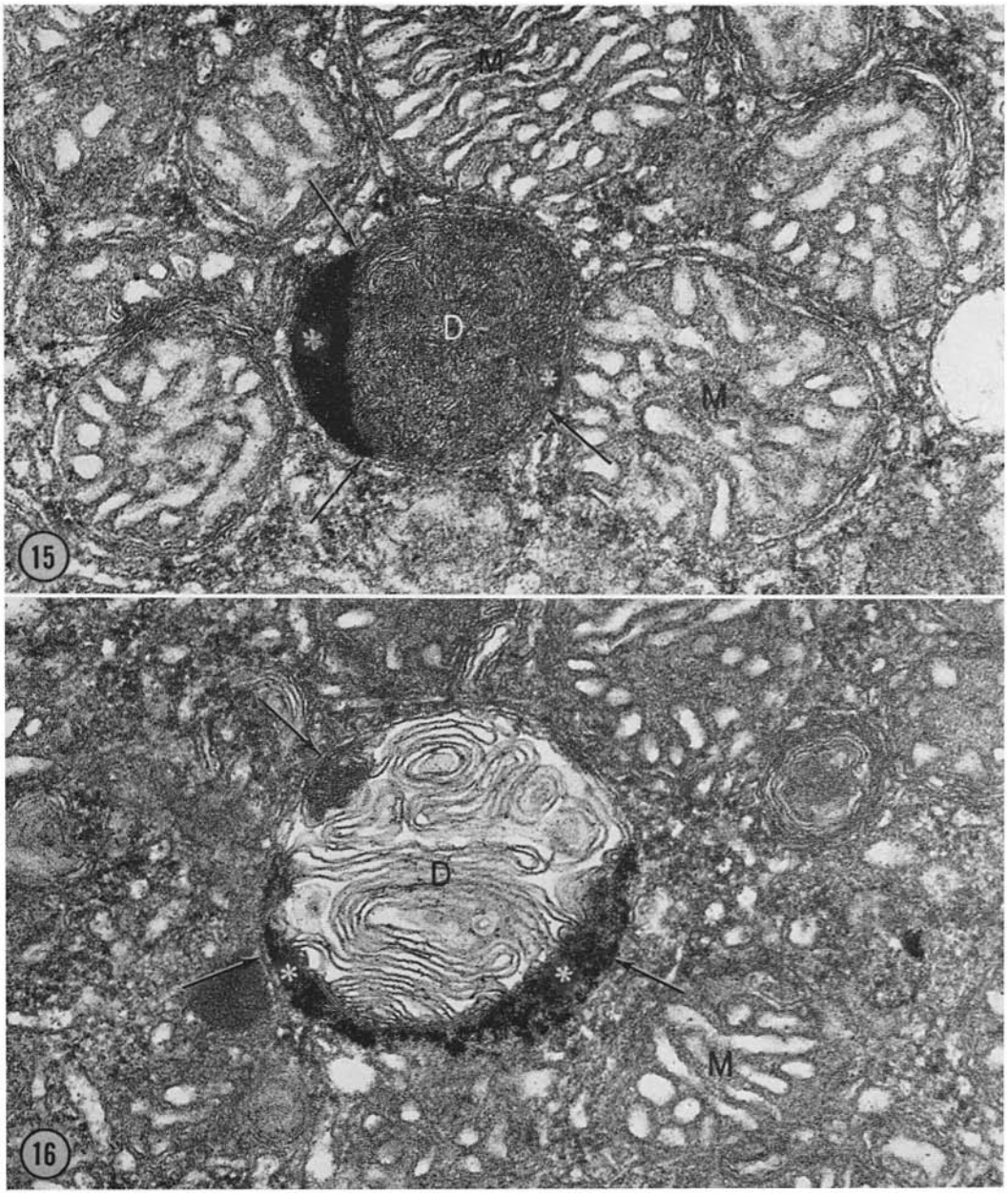
Acid Phosphatase Localization

Adrenal glands from triparanol-treated mice have been examined for the fine-structural localization of acid phosphatase. The demonstration of this hydrolytic enzyme activity within membrane-limited, cytoplasmic bodies fulfills the criteria of Essner and Novikoff (16) for the identification of such bodies as morphological lysosomes. Following the incubation of adrenal gland sections in a betaglycerophosphate medium containing lead nitrate, electron-opaque, lead phosphate reaction product (indicating acid phosphatase activity) is demonstrable within the drug-induced dense bodies (Figs. 15 and 16). The accumulation of lead reaction product is limited precisely within the membrane-defined extent of these bodies. Examples occur of enzyme-positive bodies which possess contents having most of the fine-structural forms outlined above. Fig. 15 represents a "reticular" form (Yates' type III) and Fig. 16 is a myelin figure (Yates' type I). An estimate of the relative number of dense bodies which are enzyme-positive is about 20%. Extraneous lead precipitate occurs occasionally as a nonspecific speckling over the nucleus or cytoplasm. Vigorous washing (see Methods) is effective in reducing this artifact. Control sections are incubated in a medium lacking betaglycerophosphate and in an intact medium containing 0.01 M sodium fluoride (a potent phosphatase inhibitor). Sections handled in this manner are consistently unreactive.

DISCUSSION

A comparison of the biochemical and ultrastructural effects of inhibitors of cholesterol biosynthesis provides one method for the study of cellular mechanisms of sterol metabolism. The intent of the present study is an investigation of the cytological responses of steroid-synthesizing cells to the accumulation of cholesterol precursors, normally present in only trace amounts. An attempt has been made, by the utilization of low inhibitor doses, to achieve a submaximal blockade of cholesterol biosynthesis. The demonstration of substantial quantities of cholesterol in the drug-treated organs (see Chemical Results) suggests that a submaximal effect has occurred. The authors believe that greater insight into mechanisms of sterol metabolism can be obtained by the study of cells not rendered severely toxic through maximal drug effects.

Triparanol, 20,25-diazacholesterol, and AY-9944 represent chemically distinct inhibitors. They share, however, two distinguishing characteristics: (*a*) they interrupt terminal stages of cholesterol biosynthesis by means of reductase inhibition, and (*b*) they produce identical cytological alterations in adrenal gland and testis. We conclude, therefore, contrary to the reports of Hruban et al. (24, 25, 40), that the morphological alterations following triparanol administration are neither "specific" nor unique. The accumulation of pleomorphic, membrane-limited, dense bodies



FIGURES 15 and 16 Portions of cytoplasm in two adrenal cortical cells from a mouse treated with MER-29. Incubated in a Gomori medium (pH 5) for localization of acid phosphatase activity. Lead phosphate reaction product (*) is demonstrable with in the membrane-limited confines (arrows) of these cytoplasmic dense bodies (*D*). These features fulfill the criteria of Essner and Novikoff (16) for an identification of MER-29–induced dense bodies as morphologic lysosomes. Fig. 15 represents an enzyme-positive "reticular" body. Fig. 16 is an enzyme-positive myelin figure. Mitochondrion, *M*. × 55,000.

is a general phenomenon, produced by at least three drugs. It seems probable that this common cytological response is related directly to the shared inhibitory effects which these drugs have upon cholesterol biosynthesis.

Fine-structural localization of acid phosphatase activity has been demonstrated in triparanol-induced dense bodies within the adrenal cortex. Approximately 20% of the dense bodies examined demonstrate the presence of lead phosphate reaction product. This observation does not imply that the unreactive dense bodies differ in identity from enzyme-positive bodies. Several explanations, probably acting in combination, exist for the lack of reactivity of some lysosomal dense bodies. Gahan (20), Novikoff (31), and Barka and Anderson (7) should be consulted for a detailed discussion of the problems related to the histochemical identification of lysosomes. No statement is possible regarding the acid phosphatase positivity of crystalline lattice bodies (Yates' type IV) since the use of initial osmium tetroxide fixation to facilitate their demonstration destroys enzyme activity (36). We believe that each of these membrane-limited dense bodies represents a morphological lysosome involved in a spectrum of internal reorganizations. The various fine-structural forms, which the contents assume, do not indicate a difference in identity between bodies, but probably represent physiochemical alterations of the body contents (5). We speculate that the formation of lysosomes in response to increased whole organ levels of cholesterol precursors is the result of a selective compartmentalization of accumulating precursors within these membrane-limited organelles. Since the chemical analyses have not been performed on isolated granule fractions, we have no evidence at this time regarding the non-enzymatic content of drug-induced dense bodies. However Yates et al. (45) and Arai et al. (3) report that a cell fraction from hamster adrenal gland, which contains triparanol-induced dense bodies, is rich in desmosterol, triparanol, lipoprotein, and phospholipid. Such findings are compatible with our belief that precursor sequestration is involved in the formation of these lysosomes. An indication of the effectiveness of cellular mechanisms in handling these accumulating precursors is the ability of littermate mice to reach sexual maturity, breed, and reproduce viable offspring, while receiving 0.5 mg/day of triparanol from 5 days of age. The livers of these viable offspring contain approximately 50% of their sterols in the form of desmosterol.

A group of storage diseases referred to as the lipidoses provide a possible clinical correlate to the cholesterol inhibitor-induced lysosome response reported here. Tay-Sachs' disease, an example of ganglioside storage, is considered by many investigators to result from a deficiency in one or several specific hydrolytic enzymes (39). All workers agree that metabolic intermediates accumulate in association with the appearance of membranous cytoplasmic bodies (myelin figures) in the central nervous system. Volk (42) has demonstrated the presence of acid phosphatase activity within these membranous inclusions. It is interesting to speculate that the abnormal storage of gangliosides in Tay-Sachs' disease may result from enzyme deficiencies within lysosomes. The drug-induced accumulations of cholesterol precursors, which have been reported in this study, may represent an alternative leading to lysosome accumulation, i.e., entrance of sterol substrates into a mammalian lysosome system, which lacks the degradative enzymes necessary for the catabolism of the cyclopentanoperhydrophenanthrene ring (see reference 23 for a discussion of sterol metabolic pathways and end products). The stimulation of this lysosome response suggests that the accumulating precursors are unable to substitute for cholesterol in normal cellular metabolism.

If the cholesterol biosynthesis inhibitors, which have been employed in this investigation, result in a pronounced lysosome response within the afflicted cells, the question arises regarding the origin of these dense bodies. Chesterton (9), and numerous other workers within the last decade, agree that the cellular location of most (if not all) of the enzymes involved in cholesterol biosynthesis is the endoplasmic reticulum (i.e., microsome fraction). An extensive manipulation of drug doses, administration schedules, and time sequence studies would be required to undertake a definitive morphological investigation of dense body origin. These represent some of the variables which have been kept constant in the present study in order to simplify the comparison of the cellular effects of different inhibitors of cholesterol biosynthesis. In spite of these limitations, we observe following inhibitor administration the presence of electron-opaque material within the cisternae of vesicular and tubular elements, which appear similar to the

smooth endoplasmic reticulum. From a comparison of electron opacity and lamellar organization, such cisternal contents are morphologically similar to the opaque contents of cytoplasmic dense bodies (compare Figs. 13 and 14 with Figs. 6 and 7). Although preliminary, and by no means unequivocal, these findings suggest that a possible origin for the dense bodies and their opaque contents is an elaboration from the smooth reticulum. The endoplasmic reticulum, the Golgi complex, and the GERL of Novikoff all have been implicated in lysosome production in a wide variety of cell systems (12). The authors suggest that the smooth-surfaced endoplasmic reticulum, as the location of the enzyme systems active in cholesterol biosynthesis (23, 9), is a logical organelle in which accumulating desmosterol and 7-dehydrocholesterol might reside. Sequestration of these precursors into lysosomes arising from the smooth endoplasmic reticulum would permit a remarkable economy of two diverse cellular activities.

An abbreviated report on this research has appeared earlier in abstract form (13).

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