Preparation and properties of a cell-free system from rat skin that catalyzes sterol biosynthesis

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ABSTRACT Homogenates of epidermis from rat skin were centrifuged at 10,000 × *g* for 20 min. The supernatant fraction ("whole homogenate") catalyzed the demethylation of lanosterol (C_{30}) to yield C_{27}-sterols. The rate of reaction was measured by the rate of release of $^{14}CO_2$ from the 4-methyl group of lanosterol. Conditions for maximal rates of demethylation were established.

Addition of increasing amounts of washed microsomes to a constant amount of substrate resulted in additional release of $^{14}CO_2$, but the release was not proportional to the amount of microsomes. Incubation with increasing amounts of microsomes treated with Triton WR-1339 yielded a proportional rate of release of $^{14}CO_2$. The Triton-treated microsomes were frozen and stored without loss of activity. The rate of formation of $^{14}CO_2$ was constant up to 1 hr of incubation with both Triton-treated microsomes and whole homogenate, for which the K_m for lanosterol was 5.0 and 3.0 × 10^{-5} M, respectively. Other 4-gem-dimethyl sterols were competitive inhibitors, K_i', 2.0 and 5.5 × 10^{-5} M. The enzyme system was inhibited by arsenite.

24,25-Dihydrolanosterol, 24,25-dihydrolanostenone, and squalene were demethylated by the homogenate. The whole homogenate catalyzed the incorporation of mevalonic acid, but not acetic acid, into squalene and sterols. The enzymatic properties of the sterol synthetic system from skin resemble those of similar preparations from rat liver.

KEY WORDS skin · rat · homogenate · demethylation · lanosterol · biosynthesis · cholesterol · microsomes · Triton-treated · inhibitors · sterols

RAT SKIN CONTAINS many sterols in addition to cholesterol[1] (1). The biosynthesis of these sterols has been studied with slices of skin (2), suspensions of liberated cells (3), and intact rats (4). Information about the sequence of reactions in the biosynthetic pathway has been limited to the use of either specific inhibitors (1, 5, 6) or complex time-course studies with slices of skin (7). Experiments with cell-free preparations of liver yielded most of the information about the sequence of reactions in that tissue, but cell-free preparations of skin that catalyze sterol biosynthesis have not been available. This report describes the preparation of a cell-free system of rat skin epidermis that catalyzes the formation of squalene and sterols; the subcellular localization of the enzymes; the kinetics and properties of the microsomal system that catalyzes demethylation[2] of lanosterol; and the preparation of a stabilized enzyme system in microsomes that may be frozen and stored without loss of activity. Conversion of various substrates and the effects of inhibitors are also described.

METHODS AND MATERIALS

Male weanling rats were purchased from the Holtzman Rat Co., Madison, Wis. and maintained on commercial stock ration with unlimited quantities of food and water. Rats that weighed between 175 and 275 g were selected, because of the relative ease with which epidermis is removed from samples of skin from smaller rats. Rats were killed by decapitation or by chloroform anesthesia. Comparison of activity from rats killed by the two methods suggested that there was no effect of chloroform on

Abbreviation: GLC, gas–liquid chromatography.

[1] Common names of sterols used in this report: lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; methostenol, 4α-methyl-5α-cholest-7-en-3β-ol; cholesterol, cholest-5-en-3β-ol; 4,4-dimethyl-Δ^7-cholestenol, 4,4-dimethyl-5α-cholest-7-en-3β-ol; 4,4-dimethyl-Δ^8-cholestenol, 4,4-dimethyl-5α-cholest-8-en-3β-ol; 4,4-dimethyl-$\Delta^{5,7}$-cholestadienone, 4,4-dimethylcholesta-5,7-dien-3-one; methostenone, 4α-methyl-5α-cholest-7-en-3-one; dihydrolanosterol, 4,4,14α-trimethyl-5α-cholest-8-en-3β-ol; dihydrolanostenone, 4,4,14α-trimethyl-5α-cholest-8-en-3-one; SC 12937, 20,25-diazacholesterol.

[2] Demethylation (8) refers to the oxidative cleavage of methyl groups from positions 14α, 4β, and 4α of lanosterol in the conversion of lanosterol (C_{30}) to cholesterol (C_{27}). Although other reactions are known to accompany demethylation, they were not investigated. Some of these reactions indeed may participate as obligatory components of the over-all process of demethylation.

the catalytic activity. Hair was clipped from the dorsal skin, and the skin was dissected free from underlying muscle and fat. The skin was washed with water and stored on ice. Samples of skin (approximately 5 × 10 cm) were soaked in 0.33 N ammonium hydroxide for 20 min. The skin was washed with water, 0.12 M HCl, and water until the wash water was free from acid. Epidermis was scraped from the skin with a dull scalpel (9). All procedural steps up to this point were carried out at room temperature; all subsequent operations were carried out at 4°C. The gelatinous mass of cells, hair, and surface debris was transferred to cold 0.1 M potassium phosphate buffer (pH 6.85). All buffers contained 3 mM glutathione and 30 mM nicotinamide unless specified otherwise. The volume of the suspension was diluted to 5 times the volume of tissue. The suspension was homogenized with a chilled, steel Potter-Elvehjem homogenizer that had a clearance of 1 mm. The suspension was centrifuged for 20 min with an average force of 10,000 × *g* to remove unbroken cells, debris, and mitochondria. The supernatant fraction, to be referred to as "whole homogenate," was decanted for use in isolating microsomes. A large and variable amount of solid lipid was removed from the surface of whole homogenate.

Microsomes were prepared from whole homogenate by centrifugation with an average force of 105,000 × *g* for 45 min, unless other times are specified. Microsomes were washed once with 0.1 M phosphate buffer. For incubation, the microsomes were suspended in fresh buffer by means of a conical glass homogenizer (Kontes Glass Co., Vineland, N.J.). The microsomal suspension was diluted to 25% of the volume of whole homogenate that was used originally, and the suspension of microsomes was centrifuged at low speed (5000 × *g*, 10 min) to remove aggregated material.

Supernatant fraction from high speed centrifugation was obtained by centrifuging with an average force of 105,000 × *g* for 1 hr. A thin layer of lipid was removed from the surface of the supernatant fraction before the solution was transferred from the centrifuge tube.

The solutions were incubated with labeled substrates and cofactors in 50-ml Erlenmeyer flasks that contained a center well and filter paper wick (10). The final volume of the incubation mixture was 4.5 ml, unless stated otherwise. Incubations were carried out at 37°C under 100% oxygen. Labeled carbon dioxide was collected and counted as described previously (10). Internal standards of ^{14}C radioactivity were added to all counting vials.

Labeled lanosterol was prepared from mevalonate-2-^{14}C (New England Nuclear Corp., Boston, Mass.) by the procedure of Moller and Tchen (11). The labeled lanosterol was mixed with unlabeled lanosterol ($[\alpha]_D^{25}$ + 60.1°, c = 0.3 g/ml in $CHCl_3$; mp 137–139°C) and substrate solutions were prepared with potassium phosphate buffer and Tween 80. The final concentration of lanosterol in the substrate solution was 200 mμmoles/ml (45,000–65,000 dpm/ml). The final concentration of Tween 80 in the incubation flask (<1 mg/ml) did not inhibit demethylation by similar preparations of rat liver (10).

4,4-Dimethyl-Δ^8-cholestenol and 4,4-dimethyl-Δ^7-cholestenol were prepared by the method of Gautschi and Bloch (12). The physical constants and retention times of these compounds on gas–liquid chromatography confirmed the structures and established a purity of greater than 95%.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (13). Whole homogenate contained between 3.3 and 7.5 mg of protein per ml. For ease of comparison, results are expressed as mμmoles of lanostenol demethylated per hr per 100 mg of protein. A significant error in this expression may arise when unlabeled endogenous sterols add to or compete with the labeled substrate. This error is discussed below, and maximal limits of the error are estimated.

Cofactors were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Reduced glutathione was purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol, penicillin G (K salt), and streptomycin sulfate were purchased from Calbiochem, Los Angeles, Calif. Puromycin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Triton WR-1339 is a product of Rohm and Haas Co., Philadelphia, Pa. Sources of other synthetic substrates, inhibitors, and chromatographic standards are indicated.

RESULTS

Effect of Cofactors and Antibiotics on Demethylation

Whole homogenate was incubated with labeled lanosterol, various cofactors, and antibiotics (column I, Table 1). Addition of NAD stimulated the rate of demethylation (lines 2 and 3). At the higher concentration, NADPH stimulated demethylation when homogenate was incubated without and with NAD (lines 5 and 7). Heat inactivated the enzyme system (line 8). Antibiotics did not alter the rate of demethylation (lines 9–12). When glutathione was omitted from the buffer, markedly reduced, variable activity was observed. All of the homogenates in Table 1 were prepared with buffer that contained glutathione (3 mM). Additional glutathione did not affect demethylation (line 13). NAD (1 mM) was added to all subsequent incubations with whole homogenate unless indicated otherwise.

Optimal pH

Homogenates of rat skin were prepared with 0.1 M potassium phosphate buffers of pH 5.4–9.0. The crude ho-

mogenates were centrifuged, and the pH was adjusted again to the original pH with 1 N KOH or 1 N HCl. The whole homogenates were incubated with labeled lanosterol and NAD. An optimum was observed at about pH 7.0 (●—●, Fig. 1). A sharper decline from the optimum value was observed on the acid side of neutrality. Accordingly, subsequent preparations of whole homogenate were adjusted to pH 7.2. It was observed that 0.1 M potassium phosphate buffer of pH 6.85–6.90 yielded whole homogenate of pH 7.2 when the homogenate was prepared with 4 volumes of buffer for each volume of tissue.

Effect of Length of Incubation and Enzyme Concentration

The rate of demethylation of both 50 and 100 mμmoles of lanosterol by whole homogenates remained essentially constant during the 1st hr of incubation (Fig. 2). The rate decreased markedly during the 2nd hr. Addition of NADPH did not prevent the decrease in the rate of demethylation after 1 hr of incubation. In subsequent experiments, incubations were for 1 hr or less.

Increasing amounts of whole homogenate yielded increasing rates of demethylation (○—○, Fig. 3). The response was not linear. A parallel increase was observed when increasing amounts of microsomes were added to a constant amount of supernatant fraction (●—●). With less than 1 ml of microsomal suspension (an amount that is equivalent to 4 ml of whole homogenate) the rate was essentially constant; however, the rate decreased with amounts of microsomes in excess of 1 ml. Soluble fraction alone (●—●, 0 ml of microsomes) catalyzed a very slow rate of demethylation. Addition of soluble fraction to a constant amount of microsomes (□—□) did not stimulate the rate of demethylation.

TABLE 1 EFFECT OF COFACTORS AND ANTIBIOTICS ON DEMETHYLATION OF LANOSTEROL

Addition	I Whole Homogenate*		II Triton-Treated Microsomes†	
	Concn	Demethylation	Concn	Demethylation
	mM	mμmoles/hr per 100 mg protein	mM	mμmoles/hr per 100 mg protein
1. None	—	7.5	—	18
2. NAD	1.0	11.5	2.0	139
3. NAD	5.0	12.8	10.0	145
4. NADPH	0.3	6.7	0.6	94
5. NADPH	1.5	12.5	3.0	90
6. NAD + NADPH	1.0 0.3	8.7	2.0 0.6	142
7. NAD + NADPH	5.0 1.5	13.0	—	—
8. None, enzyme boiled	—	0.25	—	<5
9. Penicillin G‡	1.0	10.9	—	—
10. Streptomycin‡	1.0	12.4	—	—
11. Puromycin‡	0.2	14.5	—	—
12. 9–11 together‡	—	13.2	—	—
13. Glutathione‡	6.0	10.6	—	—

Values are the average of duplicate results from two separate determinations.

* Whole homogenate (4 ml) and 50 mμmoles of labeled lanosterol were incubated 1 hr in a final volume of 4.5 ml. Average protein = 5.6 mg/ml.

† Triton-treated microsomes (1 ml, see Table 3) were incubated with 50 mμmoles of labeled lanosterol for 1 hr in a final volume of 2.25 ml. Average protein = 2.6 mg/ml.

‡ Contained NAD, 1 mM.

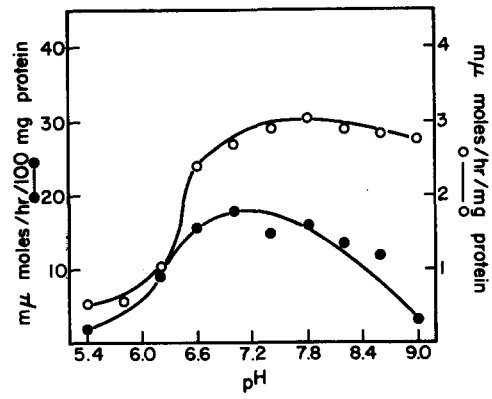

FIG. 1. Effect of pH on activity of demethylation. Each flask contained 4 ml of whole homogenate (●—●) or 1 ml of Triton-treated microsomes (○—○). The enzyme preparation was incubated with 50 mμmoles of lanosterol for 1 hr. Potassium phosphate buffer 0.1 M (30 mM nicotinamide and 3 mM glutathione) was used in the preparation of each sample; the pH of the final suspension of enzyme was adjusted with 1 N HCl or 1 N KOH. Each value is the average of at least two results from four separate incubations in each experiment.

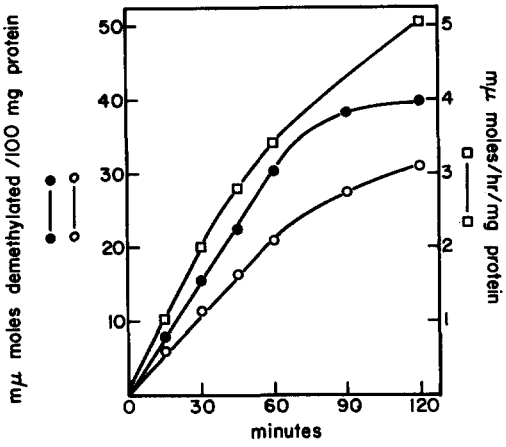

FIG. 2. Effect of length of incubation. Whole homogenate (4 ml) was incubated with 50 (○—○) or 100 (●—●) mμmoles of lanosterol. Triton-treated microsomes were incubated with 100 mμmoles of lanosterol (□—□). The experiments with whole homogenate were carried out 3 times and the results with Triton-treated microsomes are the average of duplicate experiments.

Effect of Substrate Concentration

Lanosterol, 20–200 mμmoles, was incubated with whole homogenate for 1 hr. Conventional double reciprocal expression of velocity and substrate concentration (15) yielded a linear relationship (○—○, Fig. 4). The cal-

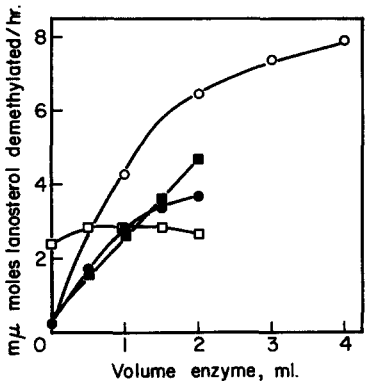

FIG. 3. Effect of enzyme concentration. Labeled lanosterol (50 mμmoles) was incubated in a final volume of 4.5 ml with 1–4 ml of whole homogenate (O—O), 0–2.0 ml of washed microsomes, and 2 ml of supernatant fraction (●—●), or 0–2.0 ml of supernatant fraction and 1 ml of washed microsomes (□—□). Triton-treated microsomes (0.5–2.0 ml, ■—■) were incubated with 50 mμmoles of lanosterol and no soluble fraction in a final volume of 2.25 ml. Results are the average of duplicate experiments.

culated kinetic values were: K_m, 3.0×10^{-5} M; and V_{max}, 83×10^{-9} mole/hr per 100 mg of protein.

Addition of 100 or 200 mμmoles of either 4,4-dimethyl-Δ^8-cholestenol or 4,4-dimethyl-Δ^7-cholestenol competitively inhibited the demethylation of labeled lanosterol (□—□, ■—■, △—△, ▲—▲, Fig. 4). The data indicate that essentially the same extent of competitive inhibition was observed with both isomers. The value of K_i' for competitive inhibition by the two compounds at the two concentrations was between 2.0 and 2.7×10^{-5} M. Difficulties in quantification arising from factors to be discussed later make this expression an approximate one. Thus K_i' rather than K_i denotes an inhibition constant obtained under these specific conditions.

Subcellular Localization of Enzymes and the Effect of Removing Endogenous Sterols

Whole homogenate was centrifuged for 20–80 min at 105,000 × *g*, and the microsomal fraction was collected after 20, 40, 60, and 80 min of centrifugation. Both demethylation activity and protein in the microsomes were assayed. The highest specific activity of demethylase was obtained in the microsomal fraction that was collected after 20 min of centrifugation (Table 2). The specific activity decreased with longer intervals of centrifugation. All of the activity of whole homogenate was recovered in the microsomes that were collected after only 20 min of centrifugation. The total activity decreased only slightly. The supernatant fraction after 80 min centrifugation contained very little enzymatic activity. The concentration of sterol that was bound to the microsomal protein was greatest in the most rapidly sedimented fraction. Some sterol was removed as a lipid layer that floated on the supernatant fraction. The lipid layer was catalytically inactive.

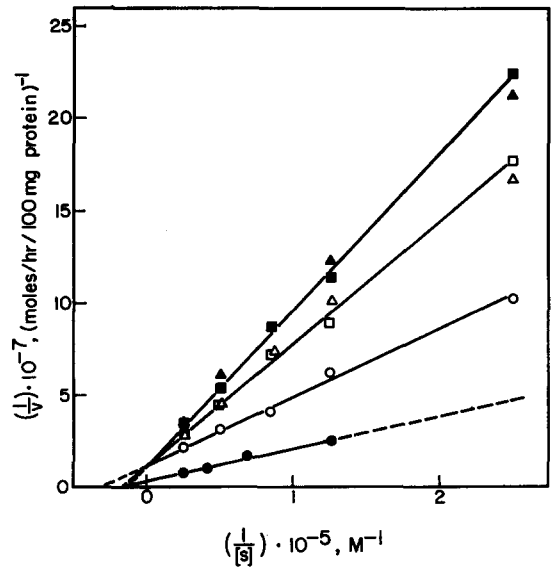

FIG. 4. Effect of substrate concentration. Whole homogenate (3.5 ml) was incubated with 20–200 mμmoles of lanosterol and 1 mM NAD in a final volume of 5.0 ml. The concentration of protein was between 3.55 and 4.75 mg/ml. The control sample (O—O) is the average of five determinations. 4,4-Dimethyl-Δ^8-cholestenol was added to similar flasks in two experiments: 100 mμmoles (□—□); and 200 mμmoles (■—■). 4,4-Dimethyl-Δ^7-cholestenol was added similarly (△—△ for 100, and ▲—▲ for 200 mμmoles, respectively). Suspensions of inhibitory sterols were prepared with Tween 80. The control flask contained an equal amount of Tween 80 solution in buffer. One milliliter of Triton-treated microsomes was incubated with 20–100 mμmoles of lanosterol and 2 mM NAD in a final volume of 2.5 ml (●—●). The concentration of protein was 2.97 and 2.98 mg/ml in the two experiments.

Microsomes were suspended in buffered solutions (pH 7.4) of the following solvents or detergents[3] at the indicated concentrations (mg/ml): Tween 80, 50 or 100; Triton WR-1339, 100; Lubrol, 50 or 100; digitonin, 10 or 20; deoxycholic acid, 10 or 15; taurocholic acid, 10; propylene glycol, 500 or 800. The microsomes were collected by centrifugation after exposure to the compounds for 15 min at 37°C, and incubated with labeled lanosterol. All of the compounds except Triton WR-1339 inhibited demethylation. Triton treatment was investigated in more detail.

Treatment of microsomes with Triton did not affect the total activity of lanosterol demethylation, but the specific activity increased from 0.69 to 0.86 mμmole/hr per mg of protein (Table 3). The concentration of microsomal-bound sterol decreased. Treatments for longer than 15 min resulted in some loss of activity, and treat-

[3] Tween 80, "Polysorbate 80", mixed polyoxyethylene ethers of sorbityl anhydride oleic acid esters, Nutritional Biochemicals Corp., Cleveland, Ohio; Lubrol WX, nonionic wetting agent (composition unknown), I.C.I. Organics, Inc., Providence, R.I.; and Triton WR-1339, oxyethylated *tert*-octylphenol polymethylene, lot AC 148, Ruger Chemical Co., Inc., Irvington-on-Hudson. N.Y.

TABLE 2 EFFECT OF DIFFERENT LENGTHS OF HIGH-SPEED CENTRIFUGATION ON MICROSOMAL DEMETHYLASE, PROTEIN, AND STEROL CONTENT*

Length of Centrifugation	Cellular Fraction	Demethylation† Specific Activity	Demethylation† Total Activity	Protein	Cholesterol‡
min		*mμmoles/hr per mg protein*	*mμmoles/hr per ml*	*mg/ml*	*μg/mg protein*
20	Microsomes	4.35	3.57	0.82	51.4
40	Microsomes	2.88	3.54	1.23	46.1
60	Microsomes	2.21	3.12	1.41	42.8
80	Microsomes	2.11	3.16	1.49	38.7
80	Supernatant fraction	0.018	0.26§	3.63	3.4
0	Whole homogenate	0.197	3.52§	4.45	6.6

* Whole homogenate was centrifuged at 105,000 × *g* for the times indicated and the pellet was suspended in 25% of the original volume. Each value is the average from three experiments. The concentration of protein in the initial whole homogenate was adjusted to approximately the same value in each experiment.

† Demethylation was assayed with 50 mμmoles of lanosterol and 1 mM NAD.

‡ Cholesterol was assayed with the differential Liebermann-Burchard procedure (14).

§ Because microsomes were diluted to 1/4 of the volume of whole homogenate, the values for total activity of supernatant fraction and whole homogenate are based on 4 ml rather than 1 ml for ease of comparison.

TABLE 3 EFFECT OF TREATMENT OF MICROSOMES WITH TRITON WR-1339 ON THE RATE OF DEMETHYLATION*

Treatment: Triton in Buffer	Treatment: Temp.	Demethylation: Specific Activity	Demethylation: Total Activity	Protein Concn.	Cholesterol Concn.
%	°C	*mμmoles/hr per mg protein*	*mμmoles/hr per ml*	*mg/ml*	*μg/mg protein*
0	4	0.69	2.51	3.58	24.6
10	4	0.71	2.95	4.16	21.9
10	37	0.86	2.77	3.24	14.4

* Microsomes from 20 min centrifugation were distributed in buffer (25% original volume). The microsomal suspension was stirred with buffer or buffer containing 10% Triton for 15 min at 4° or 37°C, 2 volumes of buffer were added, and the microsomes were collected with centrifugation (30 min). Each value is the average of four determinations except 4°C + Triton (two determinations). Glutathione (3 mM) was added only to the buffer that was used for incubation with lanosterol (50 mμmoles) and NAD (2 mM).

ment with higher concentrations of the detergent resulted in viscous suspensions and poor recovery of protein.

The enzymatic properties of the Triton-treated microsomes were investigated. The activity for demethylation of lanosterol in treated microsomes was dependent on exogenous NAD for activity (column II, Table 1). Addition of NADPH produced less stimulation than that observed with NAD. No additional activity was observed with a mixture of the two dinucleotides. Subsequent incubations of Triton-treated microsomes were carried out with 2 mM NAD; 3 mM GSH was also present in all buffers unless indicated otherwise. Triton-treated microsomes exhibited a relatively broad plateau of activity between pH 6.8 and 9.0 (○—○, Fig. 1). The sharp decrease on the acid side of the optimum resembled the response with whole homogenate. During the 2nd hr of incubation the rate of demethylation did not decrease as rapidly as with whole homogenate (□—□, Fig. 2). Subsequent incubations were for 1 hr or less. Increasing the amount of Triton-treated microsomes yielded an increase in the rate of demethylation which was constant (■—■, Fig. 3), in contrast to results with untreated microsomes.

When 20–100 mμmoles of lanosterol was incubated with Triton-treated microsomes the rate of demethylation was considerably greater than that observed with whole homogenate: K_m and V_{max} were 5.0×10^{-5} M and 290×10^{-9} mole/hr per 100 mg of protein, respectively.

Effect of Thiols, Freezing, and Storage

Cell-free preparations of epidermis from samples of frozen skin were inactive. Samples of skin microsomes were washed with buffer that contained no glutathione. Freezing without glutathione resulted in complete loss of enzymatic activity.

The effect of thiols on liver microsomes was investigated first. A suspension of liver microsomes (10) was incubated for 10 min at 37°C with labeled lanosterol, NAD, and glutathione, 1,4-dithiothreitol (Cleland's reagent, 16), or 2-mercaptoethanol. The same preparation was aged with the thiols at 0°C for 3 hr before the 10 min incubation. Glutathione (3 mM) did not inhibit demethylation. Cleland's reagent (0.1 and 0.5 mM) inhibited demethylation 10–15% both in the sample taken initially and in that taken after 3 hr's aging. Mercaptoethanol (3 mM) inhibited demethylation more than 95%. Treatment with glutathione resulted in little loss of activity during the 3 hr aging. Similar results with glutathione were observed previously (10).

The effect of thiols on skin microsomes was investigated similarly. Skin microsomes were treated with 10% Triton in 0.1 M potassium phosphate buffer (+ GSH) for 15 min at 37°C. The microsomes were washed with buffer that contained 3 mM glutathione and the microsomes were sedimented at 105,000 × *g* for 30 min. The pellet was frozen by dipping the centrifuge tube into a mixture of dry ice and acetone. The samples were stored at −15 or −25°C for various intervals of time. The samples were thawed and suspended in phosphate buffer for analysis of protein and demethylase activity. Activity decreased about 30% when the samples were stored at

−15°C (Table 4). Most of the activity was retained on storage at −25°C. Large samples of frozen, Triton-treated microsomes were prepared and stored at −25°C.

Effect of Inhibitors

Triton-treated microsomes were incubated with 8–40 μM labeled lanosterol and 20 or 40 μM unlabeled 4-methyl sterols. The value of the inhibition constant was calculated from reciprocal plots similar to those shown in Fig. 4. Strongest competitive inhibition was exhibited by methostenol (Table 5). The 4-gem-dimethyl sterols were strong inhibitors, and the inhibition constants were essentially identical with the value of K_m for the substrate, lanosterol. The isomers were equally active. Of the two keto-methyl sterols investigated, only slight inhibitory activity was observed with methostenone. Effects of C_{27}-sterols were not investigated.

Arsenite (1 mM) inhibited demethylation (Table 6). Treatment with heat essentially destroyed activity. Addition of arsenite or heat treatment yielded the same result when glutathione was added before or after the treatment. Triparanol was somewhat inhibitory, but the azasterol SC 12937[1] was without effect. A relatively high concentration of ethanol was required for 50% inhibition. Carbon monoxide did not inhibit demethylation of lanosterol.

TABLE 4 EFFECT OF FREEZING AND STORAGE ON LANOSTEROL DEMETHYLATION BY TRITON-TREATED SKIN MICROSOMES

Treatment	Length of Storage	Demethylation* and Temperature of Storage	
		−15°C†	−25°C†
	days	*mμmoles/hr per mg protein*	
Fresh	0	1.78	4.90
Frozen	0	1.69	4.92
"	3	1.67	4.46
"	7	1.52	4.53
"	10	1.15	4.62

* Average of duplicate samples from two separate experiments.
† Temperature of stored samples when removed from freezing chamber.

TABLE 5 COMPETITIVE INHIBITION OF LANOSTEROL DEMETHYLATION BY 4-METHYL STEROLS

Compound	Concentration of Inhibitor	K_i
	μM	μM
Methostenol	40	17
4,4-Dimethyl-Δ^7-cholestenol	20, 40	33–55
4,4-Dimethyl-Δ^8-cholestenol	20, 40	33–55
Methostenone*	40	120
4,4-Dimethyl-$\Delta^{5,7}$-cholestadienone†	40	no inhibition

From 20 to 100 mμmoles of labeled lanosterol was incubated with 2 mM NAD and 0, 20, or 40 μM inhibitor in a final volume of 2.5 ml. The inhibition constant was calculated from the average of duplicate sets of data and the control from the same experiment.
* Prepared by oxidation of methostenol by *t*-butyl chromate (17).
† A purified intermediate in the synthesis of the 4,4-dimethyl sterols (12).

TABLE 6 EFFECT OF INHIBITORS ON LANOSTEROL DEMETHYLATION BY TRITON-TREATED SKIN MICROSOMES

Addition*	Concentration	Demethylation	Inhibition
		mμmoles/hr per 100 mg protein	%
Control	—	50.3 ± 2.0†	0
Arsenite	1.0 mM	21.2	58
Heat‡	—	3.2	94
Triparanol	0.50 mg/ml	33.3	34
Ethanol	0.2 M	25.0	50
SC 12937[1]	0.50 mg/ml	50.4	0
96% N_2, 4% O_2§	—	63.4	0
86% N, 4% O_2, 10% CO§	—	69.7	0

* Microsomes were prepared with 0.1 M phosphate buffer (pH 7.2, 30 mM nicotinamide) that contained no glutathione. Glutathione (3 mM) was added to the incubation flasks at the time that inhibitors were added. The microsomes and inhibitor were mixed before addition of substrate.
† SEM, eight samples. Presumably the low rate may be ascribed to the preparation of microsomes without glutathione.
‡ 58°C, 5 min.
§ By volume in gaseous phase (approximately 50 ml of total volume).

Products and Substrates

The stoichiometry of release of labeled carbon dioxide and the formation of labeled C_{27}-sterols is shown in Table 7. With either whole homogenate or Triton-treated microsomes, the calculated demethylation was the same whether the amount of cholesterol formed (C_{27}) or carbon dioxide-^{14}C released was measured. With whole homogenate more than 5 times as much lanosterol-^{14}C was lost from the unsaponifiable fraction as was converted into C_{27}-sterols. Much less ^{14}C-lanosterol was lost during incubation with Triton-treated microsomes.

Various labeled substrates were incubated with either whole homogenate from rat liver (10) or Triton-treated microsomes from skin (Table 8). Lanosterol was the best substrate with either cell-free system. The efficiency of conversion of the other C_{30}-compounds was high with the skin preparation. Conversion of the 4,4-dimethyl-Δ^7-cholestenol was particularly poor with the skin preparation, whereas with the liver system this compound was demethylated efficiently. Neither preparation catalyzed the demethylation of the C_{29}-ketones.

To this point, only sterols or squalene were incubated with the skin homogenate. Incorporation of sterol precursors was investigated by incubation of samples of skin homogenate with either labeled acetate, mevalonate, or mixed prenol pyrophosphates (18). Acetate was not in-

TABLE 7 STOICHIOMETRY OF RELEASE OF $^{14}CO_2$ AND FORMATION OF C_{27}-STEROLS BY WHOLE HOMOGENATE AND TRITON-TREATED MICROSOMES

Enzyme Preparation*	Labeled Compounds isolated	Initial†	Final	Δ	Calculated Lanosterol Demethylated‡
		dpm/flask			*mμmoles/flask*
Whole homogenate (22.6–22.8 mg protein)	C_{28}- to C_{30}-sterol	65,500	53,500	−12,000	34.5
	C_{27}-sterol	6,460	8,440	1,980	6.82
	CO_2	7	369	362	6.23
Microsomes (1.6–2.3 mg protein)	C_{28}- to C_{30}-sterol	69,100	65,600	−3,500	10.1
	C_{27}-sterol	6,100	7,350	1,250	4.32
	CO_2	1	230	229	3.95

* Whole homogenate or Triton-treated microsomes were incubated with 200 mμmoles of lanosterol (69,600 dpm). The chromatographic fractionation with acid-washed alumina has been described (7). Losses from chromatography were corrected by analysis of internal standards of sterols (14). Results are averages of duplicate samples from two experiments each.

† Activity recovered from incubation with boiled enzyme.

‡ Calculated from the initial specific activity of lanosterol (348 dpm/mμmole) and the ratio of 6:5:1 radioactive centers in biosynthetic lanosterol:cholesterol:CO_2.

TABLE 8 CONVERSION OF LABELED SQUALENE AND METHYL STEROLS BY RAT SKIN MICROSOMES AND LIVER HOMOGENATE*

Substrate	Specific Activity	Demethylation Activity			
		Skin		Liver	
	dpm/mμmole	*dpm*†	*mμmoles*†	*dpm*†	*mμmoles*†
Lanosterol	335	4750	85.0	6420	115
24,25-Dihydrolanosterol‡§	43.0	266	37.0	472	67.0
24,25-Dihydrolanostenone‡§‖	60.0	256	25.6	23.8	2.4
Squalene‡	98.7	340	20.6	86	5.3
4,4-Dimethyl-Δ^7-cholestenol¶	23.3	119	5.1	751	32.2
4,4-Dimethyl-Δ^7-cholestenone‖¶	16.6	34	2.1	90	5.4
4,4-Dimethyl-$\Delta^{5,7}$-cholestadienone¶	20.4	<10	<0.5	33	1.6

* Triton-treated microsomes (3.7–4.1 mg of protein) or whole homogenate from liver (160–181 mg of protein) (7) was incubated with 100 mμmoles of substrate and 2 mM NAD for 2 hr or 20 min, respectively.

† dpm, dpm in CO_2/hr of incubation per 100 mg of protein; mμmoles, mμmoles of C_{27}-sterol formed per hr per 100 mg of protein that was calculated from the initial specific activity.

‡ Prepared biosynthetically from mevalonate-2-^{14}C.

§ Lanosterol was reduced catalytically with Raney nickel in absolute alcohol.

‖ The ketone was prepared by *t*-butyl chromate oxidation (17).

¶ Prepared synthetically (12) with labeled methyl iodide.

corporated (Table 9). Approximately 1.5% of the active isomer of DL-mevalonate was incorporated into unsaponifiable materials by incubation of the skin homogenate for 2 hr. Approximately equal ^{14}C radioactivity was associated with each chromatographic fraction. Approximately 10% of the active isomer of DL-mevalonate was incorporated into unsaponifiable materials during a 1 hr incubation of the skin homogenate when mixed prenol pyrophosphates were formed first by incubation of mevalonate with soluble liver enzymes. Most of the additional activity accumulated in squalene. The crude fractions were combined (C_{27}- to C_{30}-sterols) and the mixture was treated with digitonin (6). Incubation with mevalonate-2-^{14}C yielded 32.6–36.6% of the ^{14}C radioactivity in the precipitate with digitonin. From incubations with prenol pyrophosphates the precipitate contained from 50–63%. The remaining ^{14}C activity may be associated with 28- to 30-carbon sterols that do not precipitate well with digitonin and mixed prenols that are formed by enzymatic hydrolysis of prenol pyrophosphates (18).

Contributions of Endogeneous Sterols

The metabolism of labeled lanosterol by liver (10) and testicular tissue (19) can be studied by simply collecting $^{14}CO_2$, but rat skin contains large amounts of methyl sterols (1, 5) that could interfere with observed demethylation of lanosterol either by undergoing demethylation themselves (Table 5) or by acting as competitive inhibitors without being demethylated. The methyl sterols in the whole homogenate and other tissue fractions were analyzed by column chromatography and the Liebermann-Burchard reaction to determine the amount of sterol present and by GLC to determine the structures of the sterols.

Epidermis (1.07 ± 0.057 g/rat) was obtained from six rats. Each sample was homogenized as described under "Methods and Materials." A sample of each whole sus-

pension was removed. The remaining whole suspension was then centrifuged at 10,000 × *g* for 20 min. The floating lipid layer and precipitate were removed for analysis of sterols. A sample of the supernatant fraction was removed, and the remaining supernatant fraction was centrifuged again at 105,000 × *g* for 45 min. Samples of lipid layer, supernatant fraction, and precipitate were removed. Each sample was heated under reflux with methanolic KOH. An equal volume of water was added to the samples, and the sterols and other unsaponifiable lipids were extracted with petroleum ether. The residue from evaporation of the petroleum ether was chromatographed on acid-washed alumina (7), and two major fractions of sterols were collected, C_{27}-sterols, and C_{28}- to C_{30}-sterols. The total amount of sterol (Liebermann-Burchard reaction) and the percentage of each methyl sterol in the C_{28}- to C_{30}-sterol fraction (GLC analysis) were determined as reported in the footnote to Table 10.

In the supernatant fraction centrifuged at 10,000 × *g* the ratio of the total amounts of C_{27}-sterol and C_{28}- to C_{30}-sterol was about 5.5 to 1 (Table 10). The amounts of methyl sterol in each tissue fraction were rather variable. Extreme variation and sampling difficulties were associated with collection of the precipitate after centrifugation at 10,000 × *g* and the lipid layer after centrifugation at 105,000 × *g*. Most of the C_{28}- to C_{30}-sterol fraction was associated with discarded tissue fractions.

The percentages of the major components of the C_{28}- to C_{30}-sterols in the different tissue fractions were rather constant. The predominant fraction contained sterols with the retention times of 4α-methyl-5α-cholesta-7,24-dien-3β-ol (C_{28}-$\Delta^{7,24}$) and 4,4-dimethyl-5α-cholest-8-en-3β-ol (C_{29}-Δ^{8}). The latter compound was the predominant component of corresponding methyl sterols isolated by solvent extraction of sterols from whole rat skin (1).

Whole homogenate was prepared and incubated as described under "Methods and Materials" except that no sterol substrate was added to the incubation medium. Samples of C_{28}- to C_{30}-sterols were isolated from four incubated and three unincubated preparations. A significant decrease in the total amount of methyl sterol was not observed. Gas chromatographic analysis revealed that the percentage of 4α-methyl-5α-cholest-8-en-3β-ol decreased from 16.2 ± 2.4 to 6.8 ± 0.7 (SD) during a 1 hr incubation. Slight changes in the percentages of the combined 4α-methyl-5α-cholesta-8,24-dien-3β-ol and methostenol were observed, but the differences in this fraction, as in other methyl sterol fractions, were not greater than the standard deviations reported in Table 10.

DISCUSSION

Many procedures for the disruption of skin cells have been investigated to obtain cell-free synthesis of sterols. Enzymatic digestions of skin yield suspensions of cells (3) that catalyze the incorporation of acetate into unsaponifiable lipids. Cell-free preparations of these suspensions catalyzed the demethylation of lanosterol (unpublished results). Enzymatic digestion required much more time than the treatment with ammonium hydroxide (3), much smaller samples of skin were used, and large quantities of enzymes (collagenase and trypsin) were required. Mechanical disruption by grinding frozen samples of whole skin yielded inactive preparations. Disruption of whole skin with mechanical or sonic treatments without treatment with ammonium hydroxide yielded viscous preparations that contained little catalytic activity. The fair stability in alkali of the microsomal enzyme preparation (Fig. 1) suggests that the

TABLE 9 INCORPORATION OF MEVALONATE AND PRENOL PYROPHOSPHATES INTO STEROLS BY WHOLE HOMOGENATE OF SKIN*

Substrate	Radioactivity in Products				Recovery of Nonsaponifiable Lipid
	Nonsaponifiable Fraction	Squalene	C_{28}- to C_{30}-sterols	C_{27}-sterols	
	dpm				%
Acetate†	<70	—	—	—	—
Mevalonate‡	17,300	4,190	6,660	5,800	96
Prenol pyrophosphate§	109,000	84,100	11,900	6,890	94

*Whole homogenate of skin (4 ml) was incubated 2 hr with the labeled precursor, phosphate buffer (0.1 M), nicotinamide (30 mM) NAD (5 mg), ATP (5 mg), Mg^{++} (4 mM), and NaF (30 mM). The material was saponified, and the nonsaponifiable material was fractionated (7). Endogenous skin sterols and unincubated homogenate served as standards. Each value is the average of four samples from two experiments.

† 1 μc in 0.85 μmole of acetate-2-^{14}C. NaF was omitted from flasks with acetate-2-^{14}C in the second experiment but no activity was incorporated.

‡ 1 μc in 1.04 μmoles of mevalonate-2-^{14}C.

§ Prepared biosynthetically by incubation of 2 ml of supernatant fraction (105,000 × *g*, 2 hr) of rat liver homogenate and 1 μc of mevalonate-2-^{14}C for 2 hr with conditions described in the footnote above (18). The liver enzymes were denaturated with heat and cooled before the addition of skin homogenate. Incubation with skin homogenate was for 1 hr only. Mixed ^{14}C-prenol pyrophosphates were extracted from similar incubations of liver supernatant fraction. Hydrolysis with bacterial alkaline phosphatase yielded more than 70% of the ^{14}C in farnesol and 10% of the ^{14}C was associated with nerolidol. The supernatant fraction catalyzes little incorporation of mevalonate into squalene and digitonin-precipitate sterols. The results were not corrected for these small amounts of ^{14}C radioactivity.

TABLE 10 ENDOGENOUS SKIN STEROLS

Tissue Fraction	C_{27}-Sterols Amount	C_{28}- to C_{30}-Sterols*					
		Amount	C_{28}-Δ^8	C_{28}-Δ^7 + C_{28}-Δ^8	C_{28}-$\Delta^{7,24}$ + C_{29}-Δ^8	C_{29}-Δ^7 + C_{29}- or C_{30}-$\Delta^{8,24}$	C_{29}- or C_{30}-$\Delta^{7,24}$
	μg/flask†	*μg/flask*†			%		
Whole suspension	—	218 ± 10‡	16.0 ± 3.1	23.3 ± 2.8	22.3 ± 2.1	13.2 ± 1.8	10.0 ± 3.2
Centrifuged 10,000 × *g*							
Lipid layer	—	7.9 ± 0.92	20.1 ± 1.0	15.5 ± 5.0	38.0 ± 5.5	23.2 ± 5.0	<0.37§
Supernatant fraction	251 ± 46	42.4 ± 8.6	20.1 ± 2.6	14.0 ± 2.7	44.1 ± 4.2	17.1 ± 2.3	<1.9§
Precipitate	—	298 ± 139	23.3 ± 12.5	22.1 ± 9.3	28.2 ± 8.9	12.6 ± 7.4	4.72 ± 1.9
Centrifuged 105,000 × *g*							
Lipid layer	—	9.4 ± 5.0	24.9 ± 6.4	16.1 ± 2.7	41.0 ± 7.6	15.7 ± 2.2	<0.48§
Supernatant fraction	—	5.3 ± 0.50	14.9 ± 2.1	12.9 ± 1.6	35.4 ± 2.9	23.9 ± 3.4	4.32§
Precipitate	—	4.7 ± 1.3	20.7 ± 6.7	11.7 ± 0.4	38.0 ± 7.4	22.0 ± 4.3	<0.73§

* The C_{27}-sterols were separated from C_{28}- to C_{30}-sterols by chromatography on alumina (7). C_{28}, 4α-methyl-standard; C_{29}, 4,4-dimethyl-standard; C_{30}, 4,4,14α-trimethyl-standard. The rest of the sterol, to 100%, is accounted for by small amounts of C_{27}-sterol (for example, 3.25 ± 2.0% of cholesterol and 1.82 ± 0.90% of Δ^7-cholestenol in the supernatant fraction from centrifugation at 10,000 × *g*) and by small amounts of unidentified compounds. Gas–liquid chromatography of the C_{28}- to C_{30}-sterols was carried out at 230°C on a column of 1% SE-30 (methylpolysiloxy gum, General Electric) (5). Standards were obtained as follows: 4α-methyl-5α-cholest-8-en-3β-ol, gift from Dr. A. A. Kandutsch; 4α-methyl-5α-cholest-7-en-3β-ol, synthetic (4); 4α-methyl-5α-cholesta-8,24-dien-3β-ol, calculated, and a compound with this retention time and behavior with Liebermann-Burchard reagent was isolated from yeast; 4,4-dimethyl-5α-cholest-8-en-3β-ol, synthetic (12); 4α-methyl-5α-cholesta-7,24-dien-3β-ol, calculated from relative retention times for Δ^{24}- and 24,25-dihydrosterols; 4,4-dimethyl-5α-cholest-7-en-3β-ol, synthetic (12); 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol, calculated; 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol, isolated (7); 4,4-dimethyl-5α-cholesta-7,24-dien-3β-ol, calculated; 4,4,14α-trimethyl-5α-cholesta-7,24-dien-3β-ol, synthetic (7). The instrument was an F & M gas chromatograph, model 400, with a flame ionization detector. Relative amounts were determined with a Keuffel and Esser compensating polar planimeter, model 4236M. Standard curves and linearity of recorder response was determined with appropriate standards.

† The amount of sterol was determined with the modified Liebermann-Burchard reaction (14). The results are reported as an amount that is present in or equivalent to 4 ml of supernatant fraction (approx. 0.8 g of epidermis) from centrifugation at 10,000 × *g* (whole homogenate).

‡ Standard deviation, samples from six rats.

§ Sterol absent from more than one sample. Means without deviation are reported.

treatment with ammonium hydroxide was not detrimental. Apparently, the very brief rinse with dilute acid was not deleterious.

Bacteria do not catalyze the synthesis of sterols de novo (20). Certain microorganisms degrade steroids and similar demethylase activity may exist in bacteria. The possibility of bacterial contamination of skin preparations is apparent. The lack of effect of antibiotics (Table 1), the responses of the skin system similar to those obtained from liver and testicular tissue (see below, 10, 19), the relatively short incubation periods with linear responses, and the inhibition by arsenite and heat treatment (Table 6) suggest that bacteria have no significant effects in our system.

Treatment with Triton WR-1339 has been shown by others to enhance sterol biosynthesis in cell-free preparations of liver (21). On the other hand, many detergents are deleterious. For example, attempts to solubilize enzymes of sterol biosynthesis with Lubrol-W led either to inactivation of the enzymes or to retention of catalytic activity in the insoluble residue (22) although about 50% of the microsomal protein was rendered soluble.

The presence of radioactivity in squalene (Table 9) confirms similar observations that were made with the incorporation of labeled precursors by slices of skin (23, 24). Because the present experiments with the cell-free system were conducted in an atmosphere of 100% oxygen, the possibility that squalene accumulation in skin slices was a result of anaerobiosis due to poor penetration of oxygen into the slice is remote (see the Discussion printed as an appendix to reference 23).

The cell-free system responds enzymatically in a normal and predictable fashion. It catalyzed demethylation of lanosterol at a constant rate during 1 hr of incubation (Fig. 2). Additional amounts of preparation (microsomes) yielded proportional increases in demethylation (Fig. 3). The Lineweaver-Burk plot of the effect of substrate concentration and competitive inhibitors was linear. Similarly, responses with the whole homogenate of rat skin epidermis were reasonably reproducible. For many studies, whole homogenate is adequate.

Properties of the enzyme system that catalyzes the demethylation of lanosterol are similar in cell-free preparations of skin, liver (10), and testicular tissue (19). For example, NAD and glutathione (10, 19, 25) are required for maximal activity in each system; the enzyme system is particulate (microsomal).

Studies of hormonal and physiological effects on skin sterol metabolism (26, 27) and the recent evidence that hepatic cholesterol biosynthesis is regulated by bile acid end-product inhibition of mevalonate formation (28) raise the question of what control system is active in the

biosynthesis of sterols in skin. This cell-free preparation may allow direct investigation of these factors. Previous attempts to answer these questions with cell-free preparations of analogous sebaceous (preputial gland) tissues (26, 29), with extrapolation from results with liver enzyme systems (10), and with slices of rat skin (30) have been only partially successful.

The contribution of endogenous sterols was not as important as sterol compositions (1) of rat skin would suggest. Much of the sterol present in the homogenate is C_{27}-sterol (Table 10). Addition of cholesterol and Δ^7-cholestenol (200 μg/flask, approx. 0.1 mM) does not affect the rate of demethylation of lanosterol by any of the tissue preparations.

In the "Results" section two possible effects of methyl sterols were suggested. Either the methyl sterols may be demethylated, thus reducing the observed rate of $^{14}CO_2$ release, or methyl sterols may act as competitive inhibitors.

Demethylation of 4α-methyl-5α-cholest-8-en-3β-ol by whole homogenate of skin (see last paragraph under "Results") may account for no more than about 10–20% of the 42.4 μg/flask of methyl sterol. About 20–50 μg of lanosterol-^{14}C was added to each flask in the present experiments. However, because exogenous substrates (Table 8) and inhibitors (Table 5) are added to variable amounts of endogenous methyl sterols and the values for the observed rates of conversion may be slightly low, the effect of inhibitors is reported in the form of apparent rather than absolute inhibition constants (K_i') (31). All of the methyl sterols that have been tested as competitive inhibitors, 4α-methyl-5α-cholest-8-en-3β-ol (15), 4,4-dimethyl-5α-cholest-7-en-3β-ol (31, Table 8), and 4,4,-14α-trimethyl-5α-cholesta-7,24-dien-3β-ol (10, 19), are demethylated and K_i' is approximately equal to K_m for each compound.

The assay of $^{14}CO_2$ release is easy to manipulate, and the technique permits the study of many variables within the time that is required for fewer determinations by chromatographic resolution. However, certain observations suggest that interpretation of data from this experimental approach requires some reservation. For example, lanosterol is metabolized to compounds that are not recovered as C_{27}-sterols (Table 7). In addition, unknown and possibly variable effects of endogenous sterols, cofactors, and inhibitors (Tables 1, 5, 8, and 10) suggest that the facile technique of collection of $^{14}CO_2$ may be subject to certain errors and shortcomings.

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