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A Novel, Convenient Assay of Lanosterol 14α-Demethylase

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A rapid and sensitive kinetic assay of lanosterol 14α-demethylation has been developed and analyzed. Three substrates, [32-^{3}H]-24,25-dihydrolanosterol, [32-^{3}H]lanost-8-en-3β,32-diol, and [32-^{3}H]lanost-7-en-3β-32-diol, were studied. In all cases, the rate of tritium released into aqueous solution provided a simple and direct assay of 14α-demethylase activity. The kinetic parameters of K_m and V_{max} for each substrate have been determined in a reconstituted system from rat liver. The percentage of turnover monitored by the novel tritium release assay was comparable to that observed by conventional GC methods. Separation of unreacted sterol from tritiated formate and water via reverse-phase chromatography permitted several samples to be analyzed at once.

INTRODUCTION

Oxidative removal of the 14α-methyl group (C-32) of lanosterol[2] is a common step in the sterol biosynthetic pathway of both yeast and mammalian systems. Product analysis indicates removal of the methyl group as formic acid and conversion of the sterol to 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (*1–44*). That a single cytochrome P450 monooxygenase (14α-demethylase) is responsible for the complete oxidative removal of the methyl group has been verified by reconstitution studies with purified enzyme in both systems (*4, 5*). Recent work has clearly demonstrated the precursor/product relationship of the alcohol and aldehyde intermediates shown in Fig. 1 (*6, 7*).

Our initial endeavors to study 14α-demethylase were aimed at the identification of novel inhibitors of the enzyme. These studies were hindered by the lack of a suitable convenient kinetic assay. Difference spectra can be used to rank binding of type II inhibitors such as imidazole antifungal drugs to P450 enzymes (*8*). However, this approach is not particularly satisfactory for 14α-demethylase substrate analogs since lanosterol exhibits a relatively small type I spectral change (*9*). Analysis by gas chromatography is more sensitive than spectral binding analy-

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[2] Abbreviations and trivial names used: lanosterol, lanost-8,24-dien-3β-ol; dihydrolanosterol, lanost-8-en-3β-ol; 32-hydroxy-24,25-dihydrolanosterol (Δ8), lanost-8-en-3β,32-diol; 32-hydroxy-24,25-dihydrolanosterol (Δ7), lanost-7-en-3β,32-diol; PMSF, phenylmethylsulfonyl fluoride; BSA-TMS, *N,O*-bistrimethylsilylacetamide; DMF, *N,N*-dimethylformamide.

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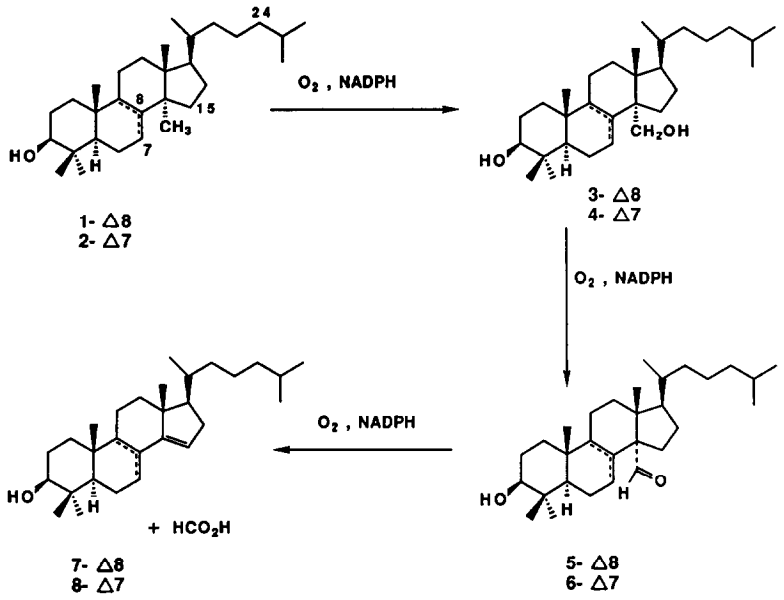

FIG. 1. Pathway for the 14α-demethylation of 24,25-dihydrolanosterol.

sis; however, it is significantly more labor intensive (*6, 10*). An analysis by HPLC has been developed which uses [24,25-^{3}H]-24,25-dihydrolanosterol (*11*), but again this method is quite time consuming. We therefore have developed a convenient kinetic assay which possesses the required sensitivity and allows for the timely analysis of multiple samples.

Early studies by Alexander *et al.* (*2*) demonstrated formation of radiolabeled 4,4-dimethylcholesta-7,14-dien-3β-ol (**8**) and formic acid, employing respectively, [3α-^{3}H]- or [32-^{3}H]lanost-7-ene-32,3β-diol (**4**) as substrates. In their assay, the formate was quantitated by a laborious bulb-to-bulb distillation method. A similar technique was used by Mitropoulos *et al.* utilizing [32-^{14}C]-24,25-dihydrolanosterol to confirm removal of the 14α-methyl group as formic acid (*1*). The rate of release of [^{14}C]formic acid provided a direct assay of lanosterol 14α-demethylase activity. Comparable results were obtained from quantitation by either distillation or extraction techniques. Although lanosterol is probably biologically the more relevant substrate, the mechanistic conclusions derived from studies with 24,25-dihydrolanosterol are still valid.

In this paper we describe a novel assay for lanosterol 14α-demethylase which capitalizes upon the normal reaction sequence to provide quantifiable water soluble products free from the lipophilic sterol precursor. In addition to 24,25-dihydrolanosterol, the alcohols (**3** and **4**) (Fig. 1) have been shown to be substrates (*2, 7*). Although the biosynthetically derived product of hydroxylation contains a double bond at position 8, the Δ7 isomers are more accessible synthetically (*12*). Therefore we have prepared for comparison purposes, both the Δ8 isomer **3** and the Δ7 isomer **4** with the tritium label at C-32 and have examined the kinetics of tritium

release. Incorporation of a tritium label at C-32 allows measurement of tritium release to water as a function of enzyme activity. Use of a small, commercially available C_{18} cartridge eliminates the need for laborious distillation or extraction procedures, making 14α-demethylase amenable to kinetic studies. Our data demonstrate that although both tritiated water and formate are produced, it is not necessary to quantitate the individual amounts of each. Analysis by tritium release based on total aqueous radioactivity has been validated by comparison with the conventional gas chromatographic assay and meets the requirements of a rapid sensitive kinetic assay for lanosterol 14α-demethylase.

EXPERIMENTAL

Lanosterol 14α-demethylase has been previously purified and reconstituted from rat liver and yeast (*4, 5*). Our protocol is based on modifications of previous methods which allow for recovery of both the reductase and the demethylase from a single preparation of microsomes. All purification steps were performed at 4°C.

Preparation of Microsomes

Male Sprague–Dawley rats (200 g) from Charles River Laboratory were quarantined and acclimated to a reverse lighting schedule for 5 days. A diet of 3% cholestyramine (Mead Laboratories) in commercial rat chow was given for 12 days. Rats were sacrificed by decapitation and livers immediately removed and put into ice-cold 0.25 M sucrose. Washed livers were homogenized (100–130 g/1000 ml) in 100 mM potassium phosphate, 2 mM reduced glutathione, 1 mM EDTA, 4 mM $MgCl_2$, and 250 mM sucrose at pH 7.4. Centrifugation at 16,000*g* for 30 min removed debris and mitochondria. The supernatant was centrifuged at 100,000*g* for 1 h to pellet the microsomes. The microsomes were washed in 100 mM potassium phosphate, 1 mM glutathione, and 0.1 mM EDTA, at pH 7.4, recentrifuged at 100,000*g* for 1 h and resuspended (40 mg/ml) in the same buffer. Microsomes were stored overnight at 4°C.

Purification of Lanosterol 14α-Demethylase and NADPH Cytochrome c Reductase

Microsomes (3–4 g protein) were solubilized in 2500 ml of 10 mM Tris–HCl, pH 7.4, 0.57% cholic acid, 20% glycerol, 1 mM DTT, 1 mM EDTA, 2 μM FMN, 0.4 mM PMSF, 1 μM pepstatin A, and 50 μg/ml soybean trypsin inhibitor. Microsomal debris was removed by centrifugation in a Ti-15 Beckman zonal rotor at 90,000*g* for 2 h. Solubilized enzymes were applied to a 5.0 × 28-cm column of DE-52 equilibrated in 25 mM Tris–HCl, 0.8% Nonidet P-40, 0.1% cholic acid, 0.05 mM EDTA, 0.05 mM DTT, and 2 μM FMN, pH 7.7 (buffer A), at a rate of about 250 ml/h. The loaded column was washed with about 800 ml of equilibration buffer. A linear gradient of 0–350 mM KCl in buffer A (3600 ml) was used to elute 14α-demethylase followed by NADPH cytochrome *c* reductase. The 14α-demethylase fractions were pooled and frozen for later use. Active reductase fractions

were pooled and applied at a rate of 250 ml/h to a 2',5'-ADP Sepharose column (2.5 × 4.7 cm) equilibrated in 10 mM potassium phosphate, 20% glycerol, 0.1% Nonidet P-40, 0.1 mM EDTA, 0.2 mM DTT, and 2 μM FMN at pH 7.7. The column was washed with 100 ml of 200 mM potassium phosphate, 20% glycerol, 0.1% Nonidet P-40, 0.4 mM EDTA, 0.2 mM DTT, and 2 μM FMN at pH 7.7. The column was then washed with 40 ml of equilibration buffer. Reductase was eluted at a rate of 10 ml/h with 10 mM potassium phosphate, 20% glycerol, 0.1% Nonidet P-40, 0.1 mM EDTA, 0.2 mM DTT, 2 μM FMN, and 5 mM 2'-AMP at pH 7.7. Active fractions were combined (30 ml) and dialyzed twice against 1000 ml of buffer containing 30 mM potassium phosphate, 20% glycerol, 0.1 mM EDTA, and 2 μM FMN at pH 7.7. Following concentration by ultrafiltration small aliquots were frozen and stored at −80°C.

Purification of 14α-Demethylase

After separation of cytochrome P450 reductase, frozen active demethylase fractions (approximately 300 ml) were thawed and the pH was adjusted to 7.25 with 1.0 M potassium phosphate. Active demethylase fractions were then applied at a rate of 100 ml/h to a hydroxylapatite column (2.5 × 13 cm) equilibrated in 10 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, and 0.1% Triton N-101, pH 7.25 (10 mM PEDGT buffer). The column was then washed with 150 ml of 50 mM PEDGT buffer, followed by another wash with 150 ml of 90 mM PEDGT. The 14α-demethylase was then eluted at a rate of 150 ml/h with 200 mM PEDGT. Active fractions were combined and concentrated by ultrafiltration and stored on ice at 4°C.

Enzyme Assays

NADPH cytochrome *c* reductase was assayed by the method of Kubota *et al.* at 30°C (pH 7.7) (*13*). Protein concentrations were determined using Pierce bicinchoninic acid reagent (*14*) with bovine serum albumin as a standard. Cytochrome P450 content was determined by the method of Omura and Sato (*15*).

Tritium Release Assay

Radioactive substrates were stored at −80°C in benzene. Prior to use, radioactive substrate was added to an ethanol solution of the carrier sterol. Solvent was removed with a stream of argon. Substrate was resuspended in ethanol and reduced to dryness to ensure complete removal of the benzene. The substrate was then resuspended in ethanol. Small quantities of stock substrate in ethanol were stable at room temperature for several days. Prior to use in the assays, the stock solution was diluted to 1 part ethanol, 1 part 2% Emulgen 913, and 8 parts 100 mM Tris–HCl, 20% glycerol at pH 7.5.

Reconstitution assays contained 100 mM Tris–HCl, 20% glycerol, 1.0 mM NADPH, 0.2 mM DTT, 0.2 mM EDTA, 10 mM glucose 6-phosphate, and 0.4 Units/ml glucose-6-phosphate dehydrogenase, pH 7.5. Lanosterol 14α-demethylase, NADPH cytochrome *c* reductase, and all buffered assay mix components

were incubated for 10 min at 30°C prior to initiation of the reaction by substrate. Unless otherwise indicated in the figure legends, reaction time was 15 min. Final ethanol and Emulgen 913 contributions from substrate were 1 and 0.02%, respectively. Reactions (0.1 ml) were quenched with 1% Emulgen 913 (0.01 ml) and 40% trichloroacetic acid containing 0.25% formic acid (0.025 ml). Following centrifugation, a 0.1-ml aliquot was put through a Sep-Pak C_{18} cartridge. Vacuum was applied and the cartridge was washed twice with 1.0 ml of water into the same collection tube. Samples were counted in Beckman Ready-Solv or subjected to bulb-to-bulb distillation.

Sep-Pak cartridges were regenerated by washing with 25 ml of methanol followed twice by 5 ml of water. Use of a Sep-Pak cartridge rack allowed for the processing of 16 samples at a time. Partitioning between water and organic solvents was tried, but the Sep-Pak protocol yielded more satisfactory backgrounds and reproducibility.

Bulb-to-Bulb Distillation

A 1-ml sample of Sep-Pak filtrate was flash frozen with a dry ice/acetone bath and distilled under vacuum. The sample side was rinsed with 0.5 ml of water and the distillation repeated. A portion of the combined volatile fraction was counted and a portion was transferred to a fresh distillation apparatus. Following the addition of KOH, the tritiated water was vacuum distilled and counted. The difference between the untreated and the base-treated samples is assumed to be due to formate production (*1–3*). The combined number of counts in the final water distillation and the formate residue (corrected for dilutions) was equal to the amount of counts in the combined volatile fraction. Control distillations using [^{14}C]formic acid indicated nearly quantitative recovery of the formate ensuring essentially complete recovery of the tritiated sample.

Gas Chromatography

Samples (0.5 ml) were prepared as in the tritium release assay quenching the reaction with 0.5 ml of 1 N HCl. Dihydrocholesterol (1 μg) was then added to each sample as an internal standard. Sterols were extracted three times with 2 ml of a diethyl ether/pentane (3 : 1) mixture and evaporated to dryness with a Savant Speed-Vac. Samples were dissolved in ethanol for GC injection. TMS-derivatized samples were prepared under argon by dissolving the sterols in DMF followed by an equal volume of *N,O*-bis-trimethylsilylacetamide and heating at 65°C overnight. This ensured complete silylation of all hydroxy groups. Samples were analyzed on a Perkin–Elmer 8500 GC fitted with an Alltech 25 m × 0.25 mm OV-17 capillary column equipped with a flame ionization detector. The temperatures of the injector, oven, and detector were 275, 270, and 320°C, respectively.

Synthesis of [32-^{3}H]-24,25-Dihydrolanosterol

3β-Benzoyloxy-4,4-dimethyl-15-trimethylsilyloxy-5α-cholest-8,14-diene. A mixture of 2.65 g (5.0 mmol) of 3β-benzoyloxy-4,4-dimethyl-5α-cholest-8(14)-en-

15-one (*16*), 1.9 ml (15 mmol) of chlorotrimethylsilane, and 4.2 ml (30 mmol) of triethylamine in 25 ml of dry DMF was heated at gentle reflux (bath 140°C) for 1 day. The bulk of the DMF was then distilled off at reduced pressure (20 mm Hg), to yield a solid mass which was then further heated at 120°C and 1 mm Hg to remove the last traces of solvent. The resulting silyl enol ether was dissolved in a minimum of 5% EtOAc in hexanes to effect separation from triethylammonium hydrochloride and then further purified by flash chromatography on silica gel to afford a mixture of silyl enol ethers (2.6 g, 85% yield) as a white crystalline solid, mp 172–177°C. ir, 1715(C=O), 1650(C=C), 1280, 1255, 1120 cm^{-1}; NMR($CDCl_3$) δ 0.82(s, 3H, H-18), 0.88(d, 6H, H-26 and -27), 0.98, 1.06, 1.09(s, 9H, H-19, -30, and -31), 0.9–2.6(m, methylene envelope), 4.75(dd, 1H, H-3), 7.4–8.1(m, 5H, aromatic); mass spectrum (DCl/CH_4), *m/e* (relative intensity) 605(M + 1, 21), 604(M^+, 28), 589(18), 484(36), 483(100). *Ana.* Calculated for $C_{39}H_{60}O_3Si$: C, 77.43; H, 10.00. Found: C, 77.60; H, 10.34.

3β-Benzoyloxylanost-8-en-15-one. To a dry argon flushed flask containing benzyltrimethylammonium fluoride (169 mg, 1 mmol), 1 g of 4A moelcular sieves, and a magnetic stirring bar was added 2 ml of THF. After this mixture was stirred vigorously for 6 h in order to activate the fluoride reagent, the flask was cooled to −5°C and a solution containing the trimethylsilyl enol ether (200 mg, 0.33 mmol) and methyl iodide (0.21 ml, 3.3 mmol) in 2 ml of THF was added to the stirring solution. After 15 min the ice bath was removed and stirring was continued for an additional 15 min at which point the reaction was diluted with hexanes (15–20 ml). The resulting solution was filtered through celite and concentrated under reduced pressure. Recrystallization from methylene chloride/hexanes afforded 94 mg (52%) of product which by GC was a 80 : 17 ratio of Δ8 : Δ7 olefin isomers plus 3% of the unalkylated Δ8,9-enone. An additional recrystallization from methylene chloride/hexanes gave analytically pure material: mp 205–207°C; ir; 1740 and 1720(C=O), 1275, 1120 cm^{-1}; NMR($CDCl_3$) δ 0.77 (s, 3H, H-18, major isomer), 0.8–1.2(m, methyl envelope), 1.2–2.8(m, methylene envelope), 4.74(dd, H-3, J = 8, 15 Hz), 6.3(m, H-7, minor isomer), 7.4–8.1(m, 5H, aromatic); mass spectrum(CDl/CH_4) *m/e*(relative intensity) 547(M + 1, 10), 529(17), 489(5), 426(29), 425(100). *Ana.* Calculated for $C_{37}H_{54}O_3$: C, 81.27; H, 9.95. Found: C, 80.87; H, 10.34.

[32-^{3}H]-3β-Benzoyloxylanost-8-en-15-one. Using tritium-labeled methyl iodide (0.33 mmol, 1.15 Ci, sp act 350 Ci/mol) the radiosynthesis was performed as described above.[3] The crystallization procedure produced 15 mCi of product which was further purified by preparative TLC (two 20 × 20-cm silica plates, 1 mm thickness, eluting with benzene) to yield 9.5 mCi of radiolabeled ketone.

24,25-Dihydrolanosterol. To a dry argon flushed flask containing the 15-oxysterol (35 mg, 0.064 mmol), hydrazine hydrochloride (300 mg, 4.4 mmol), and a magnetic stirring bar were added triethylene glycol (2.8 ml, dried over 3A sieves) and anhydrous hydrazine (1.6 ml). This solution was stirred and heated (bath 130–140°C) for 10 h to yield a homogeneous solution at which point the reflux condenser was removed and KOH (425 mg, 6.4 mmol) cautiously added. A short-path

[3] Both steps of the radiosynthesis were performed at Moravek Biochemicals, Inc., Brea, CA.

condenser was attached and the temperature of the bath gradually raised to 210°C to distill off the hydrazine. After an additional 2 h at 210°C the reaction was cooled, diluted with 10 ml each of water and ether, transferred to a seperatory funnel, and extracted with additional portions of ether. The combined organic was washed with brine, dried over $MgSO_4$, and evaporated to dryness to afford 23 mg (84%) of 24,25-dihydrolanosterol as a 3 : 1 mixture of Δ8 : Δ7 olefin isomers; mp 137–140°C for pure Δ8 isomer 148°C (*17*); mass spectrum (El, DP) gave good fit to authentic library spectra.

[32-^3H]-24,25-Dihydrolanosterol. Using the above procedure the radiolabeled ketone (4.75 mCi, 7.7 mg, 0.014 mmol) was deoxygenated to afford 3.2 mCi of crude product. Purification by preparative TLC (eluting with 10 : 10 : 1 benzene : ether : EtOAc) afforded 1.7 mCi of [32-^{3}H]-24,25-dihydrolanosterol. The compound was radiochromographically pure (HPLC) and coeluted (HPLC and TLC) with authentic dihydrolanosterol. The GC analysis further confirmed the identity of the compound as 24,25-dihydrolanosterol containing 12% of the Δ7 isomer. The final product had a specific activity of 350 mCi/mmol.

Synthesis of [32-^3H]Lanost-8- or 7-en-3β,32-diols

3β-Hydroxylanost-8-en-32-al and 3β-hydroxylanost-7-en-32-al were used to prepare the corresponding diols (*6*). The individual aldehydes (5 mg) were dissolved in a 2 : 1 mixture of methanol/methylene chloride (0.5 ml). Complete reduction of the alcohols was achieved by adding an excess (25 mCi) of [^{3}H]sodium borohydride (319 mCi/mmol). Upon completion of the reaction, the solvent was removed with a stream of argon. The excess borohydride was destroyed with 0.1 N HCl and the sterol was extracted with ethyl acetate. The organic layer was washed with water, evaporated to dryness with argon, and dried in a desiccator overnight. The dry residue was triturated with hexanes.

Final alcohol products were judged pure by TLC analysis using a 3 : 1 hexane/ethyl acetate mixture on silica gel plates. Radiochemical purity was assessed with a Bioscan system 200 imaging scanner in the same TLC system indicating a single radioactive band.

A mixture of diastereomers was formed during the reduction of the aldehydes. On the basis of the NMR of deuterated compounds prepared from reduction of the aldehyde with sodium borodeuteride, the ratios were 3.6 : 1 and 1.6 : 1 for formation of lanost-8- and lanost-7-en-3β,32-diols, respectively. Assignment of absolute stereochemistry has not been made.

Materials

Emulgen 913 was kindly supplied by Kao-Atlas Co, Tokyo. Nonidet P-40 was from LKB and cholic acid was from Atomergic Chemicals Corp. Triton N-101, PMSF, pepstatin A, soybean trypsin inhibitor, FMN, reduced glutathione, and NADPH Type III were purchased from Sigma. DE-52 was obtained from Whatman, 2′,5′-ADP Sepharose 4B was from Pharmacia Fine Chemicals, and hydroxylapatite (Bio-Gel HT) was from Bio-Rad laboratories. Commercial 24,25-dihydrolanosterol was purified by high-performance liquid chromatography.

TABLE 1

Cofactor Requirements for 14α-Demethylase Activity Monitored by Tritium Release

Conditions	Demethylase activity (nmol/mg/h)
Control	113
−NADPH	0
−Reductase	0
−Demethylase	0
Under argon	5

Note. Reconstitution conditions and tritium release assays were performed as under Experimental using 37.5 μM [32-^{3}H]-24,25-dihydrolanosterol as a substrate.

[^{14}C]Formic acid and [^{3}H]sodium borohydride were from DuPont–New England Nuclear. Sep-Pak cartridges were purchased from Waters Associates. BSA-TMS was from Regis Chemicals, and all solvents used in synthesis were Gold Label Reagents from Aldrich Chemical Co. All other chemicals were obtained from commerical sources or can be synthesized from published methods and were of the highest grade available.

RESULTS AND DISCUSSION

The purified and reconstituted 14α-demethylase from both rat liver and yeast is known to require molecular oxygen, NADPH and NADPH cytochrome *c* reductase (*4, 5*). As shown in Table 1, release of tritium from [32-^{3}H]-24,25-dihydrolanosterol is also dependent upon these factors.

Figure 2A shows the dependence of the demethylase upon NADPH cytochrome *c* reductase activity using the tritium release assay. As expected, the system was easily saturated with demethylase (Fig. 2B) and enzyme concentrations were adjusted to keep the demethylase activity limiting. The reconstituted system appeared quite sensitive to detergent (data not shown) and therefore the detergent concentration was kept to a minimum.

As shown in Fig. 3A, linearity of product formation was observed for several minutes even at the highest concentration of [32-^{3}H]-24,25-dihydrolanosterol used. Although lanosterol is the natural substrate for demethylation, rat liver demethylase will utilize 24,25-dihydrolanosterol at about a fivefold lower rate (*18*). The effect of the double bond position has been examined for 24,25-dihydrolanosterols, lanost-8-en-3β-ol (**1**), and lanost-7-en-3β-ol (**2**)[4] and although K_m does not change, V_{max} was about sevenfold higher for the Δ8 isomer (**1**).

[4] J. Trzaskos personal communication.

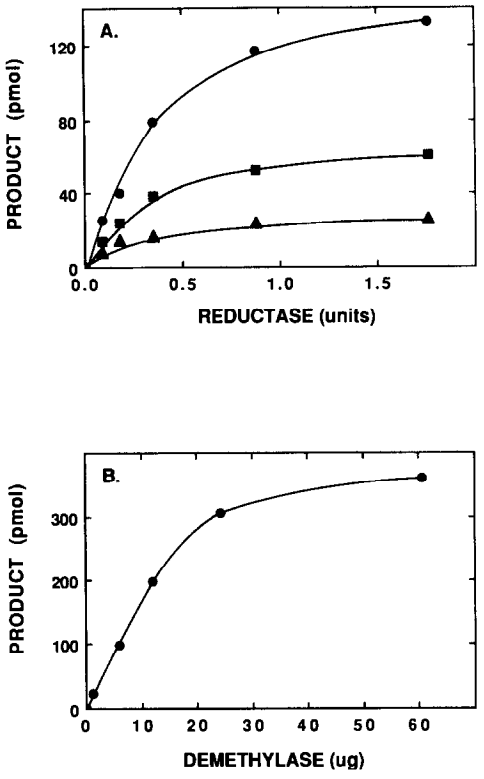

FIG. 2. Dependence of 24,25-dihydrolanosterol demethylase activity with purified components. Activity was determined using the reconstitution assay described under Experimental. The concentration of 24,25-dihydrolanosterol was 75 μM. (A) The amount of cytochrome P450 reductase (0.088–1.76 Units) was varied in the presence of 1.2 μg (▲), 2.4 μg (■), and 6.1 μg (●) 14α-demethylase. (B) The amounts of 14α-demethylase was varied in the presence of 0.7 Unit of cytochrome P450 reductase.

We found that lanost-7-en-3β,32-diol (**4**), displayed comparable kinetic behavior to 24,25-dihydrolanosterol (**1**) (Fig. 3B). In contrast, lanost-8-en-3β,32-diol (**3**) turned over so rapidly that substrate depletion caused nonlinearity at high enzyme levels (Fig. 3C).

The kinetic parameters of K_m and V_{max} determined by the tritium release assay for reconstituted rat liver enzyme are shown in Table 2. The K_m value for 24,25-dihydrolanosterol was considerably lower than that obtained using microsomal preparations (312 μM) reported by Trzaskos *et al.* (*18*) but was comparable to the microsomal K_m of Frye and Robinson (23 μM) (*19*).

The data obtained by Aoyama *et al.* (*7*) using a reconstituted system from *Saccharomyces cerevisiae* indicate relatively little change in V_{max} between 24,25-dihydrolanosterol and lanost-8-en-3β,32-diol but a 19-fold reduction in K_m for the C-32 alcohol. Our data show a similar trend with respect to K_m in that we observed a drop in K_m for lanost-8-en-3β,32-diol compared to 24,25-dihydrolanosterol. However, we observed an an almost 4-fold increase in V_{max} for lanost-8-en-3β,32-

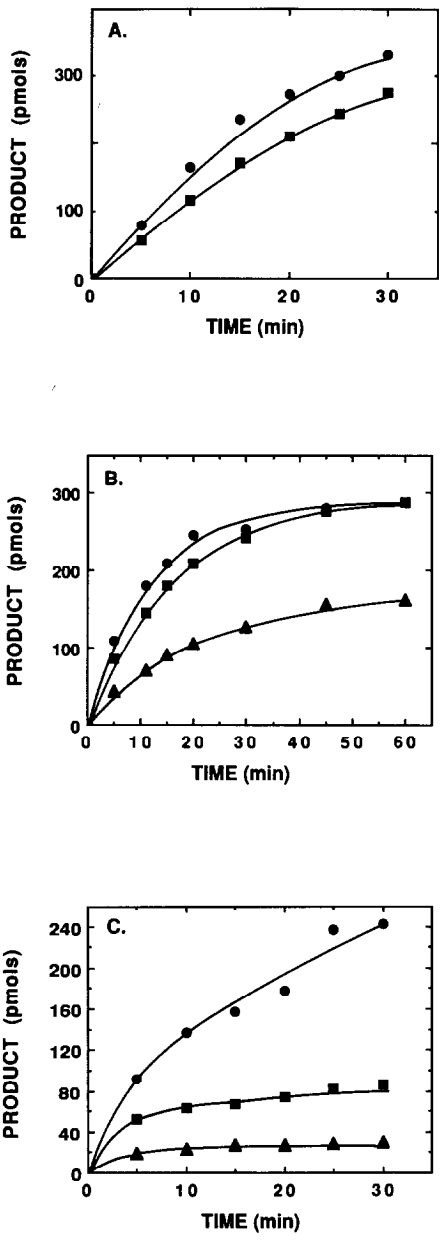

FIG. 3. Linearity of tritrium release with three different substrates. Activity was determined using the reconstitution assay described under Experimental. The amount of cytochrome P450 reductase used in all experiments was 0.7 Units. (A) In the presence of 15 μg of 14α-demethylase the concentration of 24,25-dihydrolanosterol was 37.5 μM (■) and 75.0 μM (●). (B) In the presence of 12 μg of 14α-demethylase the concentration of lanost-7-en-3β,32-diol was 3.0 μM (▲), 15 μM (■), and 74 μM (●). (C) In the presence of 12 μg of 14α-demethylase the concentration of lanost-8-en-3β,32-diol was 2.8 μM (▲), 14 μM (■), and 69 μM (●).

TABLE 2

Kinetic Constants Determined by Tritium Release

Substrate	K_m (μM)	V_{max} (nmol/mg/h)
24,25-Dihydrolanosterol[a]	35.2	178
Lanost-8-en-3β,32-diol[b]	5.1	688
Lanost-7-en-3β,32-diol[c]	5.7	129

Note. Kinetic assays were performed using the tritium release assay described under Experimental. The amount of cytochrome P450 reductase used in all experiments was 0.7 Units. K_m and V_{max} values were determined from double reciprocal plots.

[a] 24,25-Dihydrolanosterol concentrations were varied between 7.5 and 75 μM. The amount of 14α-demethylase used was 12 μg.

[b] Lanost-8-en-3β,32-diol concentrations were varied between 0.63 and 10 μM to minimize substrate depletion. The amount of 14α-demethylase used was 1.2 μg.

[c] Lanost-7-en-3β,32-diol concentrations were varied between 1.9 and 74 μM. The amount of 14α-demethylase used was 12 μg.

diol. The difference in kinetic parameters between the Δ7 and the Δ8 diols seems to be primarily a difference in V_{max}.

Oxysterol intermediates may accumulate during demethylation, depending upon the reaction conditions employed (*18*). Since the tritium release assay would not distinguish these intermediates, independent analysis of the reaction was performed by GC. Derivatization of sterols was not mandatory for monitoring the Δ8,14-diene formation by GC. Hydroxy sterols, however, gave a broad signal which could not be integrated. Derivatization with bistrimethylsilylacetamide improved the resolution and permitted accurate integration. Except for commercially available dihydrocholesterol, all other standards were synthesized and used to verify the identity of enzyme-produced products.

Analysis of substrate turnover by tritium release could potentially give a low estimate of the amount of turnover in the event of a large isotope effect on the removal of the tritium. Table 3 compares the results of turnover for the three radiolabeled substrates measured by tritium release with that of gas chromatographic analysis (which monitors primarily unlabeled carrier sterol) at a single concentration and incubation time point. The data for the 32-hydroxylated substrates was identical for the two analyses. Analysis by tritium release gave lower values than those of the GC analysis only for TMS-derivatized reaction products from [^{3}H]-24,25-dihydrolanosterol and not the underivatized reaction products. Since we were unable to detect formation of any intermediates we conclude these differences were not significant.

14α-Demethylase has mechanistic similarities to aromatase. Aromatase, another cytochrome P450 enzyme, successively hydroxylates C-19 of androstenedione prior to expulsion of C-19 as formic acid and aromatization of the ring. A

TABLE 3

Comparison of Tritium Release and Gas Chromatography Assays

Substrate	% turnover radioassay	% turnover GC
24,25-Dihydrolanosterol	14	15
24,25-Dihydrolanosterol[a]	11	17
Lanost-8-en-3β,32-diol[a]	32	33
Lanost-7-en-3β,32-diol[a]	9	9

Note. Analyses by tritium release and gas chromatography were performed as described under Experimental. The ratio of 14α-demethylase to cytochrome P450 reductase used in these experiments was 12.1 μg/1.0 Unit. Incubations were for 10 min followed by 1 h reaction times at 30°C. The concentrations and specific activities of 24,25-dihydrolanosterol, 75 μM (60,000 dpm/nmol); lanost-8-en-3β,32-diol, 70 μM (60,000 dpm/nmol); and lanost-7-en-3β,32-diol 74 μM (20,000 dpm/nmol).

[a] Derivatized as the Bis-TMS ether for GC analysis.

tritium isotope effect was observed in the first, but not the second hydroxylation at C-19 of aromatase (*20*). The aromatase studies were performed in a microsomal system and it has been suggested that the involvement of different mechanisms or even different catalytic sites may be responsible for the distinction between successive hydroxylations. In the case of 14α-demethylase, it is clear that a single enzyme is responsible for all three oxidations in the purified reconstitution system. For the purpose of validation of the assay, the absence of observed isotope effects is an advantage in that tritium substitution causes no loss in sensitivity and no apparent changes in observed products.

Product analysis of all three substrates indicated production of both tritiated water and tritiated formate in the course of substrate turnover. The ratio of water to formate (data not shown) did not change substantially over a 2-h time period for each substrate. Table 4 lists the average ratios of the water soluble products formed by the demethylation sequence for all three substrates. One would predict that [32-^{3}H]-24,25-dihydrolanosterol would yield a ratio of two tritiated waters for every labeled formate in the absence of (1) an isotope effect, (2) a stereochemical preference for initial hydrogen abstraction, and (3) an accumulation of oxysterol intermediates. If the 32-hydroxy substrates were an equal mixture of isomers, and assuming stereospecific H abstraction in the formation of the C-32 aldehyde as has been seen for aromatase (*21*), the 32-hydroxy substrates would be expected to yield a 1 : 1 mixture of labeled water and formate. Initial experiments using a single substrate concentration at a single time point were at variance with this prediction. Since it was known that incubation parameters can influence the production of oxysterol intermediates (*18*) a complete time course was performed at K_m substrate concentrations. In contrast to a similar experiment with aromatase

TABLE 4

Ratio of the Water Soluble Products Produced from 14α-Demethylation

Substrate	H_2O/formate	Diastereomer ratio
24,25-Dihydrolanosterol	1.2	
Lanost-8-en-3β,32-diol	0.37	3.6/1
Lanost-7-en-3β,32-diol	1.2	1.6/1

Note. Bulb-to-bulb distillation which was described under Experimental was used to quantitate the formation of water and formate. Except for the length of reaction, conditions were adjusted to mimic initial velocity conditions and substrate concentrations were at K_m.

(*20*), the ratios of radioactive water and formate did not change with time. An earlier experiment by Alexander *et al.* (*2*) using [32-^{3}H]lanost-7-en-32,3β-diol to verify the production of formate by 14-demethylase (at a single time point using microsomes) yielded close to 50% of the radioactivity in the formate (no stereochemical information was given regarding starting substrate). NMR analysis of C-32-deuterated lanost-(7- or 8-en)-3β,32-diols, formed by [^{2}H]sodium borohydride reduction of the corresponding aldehydes, indicated diastereomer mixtures of 3.6:1 and 1.6:1. The stereochemistry of hydrogen abstraction is not known for 14α-demethylase. The data in Table 4 show that in the case of [32-^{3}H]lanost-8-en-3β,32-diol the stereochemistry at C-32 is such that ^{1}H is preferentially abstracted over ^{3}H and suggest that the diastereomers may be processed stereospecifically. In contrast, ^{3}H is abstracted from the major isomer of [32-^{3}H]lanost-7-en-3β,32-diol. The lower than expected water/formate ratio observed with [^{3}H]-24,25-dihydrolanosterol is probably a reflection of the enzyme's ability to stereoselectively process the prochiral C-32 methyl.

SUMMARY

We have developed a radiochemical assay for 14α-demethylase based upon [32-^{3}H]-24,25-dihydrolanosterol metabolism. Previous studies using ^{14}C- and ^{3}H-labeled substrates had demonstrated the feasibility of a direct assay for 14α-demethylase but neither system was amenable to processing a multitude of samples (*1*, *2*). Using the Sep-Pak separation system, it is possible to process several dozen samples a day. The degree of turnover for all three substrates estimated by this assay are in excellent agreement with that estimated by gas chromatography and the kinetic parameters observed are in reasonable agreement with published information. Although we made three radiolabeled substrates, given the relatively small differences in kinetic parameters versus the synthetic difficulties for the preparation of the Δ8 compounds, [^{3}H]lanost-7-en-3β,32-diol would be the compound of choice for future assays.

As indicated in Table 4, a mixture of labeled water and formate is produced from the three substrates. However, since the water/formate ratio remains constant with time it was not necessary to quantitate the individual components and Michaelis–Menten kinetics were observed using total aqueous counts. Note, however, that it is possible to generate a number of reaction intermediates depending upon the reaction conditions (*18*) and consequently the ratio of water to formate would be expected to vary under conditions where intermediates accumulate. Although the absolute stereochemistry at C-32 of the tritiated 32-alcohols has not been assigned, this does not preclude the use of these substrates to monitor enzyme activity.

In these studies we used about fivefold less enzyme for the tritium release assay than for the GC assay and allowed a minimum of 5% turnover by GC to make a direct comparison between the two methods. Since all three substrates used have specific activities in excess of 300 mCi/mmol we can detect less than 1% turnover and gain two orders of magnitude increase in sensitivity while maintaining low backgrounds.

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