Oxidative demethylation of lanosterol in cholesterol biosynthesis: accumulation of sterol intermediates[1]

Ali Shafiee,[2] James M. Trzaskos,[3] Young-Ki Paik,[4] and James L. Gaylor

Central Research and Development Department, E. I. du Pont de Nemours and Company, Wilmington, DE 19898

Abstract With [^{3}H-24,25]-dihydrolanosterol as substrate, large-scale metabolic formation of intermediates of lanosterol demethylation was carried out to identify all compounds in the metabolic process. Utilizing knowledge of electron transport of lanosterol demethylation, we interrupted the demethylation reaction allowing accumulation and confirmation of the structure of the oxygenated intermediates lanost-8-en-3β,32-diol and 3β-hydroxylanost-8-en-32-al, as well as the demethylation product 4,4-dimethyl-cholesta-8,14-dien-3β-ol. Further metabolism of the $\Delta^{8,14}$-diene intermediate to a single product 4,4-dimethyl-cholest-8-en-3β-ol occurs under interruption conditions in the presence of 0.5 mM CN^-[1]. With authentic compounds, each intermediate has been rigorously characterized by high performance liquid chromatography and gas–liquid chromatography plus mass spectral analysis of isolated and derivatized sterols. Intermediates that accumulated in greater abundance were further characterized by ultraviolet, ^{1}H-NMR, and infrared spectroscopy of the isolated sterols. — **Shafiee, A., J. M. Trzaskos, Y-K. Paik, and J. L. Gaylor.** Oxidative demethylation of lanosterol in cholesterol biosynthesis: accumulation of sterol intermediates. *J. Lipid Res.* 1986. **27:** 1–10.

Supplementary key words lanosterol demethylation • cytochrome P-450 • steroid demethylation

The metabolic process of lanosterol demethylation and subsequent conversion of demethylated intermediates to cholesterol has been studied in several laboratories (1–5). In most instances, these laboratories have shown conversion of synthetic compounds to the desired end-product sterols by metabolically active systems (2–4). Verification of intermediacy of each sterol in the complex biosynthetic process, however, depends not only upon demonstrated metabolism of synthetic compounds, but also upon generation of the presumed intermediate in comparable metabolically active systems. We have previously demonstrated by manipulation of microsomal electron transport, either through solubilization of membrane-bound electron transport components or by limiting reduction potential with low pyridine nucleotide concentration, that 14α-demethylation of dihydrolanosterol can be separated experimentally into sequential component reactions: cytochrome P-450-dependent hydroxylations, formation of $\Delta^{8,14}$-sterol diene, and reduction of the diene to a Δ^8 sterol monoene (1, 5). Accordingly, we now report conditions permitting sufficient accumulation of each intermediate generated during demethylation, thus allowing their characterization. In addition, with knowledge of the microsomal electron transport process (1, 6), we can now propose a metabolic pathway based upon formation of biosynthetic intermediates.

EXPERIMENTAL PROCEDURES

Incubation conditions

Preparation of microsomes and incubation conditions were as described previously (1). Assays of dihydrolanosterol oxidative demethylation employing purified dihydrolanosterol and [^{3}H-24,25]-dihydrolanosterol (New England Nuclear, Boston, MA, lot #1574-252) electron carriers and protein also were as conducted previously (1).

Preparation of sterol standards and substrates

Cholesta-4,7-dien-3-one, which was used as starting material for most sterol syntheses, was prepared from 7-dehydrocholesterol in 80% yield as previously described (7, 8). The isolated product displayed mp 85–87°C; UV

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectroscopy; EIMS, electron impact mass spectroscopy; CIMS, chemical ionization mass spectroscopy; DHL, 24,25-dihydrolanosterol. Sterol common names are: lanostane, 4,4,14α-trimethyl-5α-cholestane; zymosterol, 5α-cholesta-8,24-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol.

[1]Initial studies carried out during the tenure of Fellowship 5F32HL06177 from the United States Public Health Service (J.M.T.).

[2]Present address: Medicinal Chemistry Department, College of Pharmacy, University of Wisconsin, Madison, WI 53706.

[3]To whom reprint requests should be addressed at: E. I. du Pont de Nemours and Company, Experimental Station E400/3251, Wilmington, DE 19898.

[4]Present address: Gladstone Foundation Laboratory, University of California, San Francisco, CA 94140.

spectrum in ethanol gave λmax 238 nm (ε 15800), literature (8) mp 86–88°C; λmax 238 nm (ε 15500).

Synthesis of 4,4-dimethyl-5α-cholesta-8,14 and -7,14-dien-3β-sterols

4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol and 4,4-dimethyl-5α-cholesta-7,14-dien-3β-ol were prepared and resolved by previously published procedures (9, 10) with published modifications (5).

Synthesis of 4,4-dimethyl-5α-cholest-8-en and -7-en-3β-sterols

Modifications of previously described procedures (10–12) were employed for the production and isolation of the substituted monoene sterols (5).

Synthesis of 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol

Two methods were employed for the synthesis of this monoene sterol adapted from Gautschi and Bloch (10) and Iida (13). The desired sterol was crystallized from ethyl acetate–methanol to give 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol, mp 136–141°C; $[\alpha]_D$ + 15° (*c*,1.95). High resolution mass spectral analysis showed precise mass of 414.3840 (calculated for $C_{29}H_{50}O$: 414.3861). Prominent ions in the high mass region of the spectrum were: 414 (100%; M), 399 (15%; M–CH_3), 396 (3%; M–H_2O), 301 (3%; M–C_8H_{17}). ^{1}H-NMR (360 MHz): 0.76 (s, 3H,19-H), 0.81 (s, 3H, 18-H), 0.84 (s, 3H, 4β-H), 0.87 (d, 6H, 26, 27-H), 1.01 (s, 3H, 4α-H), 3.26 (d, 1H, 3α-H).

Synthesis of lanost-8-en-3β,32-diol and lanost-7-en-3β,32-diol

A mixture of lanost-8-en- and -7-en-3β,32-diol was prepared from 3β-acetoxy-7α,32α-epoxy lanosterol by the reported procedure of Parish and Schroepfer (14). Characterization of the synthesized starting material gave, mp 202–206°C [lit. (14), mp 201–203°C]. Mass fragmentation ions included 455 (44%; M–CH_2OH), 395 (41%; M–CH_3COOH–CH_2OH), 381 (8%; M–CH_3–CH_2O–CH_3COOH), 341 (100%; M–$C_9H_{21}O$), 281 (15%; M–$C_{11}H_{25}O_3$). ^{1}H-NMR (360 MHz): 0.81 (s, 3H, 18-H), 2.04 (s, 3H, CH_3 of acetoxy function), 3.36 (d, 1H, 32-H), 3.98 (d, 1H, 32-H), 4.16 (m, 1H, 7-H), 4.48 (m, 1H, 3α-H). IR: 1735, 1258, 1038 cm^{-1}.

The desired diol mixture was isolated by preparative silica gel G thin-layer chromatography. Plates were developed in ethyl acetate–toluene 50:50, and the fraction with R_f = 0.48 was recovered with 94.8% purity as determined by GLC. High resolution mass spectral analysis showed a precise mass for the M^+–H_2O ion of 426.3860 (calculated for $C_{30}H_{50}O$: 426.3862). Prominent fragmentation ions in the high mass region included: 426 (7%; M–H_2O), 413 (100%; M–CH_2OH), 399 (5%; M–CH_3–CH_2O), 395 (63%; M–H_2O–CH_2OH), 313 (15%; M–H_2O–C_8H_{17}). TMS-derivatization resulted in separation of the two isomers of molecular weight 588 (Table 4) consistent with the addition of two TMS-groups to a diol of molecular weight 444. EIMS showed prominent fragments at 485 (25%, M–CH_2OTMS) and 395 (100%, M–CH_2OTMS–HOTMS) for the Δ^8-isomer[5] and 498 (3%, M–HOTMS), 485 (30%, M–CH_2OTMS), 395 (100%, M–CH_2OTMS–HOTMS) and 393 (5%, M–CH_3–2(HOTMS)) for the Δ^7-isomer. CIMS fragmentation showed high mass ions at 587 (8%, M–H), 573 (50%, M–CH_3), 497 (30%, M–H–HOTMS), 485 (40%, M–CH_2OTMS), 409 (100%, M+H–2(HOTMS)) and 395 (10%, M–CH_2OTMS–HOTMS) also for the Δ^8-isomer with the Δ^7-isomer showing a similar pattern including 471 (15%, M+H–CH_3–CH_2OTMS) and 397 (40%, M+H–CH_2OTMS–OTMS), but not the 395 fragment. ^{1}H-NMR (360 MHz): 0.72 (s, 3H, 18-H), 3.22 (m, 2H, 3α-H and 32-H), 3.62 (d, 1H, 32-H), 5.37 (m, 0.56H, 7-H). IR: 3410, 1059, 1024, cm^{-1}. Both GLC–MS and ^{1}H-NMR indicated a 60:40 mixture of the Δ^7- and Δ^8-sterols.

Preparation of 3β-hydroxy-lanost-7-en-32-al and 3β-hydroxy-lanost-8-en-32-al

Three mg of lanost-7 and -8-en-3β,32-diol were oxidized by Jones reagent (5 μl) (15) at −10°C for 2 min. After work-up in the cold, TLC plates were developed in methylene chloride. The band with R_f = 0.29 was eluted and collected. The isolate was reduced with $NaBH_4$ in 90% dioxane as described by Gibbons, Mitropoulos, and Pullinger (2) and the corresponding aldehydes were isolated in the TLC system above. EIMS fragmentation ions included: 442 (0.5%; M), 413 (100%; M–CHO), 395 (20%; M–CHO–H_2O). CIMS ions were 443 (75%; M+H), 427 (55%; M–CH_3), 425 (100%; M+H–H_2O), 413 (60%; M–CHO), 397 (65%; M–H–2H_2O). ^{1}H-NMR (360 MH_z): 3.2 (m, 1H, 3α-H), 5.5 (m, 0.8H, 7-H), 9.45 (s, 0.2H, 32-H), 9.64 (s, 0.8H, 32-H). IR: 3270, 2720 (weak), 1705 cm^{-1}.

Biosynthesis of 4,4-dimethyl-5α-cholest-8-en-3β-ol and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol by rat liver microsomes

The reaction mixture contained in a final volume of 20 ml: 100 mg of microsomal protein; 0.5 mM NaCN; 2 mM NADPH; 50 mg of isocitrate; 0.4 mM $MgCl_2$; 3.5 units of isocitrate dehydrogenase; 0.1 M potassium phosphate buffer (also containing 1 mM glutathione, 0.1 mM EDTA, and 20% glycerol, pH 7.4). The reaction was initiated by the addition of 250 μM dihydrolanosterol (suspended in Triton WR-1339, 75:1 w/w detergent–sterol at a sterol

[5]Based upon data presented in Table 1 showing the relative elution of Δ^8 versus Δ^7-monoene sterols, we have made these tentative assignments.

concentration of 1000 nmol/ml); incubation was continued for 2 hr at 37°C. The reaction was terminated by the addition of 20 ml of 15% KOH in 95% methanol, and the mixture was saponified under reflux for 1 hr. The saponified mixture was extracted three times with a total volume of 150 ml of petroleum ether, the extraction solvent was removed under reduced pressure at 50°C, and the residue was dissolved in 0.5 ml of ethanol for purification by reverse-phase HPLC (see below). This procedure resulted in 60% metabolism of substrate to two isolable products as judged by GLC analysis.

Biosynthesis of lanost-8-en-3β,32-diol and 3β-hydroxy-lanost-8-en-32-al

Rat liver microsomes (50 mg) were incubated in a final volume of 25 ml in 0.1 M potassium phosphate buffer (also containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, pH 7.4) with the following components: 10 mM ATP; 0.1 mM NADPH; 0.1 mM NADH, 0.4 mM $MgCl_2$; 62.5 mg of isocitrate; 4 units of isocitrate dehydrogenase; 46 mg of β-hydroxybutyric acid; 5 units β-hydroxybutyrate dehydrogenase; and 1250 nmol [^{3}H-24,25]dihydrolanosterol (10,000 dpm/nmol) suspended as above. The reaction was initiated by the addition of substrate and continued for 2 hr at 37°C. The incubation was terminated by the addition of 25 ml of 15% KOH in 95% methanol, and the mixture was allowed to stand at room temperature overnight. The reaction mixture was extracted as above with a total volume of 200 ml of petroleum ether. The isolated residue was dissolved in 0.5 ml of ethanol for purification by HPLC. This procedure resulted in a 30% conversion of substrate to isolable products as judged by radio-HPLC analysis with the C-32 alcohol and aldehyde in overall 4% yield.

Alternatively, solubilized rat liver microsomes were also employed to accumulate oxygenated polar metabolites of lanosterol demethylation. Rat liver microsomes were suspended to a final concentration of 30 mg/ml in 0.1 M potassium phosphate buffer (also containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol, pH 7.4). This microsomal suspension (12.6 ml) was then treated with an equal volume of ocytylglucoside solution (45 mg/ml) in the same buffer by dropwise addition of the detergent solution with stirring at 4°C. The mixture was allowed to stir at 4°C for an additional 20 min when the solubilized suspension was clarified by centrifugation at 105,000 *g* for 1 hr. The isolated soluble fraction was used for the production of the desired sterol under the following incubation conditions. In a final volume of 50 ml, the reaction mixture contained: 6.7 ml of clarified soluble fraction (50 mg of protein); 0.05% Triton X-100; 1 mM NADPH; 125 mg of isocitrate; 0.4 mM $MgCl_2$; 8.75 units of isocitrate dehydrogenase; and 0.1 M potassium phosphate buffer (also containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol, pH 7.4) to volume. The reaction was initiated by the addition of [^{3}H-24,25]dihydrolanosterol substrate (10,000 dpm/nmol) suspended in detergent as above to a final concentration of 50 μM and continued for 2 hr at 37°C. The incubation was terminated by the addition of 50 ml of 15% KOH in 95% methanol. The next day, the mixture was extracted three times with a total volume of 375 ml of petroleum ether, the extraction solvent was removed under reduced pressure at 50°C, and the residue was dissolved in 1.0 ml of ethanol for purification by HPLC. This procedure resulted in the production of both lanost-8-en-3β,32-diol and 3β-hydroxy-lanost-8-en-32-al, the latter in greater amounts with an overall conversion of 3%. Because of the low total conversion, the isolated sterols from this preparation and those from the preceeding preparation were combined prior to HPLC purification.

HPLC purification of DHL oxidase metabolites

Purification of dihydrolanosterol (DHL) metabolites was accomplished by reverse-phase chromatography employing an Ultrasphere-octyl column and chromatographic conditions as described for the radio-HPLC assay of DHL demethylase (1). Repeated 50-μl injections of extracted incubation mixtures were made, and 0.5-ml or 0.25-ml fractions were collected starting at a run time of 11 min. Fractions were combined as indicated in Fig. 1, dried under N_2, and analyzed by GLC–MS techniques described below.

Complete resolution of lanost-8-en-3β,32-diol from 3β-hydroxylanost-8-en-32-aldehyde for NMR characterization of the isolated aldehyde was accomplished by reverse-phase separation as above, employing a mobile phase of acetonitrile–methanol–water 41:41:18.

Gas–liquid chromatography of sterols

Gas–liquid chromatography was performed with a Hewlett-Packard 5880 gas chromatograph using a 6-ft column of 3% OV-17 on Chromasorb WHP. Analyses were conducted at 260°C with a N_2 carrier gas flow rate of 30 ml/min. Sterols were quantitated relative to a standard of 5α-cholestane using a flame ionization detector.

GLC–MS analysis of isolated metabolite fractions and synthetic sterols

GLC–MS analysis was performed at Haskell Laboratory (Newark, DE) on a Finnigan 4021 GLC–MS system. GLC separation conditions were: 6-ft column of 3% SP-2250 on Supelcoport (100/120 mesh); oven temperature, 275°C; injection port, 275°C; zone, 250°C; carrier gas, helium; flow rate, 25 ml/min. MS conditions were: ionization mode, EI and CI; scan range, 40–600, 60–600 AMU; scan time, 2.0 sec; source temperature, 270°C; multiplier voltage, 1300 V; electron volts, 70, 125 EV; ionizing gas, methane. Sterol metabolites were dissolved in methylene chloride, and 1- to 2-μl aliquots were in-

jected for analysis. Direct probe analysis was performed under the following conditions: initial temperature, 40°C for 0.5 min.; ramp speed 50°C/min; final temperature, 300°C; all other parameters where appropriate, as above.

Trimethylsilyl ether derivatization was performed with Trisil/BSA (Pierce, Rockford, IL) in dimethylformamide at 60°C for 30 min. Methoxime derivatization was performed with MOX reagent (Pierce) in pyridine at 60°C for 1 hr.

High resolution mass spectroscopy was performed on a VG-MM 7070H mass spectrometer.

Other spectroscopic techniques

NMR analysis was performed on either a Varian EM 390 (90 MHz) or a Nicolet 360 WB (360 MHz) using $CDCl_3$ as solvent.

Diffuse reflectance IR analysis was performed on a Nicolet 7199 FTIR.

Materials

All chemicals were reagent grade or the best grade commercially available. Source and types of reagents employed were as previously reported (1).

RESULTS

HPLC-GLC separation of intermediates in cholesterol biosynthesis

The utility of the reverse-phase C_8-column separation employed throughout the course of this investigation and the GLC separations used during this study are summarized in **Table 1** and **Table 2**. It can be seen that the chromatographic conditions employed provide a rapid convenient separation and means of identification of the sterol intermediates of the cholesterol biosynthetic sequence.

TABLE 2. GLC analysis of various sterols

Sterol	Retention Time	Relative Retention
	min	
5α-Cholestane	6.61	0.426
Cholesterol	15.50	1.000
5α-Cholesta-7,14-dien-3β-ol	16.47	1.062
5α-Cholest-8-en-3β-ol	16.61	1.071
5α-Cholesta-8,14-dien-3β-ol	16.80	1.084
4,4-Dimethyl-cholesta-5,7-dien-3β-ol	17.20	1.109
5α-Cholest-7-en-3β-ol	18.06	1.156
7-Dehydrocholesterol	18.10	1.167
Desmosterol	18.32	1.182
4α-Methyl-5α-cholesta-7,14-dien-3β-ol	18.42	1.208
Zymosterol	19.98	1.289
4,4-Dimethyl-cholest-5-en-3β-ol	21.39	1.380
5α-Cholesta-7,24-dien-3β-ol	22.04	1.422
Dihydrolanosterol	22.09	1.425
4,4-Dimethyl-5α-cholest-8(14)-en-3β-ol	22.39	1.444
4,4-Dimethyl-5α-cholest-8-en-3β-ol	23.14	1.493
4,4-Dimethyl-5α-cholesta-7,14-dien-3β-ol	24.26	1.565
4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol	24.66	1.591
Lanosterol	26.09	1.683
4,4-Dimethyl-5α-cholest-7-en-3β-ol	26.71	1.723

Sterols were analyzed by gas-liquid chromatography employing a packed column of OV-17, 3% w/w on WHP, 100/120 mesh (2 mm i.d. × 6 ft). A nitrogen carrier gas flow rate of 30 ml/min was employed with an oven temperature of 260°C and detector and injection port temperatures of 300°C. Detection was made with a flame ionization detector. Relative retention is with respect to cholesterol.

Identification of cholesterol biosynthetic sterol intermediates

Fig. 1 shows the metabolite profile obtained when radiolabeled dihydrolanosterol was incubated as described

TABLE 1. HPLC chromatographic behavior of various C_{30}, C_{29}, and C_{27} sterols

Sterol	Retention Time	x'	Relative Retention
Lanost-8-en-3β,32-diol	13.33	5.09	0.455
3β-Hydroxy-lanost-8-en-32-al	14.31	5.53	0.488
Cholesta-5,7-dien-3β-ol	16.00	6.31	0.546
5α-Cholest-7-en-3β-ol	18.92	7.64	0.645
Cholest-5-en-3β-ol (cholesterol)	19.73	8.01	0.673
4,4-Dimethyl-5α-cholesta-7,14-dien-3β-ol	21.65	8.89	0.738
4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol	21.70	8.91	0.740
4,4-Dimethyl-cholesta-5,7-dien-3β-ol	22.43	9.24	0.765
4,4-Dimethyl-5α-cholest-7-en-3β-ol	26.31	11.01	0.897
4,4-Dimethyl-5α-cholest-8(14)-en-3β-ol	26.81	11.24	0.950
4,4-Dimethyl-5α-cholest-8-en-3β-ol	27.85	11.72	0.950
4,4,14α-Trimethyl-5α-cholest-8-en-3β-ol (dihydrolanosterol)	29.32	12.59	1.00

HPLC separation of sterols was performed on an Ultrasphere-octyl column (4.6 mm × 25 cm) with a mobile phase of acetonitrile–methanol–water 45:45:10 at 45°C with a flow rate of 1 ml/min. Injections (10–50 μl) were made of sterols dissolved in ethanol at 1–5 mg/ml with detection by UV absorbance at 214 nm. Relative retention is with respect to dihydrolanosterol.

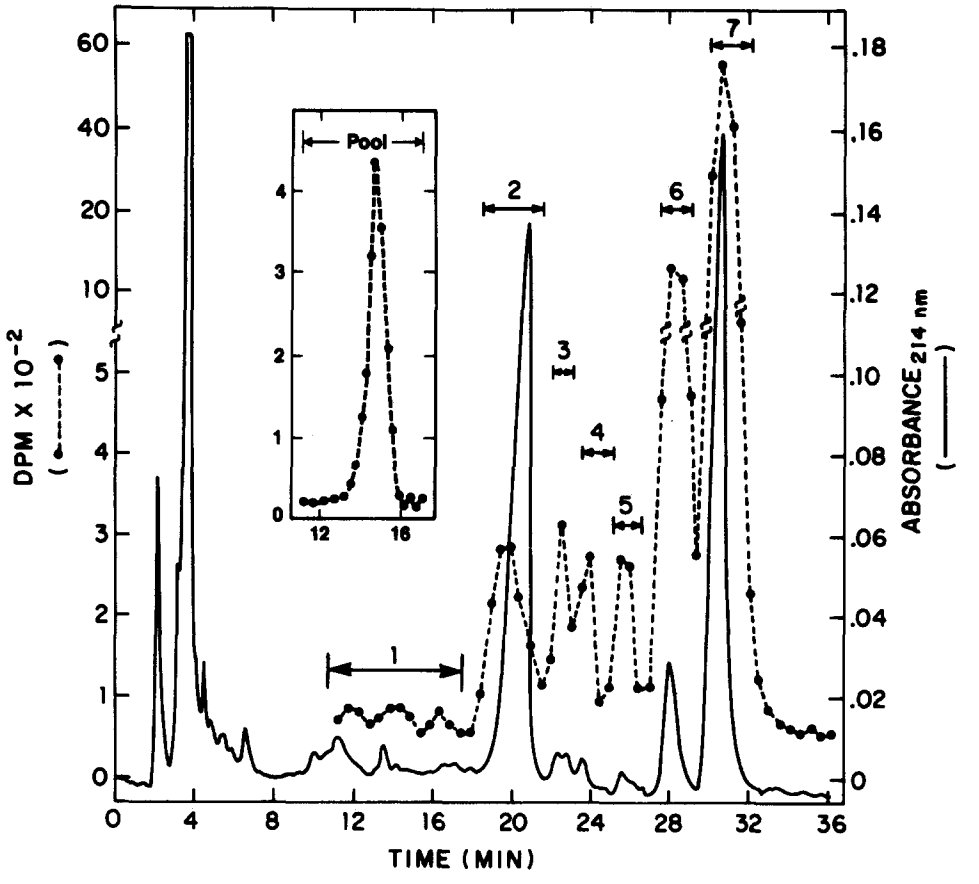

Fig. 1. Preparative reverse-phase HPLC separation of dihydrolanosterol metabolites isolated from rat liver microsomal incubations. Reaction mixture extracts from approximately 30 dihydrolanosterol demethylase assays (1) utilizing [^{3}H-24,25]-dihydrolanosterol as substrate were combined and dried under a stream of N_2. The residue was dissolved in 0.5 ml of ethanol, and sterols were separated by reverse-phase HPLC and isolated as described in Experimental Procedures. Twenty-μl aliquots of isolated fractions were used to determine radioactivity by liquid scintillation counting. Fractions were combined as indicated and these were used for GLC–MS analysis shown in Fig. 2 and Table 3. The insert shows the chromatographic behavior of accumulated, polar sterols generated only under conditions of limiting pyridine nucleotide concentration or with solubilized preparations. These fractions were combined for the GLC–MS analysis described in Table 4.

for the DHL demethylase assay (1) and the accumulated sterol intermediates were separated by reverse-phase HPLC. GLC–MS analyses of the indicated fractions (**Fig. 2**) showed that the pooled HPLC fractions contained some overlap of unresolved sterols, but as indicated in **Table 3,** the isolated components could be identified by combined mass spectroscopy, GLC chromatographic behavior, and HPLC with limited difficulty when the three techniques were used in combination. For example, the shift in the radioactivity profile toward more polar sterols in the cholesterol area of the HPLC chromatogram (fraction 2, Fig. 1) suggested the accumulation of isomeric 5α-cholest-7-en-3β-ol based upon HPLC behavior (Table 1). The detection by GLC–MS of a sterol with chromatographic behavior identical to the Δ^7-sterol and a molecular ion of 386 (Table 3) was indeed confirmatory of the presence of the anticipated 5α-cholest-7-en-3β-ol metabolite.

Identification of C-32 oxygenated intermediates

The metabolically formed lanost-8-en-3β,32-diol and 3β-hydroxylanost-8-en-32-al accumulate only under incubations with either limiting NADPH concentrations or in a solubilized reconstituted system (1). The preparative HPLC chromatogram obtained when limiting reduced pyridine nucleotide or solubilized DHL demethylase was used to accumulate C-32 oxidation products is shown in Fig. 1 (see insert). HPLC behavior alone of the oxidized polar metabolites suggests the production of the C-32 alcohol and C-32 aldehyde, respectively. Verification of the HPLC chromatographic data by GLC–MS analyses of the pooled polar fractions is presented in **Table 4.** GLC–EIMS of underivatized sample and standard C-32 alcohol and C-32 aldehyde displayed a single component characterized by an apparent molecular ion of 414 (100%, M). The results reflect the thermal lability of the C-32

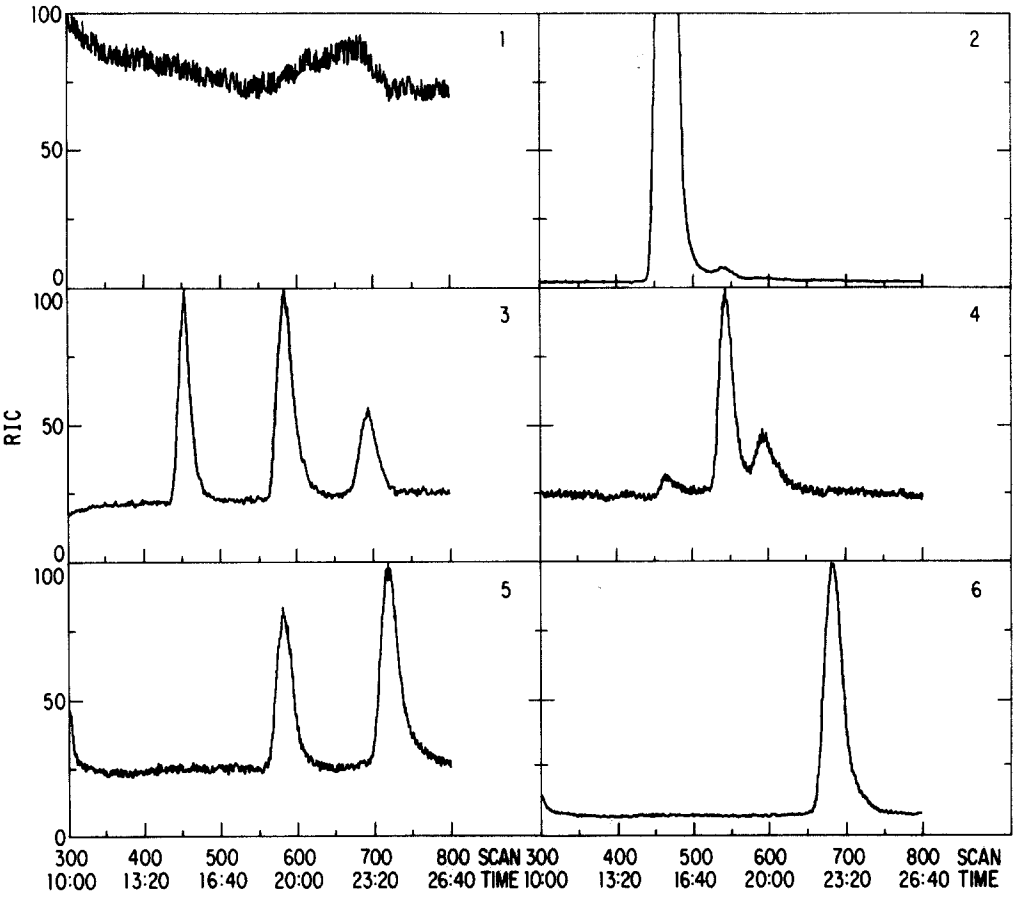

Fig. 2. GLC–MS analysis of dihydrolanosterol metabolites isolated by reverse-phase HPLC. Fractions 1 to 6 prepared by HPLC separation, as described in the legend to Fig. 1, were analyzed by GLC–MS as described in Experimental Procedures. Relative ion count (RIC) is plotted against scan number and retention time. Panel numbers correspond to HPLC pooled fractions from Fig. 1.

alcohol and aldehyde which decompose to the corresponding $\Delta^{8(14)}$-monoene sterols with loss of the functionalized 14α-methyl group under the GLC conditions employed in the analysis (4, 16). Trimethylsilyl ether (TMS) derivatization of the isolated polar fraction prior to GLC–MS analysis resulted in resolution of the two major metabolic products and a third minor component. The first major component displayed chromatographic behavior identical with that of the resolved Δ^{8}-isomer of standard C-32 alcohol with EIMS and CIMS fragmentation consistent with the addition of two TMS derivatives to a steroidal diol of mass 444. Analogous behavior was seen with the standard lanost-8-en-3β,32-diol that was carried through identical analytical procedures (Table 4).

Chromatographic behavior of the second major component in the pooled polar fractions following TMS derivatization was coincident with TMS-derivatized C-32 aldehyde (Table 4). Prominent ions in the high mass region of the EIMS spectrum reflected addition of a single TMS derivative to the C-32 aldehyde with expected collapse of the 14α-aldehyde to the $\Delta^{8(14)}$-monoene of MW 414 under GLC separation conditions.

$NaBH_4$ reduction of the isolated sterols prior to GLC–EIMS analysis resulted in generation of a single component with a molecular ion of 414 (100%, M). TMS derivatization of the reduced sample yielded a single component with chromatographic behavior coincident with the standard C-32 alcohol. EIMS and CIMS fragmentation patterns were also identical to the standard steroidal 3β,32-diol (Table 4). Further characterization of the isolated aldehyde which accumulates in greater abundance than the C-32 alcohol was possible following separation of the oxygenated intermediates from each other by further HPLC. The 360 MHz ^{1}H-NMR of the resolved C-32 aldehyde showed prominent peaks at 0.76 (s,3H,18–H), 0.83 (s,3H,4β–H), 0.86 (m,6H,25,26–H), 0.91 (d,3H,21–H), 0.98 (s,3H,4β–H), 1.07 (s,3H,19–H), 3.23 (m,1H,3α–H), and 9.45 (s,1H,32–H). High resolution EIMS gives a precise mass for the M^+–CHO ion of 413.3790 (calculated for $C_{29}H_{49}O$: 413.3785). CIMS

TABLE 3. Component assignment of HPLC-purified dihydrolanosterol metabolite fractions

HPLC Fraction Number[a]	Component Description	Molecular Ion	Component Assignment
1			none detected
2	A[b]	386	cholest-5-en-3β-ol (cholesterol)
	B	386	5α-cholest-7-en-3β-ol
3	A	386	cholesterol
	B	400	4α-methyl-5α-cholest-7-en-3β-ol
	C	412	4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol
4	A	386	cholesterol
	B	400	4α-methyl-5α-cholest-8-en-3β-ol
	C	400	4α-methyl-5α-cholest-7-en-3β-ol
5	A (methoxime)	398 (427)	4α-methyl-5α-cholest-8-en-3-one
	B (TMS)	414 (486)	4,4-dimethyl-5α-cholest-7-en-3β-ol
6	A (TMS)	414 (486)	4,4-dimethyl-5α-cholest-8-en-3β-ol
7	A	428	4,4,14α-trimethyl-5α-cholest-8-en 3β-ol (dihydrolanosterol)

Fractions isolated as indicated in Fig. 1 were analyzed by GLC and GLC-MS employing both electron impact and chemical ionization techniques as described in Experimental Procedures. In certain instances, trimethylsilylether or methoxime derivatization was performed to enhance GLC resolution and confirm functional group presence in certain sterol components. Components are listed in increasing elution order as detected during GLC and GLC-MS analysis. Assignments are based upon GLC-MS molecular ion data and comparison of migration properties with known standards in the GLC and HPLC systems used for analysis and isolation.

[a] See Fig. 2.

[b] Detection of cholesterol in several HPLC-purified fractions reflects the abundance of this sterol in mammalian microsomes and the difficulty in resolving cholesterol from the other metabolites by a single chromatographic technique.

shows prominent ions at 443 (50%; M+H), 425 (100%; M+H-H_2O), 413 (75%; M-CHO), 397 (50%; M+H-CHO-OH) and 395 (30%; M-CHO-H_2O). IR: 3270,2720 (weak) and 1705 cm^{-1}. Thus, the identification of the accumulated intermediates as lanost-8-en-3β,32-diol and 3β-hydroxy-lanost-8-en-32-al was established by a combination of HPLC and GLC behavior in addition to chemical derivatization, mass spectral analysis, IR and ^{1}H-NMR characteristics.

Characterization of the immediate decarbonylation products of C-32 demethylation

As previously described (1), when metabolism of dihydrolanosterol is interrupted by cyanide inhibition, two sterol metabolites accumulate. Based solely upon chromatographic behavior, these two metabolites were identified as 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol and 4,4-dimethyl-5α-cholest-8-en-3β-ol, respectively. However, because of the importance in establishing the identity of the demethylation products of DHL demethylase, the two demethylated sterols were subjected to further, more rigorous, criteria of identity. High resolution mass spectra and 360 MHz ^{1}H-NMR, respectively, of the two sterols generated by rat liver microsomes in the presence of 0.5 mM NaCN and isolated by reverse-phase HPLC was performed. High resolution mass spectroscopy for the isolated 4,4-dimethyl-5α-cholest-8-en-3β-ol yielded a precise mass of 414.3861 (calculated for $C_{29}H_{50}O$: 414.3861). Prominent ions in the high mass region of the spectrum were: 414 (80%, M), 399 (20%, M-CH_3) 381 (10%, M-CH_3-H_2O), and 283 (15%, M-$C_8H_{19}O$). 360 MHZ ^{1}H-NMR displayed prominent peaks at 0.62 (s, 3H, 18-H), 0.86 (s, 3H, 4β-H), 0.91 (m, 6H, 25,26-H), 0.96 (d, 3H, 21-H), 1.02 (s, 3H, 19-H), 1.04 (s, 3H, 4α-H), and 3.28 (m, 1H, c-3α-H). IR spectroscopy gave maxima at 3475, 2935, 1495, 1370, 1098, and 1040 cm^{-1}.

Similarly, spectral data confirm the isolation of 4,4-dimethyl-5α-cholesta-8,14-diene-3β-ol. High resolution mass spectroscopy yielded a precise mass of 412.3729 (calculated for $C_{29}H_{48}O$: 412.3705). Prominent ions in the high mass region of the spectrum included: 412 (100%, M), 397 (50%, M-CH_3), 379 (45%, M-CH_3-H_2O), 299 (10%, M-C_8H_{17}) and 285 (20%, M-$C_8H_{15}O$). 360 MHz ^{1}H-NMR analysis showed peaks at 0.82 (s,3H,18-H), 0.84 (s,3H,4β-H), 0.87 (m, 6H,25,26-H), 0.93 (d,3H,21-H), 1.02 (s,3H,19-H), 1.04 (s,3H,4α-H), 3.26 (m,1H,3α-H), and 5.36 (m,1H,15-H). IR analysis displays maxima at 3220, 3130, 1465, and 1365 cm^{-1} with characteristic peaks due to the C-15-H at 3045 and 795 cm^{-1} which are absent in the reduction product. In addition, λmax 248 in ethanol (calculated, λmax 249) in the UV absorbance spectrum substantiated the identity of the heteroanular diene as the $\Delta^{8,14}$ isomer. HPLC and GLC-MS analysis of the isolated sterols showed only single components and spectral data were in complete agreement with synthetic standards (5), thus essentially assuring purity and structure assignments.

TABLE 4. GLC-MS analysis of isolated polar DHL metabolites and comparison with synthetic lanost-8-en-3β,32-diol and 3β-hydroxylanost-8-en-32-al

Sample	GLC Retention	Ionization Mode	Molecular Ion	Prominent Fragmentation Ions (m/z) (% Relative Abundance)
	min			
Lanost-8-en-3β,32-diol	20.6	EI	414	414, 399, 381 (100) (5) (5)
TMS derivative	12.9	EI	485	485, 395 (35) (100)
		CI	588	587, 573, 497, 485, 409, 395 (5) (45) (40) (40) (100) (10)
3β-Hydroxylanost-8-en-32-al	20.7	EI	414	414, 399, 381 (100) (5) (5)
TMS derivative	15.6	EI	486	486, 471, 396, 381 (100) (5) (20) (20)
Polar isolate	20.6	EI	414	414, 399, 381 (100) (5) (5)
TMS derivative				
Component A (C-32 alcohol)	12.9	EI	485	485, 395 (35) (100)
		CI	588	587, 573, 497, 485, 409, 395 (5) (50) (30) (45) (100) (10)
Component B (C-32 aldehyde)	15.6	EI	486	486, 471, 396 (100) (5) (25)
		CI	486	485, 471, 397, 381 (45) (55) (100) (10)
Polar isolate $NaBH_4$ Reduced[a]	20.6	EI	414	414, 399, 381 (100) (10) (10)
TMS derivative	12.9	EI	486	485, 395 (25) (100)
		CI	588	587, 573, 497, 485, 409, 395 (10) (75) (35) (55) (100) (10)

Polar DHL metabolites were accumulated under appropriate conditions as described in Experimental Procedures. The polar sterols were isolated by HPLC as described in the legend to Fig. 1 and were analyzed by GLC-MS techniques as in Table 3.

[a]Ten μg of sterol was reduced with excess $NaBH_4$ in methanol for 3 hr at room temperature. The reduced sample was extracted with ether and analyzed.

DISCUSSION

Identification of cholesterol biosynthetic intermediates generated by rat liver microsomal incubations with [^{3}H-24,25]dihydrolanosterol substrate has been accomplished by combined chromatographic analysis employing HPLC and GLC-MS techniques. Chromatographic analysis by HPLC allows identification of most sterol biosynthetic intermediates generated during in vitro incubations; however, where minor ambiguities result from overlap of sterols, GLC and GLC-MS analyses readily separate and identify the components in the isolated mixtures of metabolites. By means of preparative HPLC, GLC, and GLC-MS analyses, identification of the radiolabeled sterols generated and detected by the radio-HPLC assay of DHL demethylase activity previously described (1) was made (Table 3).

The assignments made in Table 3 are quite revealing in another sense, as most known or proposed intermediates of the sterol synthetic pathway between dihydrolanosterol and cholesterol are detected by our combined analyses. The conspicuous absence of cholesta-5,7-dien-3β-ol, 5α-cholest-8-en-3β-ol, and 4,4-dimethyl-5α-cholesta-7,14-dien-3β-ol provide insight into the primary metabolic pathway of cholesterol synthesis (17) and can be explained on the basis of enzyme substrate specificity and known enzymic reaction rates. The absence of 4,4-dimethyl-5α-cholesta-7,14-diene-3β-ol and 5α-cholest-8-en-3β-ol may be due to the facts *1*) that the $\Delta^{8\rightarrow7}$ isomerase is not active against $\Delta^{8,14}$-diene substrates (18) and *2*) that the isomerization of 4,4-bisnormethyl Δ^8-monoene sterol to the corresponding Δ^7-isomer is one of the fastest reactions in the cholesterol biosynthetic sequence (19). The absence of the $\Delta^{5,7}$-diene sterol may reflect its rapid conversion to the Δ^5-monoene-sterol as well as a relatively slow synthetic rate of formation. Since the Δ^7-sterol 5-desaturase enzyme has properties very similar to 4-methyl sterol oxidase (20), it is consistent with our earlier suggestion that the Δ^7-sterol

5-desaturase activity may be rate-limiting in the later stages of the cholesterol synthetic sequence as 4-methyl sterol oxidase is earlier in the pathway (21). This possibility is reflected by accumulation of 5α-cholest-7-en-3β-ol under our incubation conditions (Table 3).

Conditions for accumulation and extensive characterization of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol and 4,4-dimethyl-5α-cholest-8-en-3β-ol, the immediate 14α-demethylation products of dihydrolanosterol metabolism (1, 5), have been presented in this report. Our results are in agreement with previous reports that demonstrated accumulation of the $\Delta^{8,14}$-diene under "trapping" conditions (22, 23). The extensive characterization of the two isolated sterols is of importance since debate over the actual, immediate decarbonylation intermediate exists. Pascal, Chang, and Schroepfer (24) observed conversion of relatively small amounts of 14α-hydroxymethyl-5α-cholest-7-en-3β-ol to 5α-cholest-8(14)-en-3β-ol with rat liver microsomes incubated under anaerobic conditions. However, in our studies, in which we have achieved 60% demethylation, we have failed to detect any $\Delta^{8(14)}$-monoene sterol either in complete reaction mixtures (Fig. 1, Table 3) or under interruption conditions (1, 5). Characterization of the isolated monoene sterol as the $\Delta^{8(9)}$-isomer was done by GLC–MS and ^{1}H-NMR techniques which readily distinguish the $\Delta^{8(9)}$- from the $\Delta^{8(14)}$-monoene sterol. Furthermore, we have not detected the $\Delta^{8(14)}$-isomer in any of our previous studies dealing with the $\Delta^{8,14}$-sterol 14-reductase enzyme (5). Our failure to detect such a sterol, although suggestive of non-involvement of the $\Delta^{8(14)}$-monoene sterol in the pathway, may reflect a combination of aerobic incubation conditions necessary to generate the proposed oxygenated intermediates as well as use of the natural Δ^8-isomeric substrate. Furthermore, exogenously supplied diol substrate may result in preferential metabolism via different enzymes of the microsomal system. Also, the substrate used by Pascal et al. (24) did not contain the 4-gem-dimethyl group of the natural substrate. When we used authentic lanost-8-en-3β,32-diol as substrate and incubated under identical anaerobic conditions as those of Pascal et al. (24), we did not detect conversion to any sterol products when assayed by reverse-phase HPLC and GLC–MS (J. M. Trzaskos and J. L. Gaylor, unpublished results). We would still withhold interpretation of this seeming paradox until after purification and reconstitution of the decarbonylase enzyme that is free of contaminating enzymes that may metabolize any of the possible intermediates.

Generation of small amounts of polar sterol metabolites under limiting NADPH concentration (in the presence of inhibitors of the nucleotide pyrophosphatase of microsomes) or with the solubilized enzyme systems has been presented (1). Accumulation, isolation, and characterization of the polar metabolites described in this report establish the identity of these two sterols as lanost-8-en-3β,32-diol and 3β-hydroxylanost-8-en-32-al. Both sterols have been suggested as intermediates of the 14α-demethylation reaction sequence since both sterols are converted to cholesterol by cell-free systems (2–4), and recently the aldehyde has been isolated from tissue sources (25). The demonstrated accumulation of these two oxygenated sterols by interruption through manipulation of incubation conditions represents a method which allows single catalytic turnover cycles of substrate-bound cytochrome P-450, analogous to that observed for cytochrome P-450scc in pregnenolone synthesis (26). These results show that these conditions allow detection of oxidative products formed from sterol substrates which essentially assures their role as metabolic intermediates.

Finally, since the characterization of intermediates generated by interruption conditions described in this report, purification of the lanosterol demethylase cytochrome P-450 to homogeneity has been accomplished (6). When the demethylase is reconstituted with purified components, only three metabolic products are detected. Based upon chromatographic behavior in the HPLC and GLC–MS systems described in this report, these intermediates have been identified as lanost-8-en-3β,32- diol, 3β-hydroxylanost-8-en-32-aldehyde, and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol. Continued investigations of the oxidative demethylation reaction and of the mechanisms of the lyase reaction are underway.

The authors wish to thank Ms. Andrea Daulerio for her technical assistance with the production and isolation of sterols, and Dr. Daniel Kasprzak for performing the GLC–MS analyses and his help in interpretation of mass spectral data. Supported in part by grant AM-21336 from the United States Public Health Service. Contribution No. 3286 from the Central Research and Development Department, E. I. du Pont de Nemours and Company.

Manuscript received 6 September 1984.

REFERENCES

1. Trzaskos, J. M., W. D. Bowen, A. Shafiee, R. T. Fischer, and J. L. Gaylor. 1984. Cytochrome P-450-dependent oxidation of lanosterol in cholesterol biosynthesis: microsomal electron transport and C-32 demethylation. *J. Biol. Chem.* **259:** 13402–13412.
2. Gibbons, G. F., K. A. Mitropoulos, and C. R. Pullinger. 1976. Lanosterol 14α-demethylase. The metabolism of some potential intermediates by cell-free systems from rat liver. *Biochem. Biophys. Res. Commun.* **69:** 781–789.
3. Gibbons, G. F., C. R. Pullinger, and K. A. Mitropoulos. 1979. Studies on the mechanism of lanosterol 14α-demethylation. A requirement for two distinct types of mixed-function-oxidase systems. *Biochem. J.* **183:** 309–315.
4. Akhtar, M., C. W. Freeman, D. C. Wilton, R. B. Boar, and D. B. Copsey. 1977. The pathway for the removal of the

15α-methyl group of lanosterol. The role of lanost-8-ene-3β,32-diol in cholesterol biosynthesis. *Bioorg. Chem.* **6:** 473–481.
5. Paik, Y. K., J. M. Trzaskos, A. Shafiee, and J. L. Gaylor. 1984. Microsomal enzymes of cholesterol biosynthesis from lanosterol: characterization, solubilization and partial purification of NADPH-dependent $\Delta^{8,14}$-steroid 14-reductase. *J. Biol. Chem.* **259:** 13413–13423.
6. Trzaskos, J. M., S. Kawata, A. Daulerio, and J. L. Gaylor. 1984. Purification and characterization of lanosterol 14α-demethylase—an endogenous cytochrome P-450-dependent enzyme. *Federation Proc.* **43:** 2034 (Abstract).
7. Teranishi, R., and F. J. Eastman. 1963. Δ^4-Cholesten-3-one (cholest-4-en-3-one). *Org. Synth. Collect.* **IV:** 192–195.
8. Antonucci, R., S. Bernstein, D. Giancola, and K. J. Sox. 1951. The preparation of $\Delta^{4,7}$- and $\Delta^{4,7,9}$-3-ketosteroidal hormones. *J. Org. Chem.* **16:** 1453–1457.
9. Watkinson, I. A., D. C. Wilton, K. A. Munday, and M. Akhtar. 1971. Formation and reduction of the 14,15-double bond in cholesterol biosynthesis. *Biochem. J.* **121:** 131–137.
10. Gautschi, F., and K. Bloch. 1958. Synthesis of isomeric 4,4-dimethylcholestenols and identification of a lanosterol metabolite. *J. Biol. Chem.* **233:** 1343–1347.
11. Woodward, R. B., A. A. Patchett, D. H. R. Barton, D. A. J. Ives, and R. B. Kelley. 1957. Synthesis of lanosterol (lanostadienol). *J. Chem. Soc. (Lond.)* **I:** 1131–1144.
12. Adkins, H., and A. A. Pavlic. 1947. Hydrogenation of esters to alcohols over Raney nickel. *J. Am. Chem. Soc.* **69:** 3039–3041.
13. Iida, T. 1977. Substituent effects on the proton-NMR signals of methyl groups in methyl steroids. *Chem. Phys. Lipids.* **19:** 169–178.
14. Parish, E. J., and G. J. Schroepfer, Jr. 1981. Sterol synthesis. A simplified method for the synthesis of 32-oxygenated derivatives of 24,25-dihydrolanosterol. *J. Lipid Res.* **22:** 859–868.
15. Djerassi, C., R. R. Engle, and A. Bowers. 1956. Direct conversion of steroidal Δ^5-3β-alcohols to Δ^5- and Δ^4-3-ketones. *J. Org. Chem.* **21:** 1547–1549.
16. Fried, J., and J. W. Brown. 1967. Rearrangement of 32-oxygenated lanostanes. *Tetrahedron Lett.* **10:** 925–930.
17. Gaylor, J. L. 1981. Formation of sterols in animals. *In* Biosynthesis of Isoprenoids. J. W. Porter and S. L. Spurgeon, editors. J. Wiley and Sons, Inc., New York. 481–543.
18. Lutsky, B. N., J. A. Martin, and G. J. Schroepfer, Jr. 1971. Metabolism of 5α-cholesta-8,14-dien-3β-ol and 5α-cholesta-7,14-dien-3β-ol in rat liver homogenate preparations. *J. Biol. Chem.* **246:** 6737–6744.
19. Gaylor, J. L., C. V. Delwiche, and A. C. Swindell. 1966. Enzymic isomerization ($\Delta^8 \rightarrow \Delta^7$) of intermediates of sterol biosynthesis. *Steroids.* **8:** 353–363.
20. Grinstead, G. F., and J. L. Gaylor. 1982. Total enzymic synthesis of cholesterol from 4, 4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol. Solubilization, resolution and reconstitution of Δ^7-sterol 5-desaturase. *J. Biol. Chem.* **257:** 13937–13944.
21. Williams, M. T., J. L. Gaylor, and H. P. Morris. 1977. Investigation of the rate-determining microsomal reaction of cholesterol biosynthesis from lanosterol in Morris hepatomas and liver. *Cancer Res.* **37:** 1377–1383.
22. Fiecchi, A., M. Galli Kienle, A. Scala, E. Grossi Paoletti, F. Cattabeni, and R. Paoletti. 1972. Hydrogen exchange and double bond formation in cholesterol biosynthesis. *Proc. R. Soc. Lond. B.* **180:** 147–166.
23. Akhtar, M., D. C. Wilton, I. A. Watkinson, and A. D. Rahimtula. 1972. Substrate activation in pyridine nucleotide-linked reactions. *Proc. R. Sco. Lond. B.* **180:** 167–178.
24. Pascal, R. A., Jr., P. Chang, and G. J. Schroepfer, Jr. 1980. Possible mechanisms of demethylation of 14α-methyl sterols in cholesterol biosynthesis. *J. Am. Chem. Soc.* **102:** 6599–6601.
25. Tabacik, C., S. Aliau, B. Serrou, and A. Crastes de Paulet. 1981. Post-HMG-CoA reductase regulation of cholesterol biosynthesis in normal human lymphocytes: lanosten-3β-ol-32-al, a natural inhibitor. *Biochem. Biophys. Res. Commun.* **101:** 1087–1095.
26. Hume, R., R. W. Kelley, P. L. Taylor, and G. S. Boyd. 1984. The catalytic cycle of cytochrome P-450scc and intermediates in the conversion of cholesterol to pregnenolone. *Eur. J. Biochem.* **140:** 583–591.