# Enzymic formation of esters of methyl sterol precursors of cholesterol

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ABSTRACT For investigation of the reactions of cholesterol biosynthesis, a number of workers use the 10,000 *g* supernatant fraction (or similar preparations) obtained from cell-free homogenates of rat liver. We have found that esters of methyl sterol biosynthetic intermediates are formed by this crude source of enzymes. Esters of $C_{30}$-, $C_{29}$-, $C_{28}$-, and $C_{27}$-sterol intermediates have been isolated by silicic acid chromatography of an acetone extract of incubation mixtures. Competition between ester formation and demethylation of the $C_{28}$-sterol intermediate has been demonstrated. With 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol as substrate, maximal velocities of ester formation (0.36 nmole/30 min per mg of protein) were almost equivalent to maximal velocities of demethylation (0.45 nmole/30 min per mg of protein). Ester formation may be eliminated by carrying out incubations with microsomal preparations; ester formation may be restored completely upon addition (to the microsomes) of either coenzyme A and ATP or the supernatant fraction resulting from centrifugation at 105,000 *g*.

Ester formation has been examined similarly with broken-cell preparations of rat skin. With 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol as substrate, the rate of ester formation was more than six times the rate of methyl sterol demethylation. The very significant competition between esterification and demethylation of methyl sterol intermediates of skin suggests that sterol intermediates accumulate in rat skin because of the rapid formation of esters that may not be further metabolized.

SUPPLEMENTARY KEY WORDS sterol esters · cholesterol biosynthesis · competition between esterification and biosynthesis · homogenates · skin sterol accumulation

THE TERMINAL REACTIONS of cholesterol biosynthesis have not been fully elucidated. Two types of investigation have been most fruitful and widely used. They are either study of the metabolism of proposed intermediates obtained by organic synthesis or isolation of naturally occurring metabolites from appropriate tissues (1). For both approaches, the purity of the enzymic system has not been a matter of particular concern.

In studies using broken-cell preparations of rat liver, the 10,000 *g* supernatant fraction, or a similar fraction frequently called "Bucher homogenate" (2), has been used extensively (1, 3). Because the terminal reactions of cholesterol biosynthesis are catalyzed by enzymes found in the microsomal pellet (3), use of microsomes for these studies rather than Bucher homogenate may be advantageous, particularly since competing reactions may be prevented, or complicating factors may be eliminated by the removal of the 105,000 *g* supernatant fraction.

This report describes one such complication: the formation of esters of methyl sterol intermediates in Bucher homogenates. Furthermore, competition of this hepatic esterification reaction with demethylation of methyl sterol precursors of cholesterol is described. Kinetic measurements of rates of esterification and demethylation are compared. Finally, since esters of methyl sterol precursors of cholesterol are very abundant in rat skin (4–6), relative rates of esterification and demethylation in a cell-free preparation of rat skin were compared.

## METHODS AND MATERIALS

### *Enzyme Preparation*

Adult, male Sprague–Dawley rats (200–400 g) were purchased from Holtzman Co., Madison, Wis. Rats

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were maintained on commercial stock ration with unlimited quantities of food and water. The rats were killed by decapitation. The liver was removed, minced with scissors, and homogenized with a TenBroeck glass homogenizer; 2 volumes of 0.1 M potassium phosphate buffer (pH 7.4, containing 4 mM glutathione and 30 mM nicotinamide) were used for each volume of liver. The supernatant fraction resulting from 10,000 *g* centrifugation for 20 min was collected. Microsomes were isolated by centrifugation of this supernatant fraction for 1 hr at 105,000 *g*. The microsomes were suspended in a volume of buffer equal to one-fourth of the volume of the original supernatant fraction from the 10,000 *g* centrifugation. The protein concentration of the 10,000 *g* supernatant fraction and microsomes averaged 40 mg/ml and 20 mg/ml, respectively, as determined by the method of Lowry, Rosebrough, Farr, and Randall (7).

### *Incubation Conditions*

Supernatant fraction from the 10,000 *g* centrifugation or microsomes were incubated aerobically at 37°C. For experiments requiring anaerobic conditions, incubation flasks were flushed with nitrogen for about 20 sec and capped with serum stoppers. Solutions of glucose and glucose oxidase were injected. Following incubation for 5 min at 4°C, the flask contents were incubated at 37°C. Final volume was 6.0 ml unless stated otherwise. The enzymic process was terminated either by the addition of acetone or, if labeled carbon dioxide was to be collected, by addition of 0.5 ml of 10 N sulfuric acid. Carbon dioxide was collected by a procedure described earlier (8). Demethylation was measured by $^{14}CO_2$ release from the $^{14}C$-labeled substrate used (8). Following termination of the enzymic process with acetone, the sterol was extracted with an additional 150 ml of acetone; the resulting protein precipitate was removed by filtration. The acetone extract was dried and evaporated. The resulting residue was chromatographed on a column (2 × 2 cm) containing 5 g of silicic acid; the column was developed with 30 ml of petroleum ether–benzene 1:1, 70 ml of benzene, and 30 ml of methanol. More than 95% of the $^{14}C$ radioactivity was recovered. Further analysis of the petroleum ether–benzene and benzene fractions was performed on thin-layer sheets (Eastman Chromagrams No. 6060 with fluorescent indicator).

For rat skin, the homogenate was prepared according to a method described earlier (9). Enzymic action was terminated, and product formation was measured as described above for liver.

### *Substrate Preparation*

All substrates were suspended in buffer with the aid of the detergent Triton WR-1339. Concentrations in the substrate solution were 300 nmoles of sterol per ml and 10 mg of Triton WR-1339 per ml unless indicated otherwise. Within this range of concentration the detergent has little effect on the rate of demethylation (10).

4α-Methyl-5α-cholest-7-en-3β-ol (mp 146–148°C) (5); 4,4-dimethyl-5α-cholest-7-en-3β-ol (mp 146–147°C) (11); 4,4,14α-trimethyl-5α-cholest-7-en-3β-ol (mp 141–142°C) (12); and 5α-cholest-7-en-3β-ol (mp 125–126°C) (12) were prepared as described in the procedures indicated. Relative retention times on gas–liquid chromatography (1% SE-30, 230°C) were equivalent to those obtained with authentic samples.

Both 30-$^{14}C$-4α-methyl-5α-cholest-7-en-3β-ol and 30, 31-$^{14}C$-4,4-dimethyl-5α-cholest-7-en-3β-ol were prepared using $^{14}C$-labeled methyl iodide. 24,25-$^{3}H$-4,4,14α-Trimethyl-5α-cholest-7-en-3β-ol was prepared according to Swindell and Gaylor (12). The specific activities of the three substrates were 3.5 × $10^5$ dpm/mg, 1.8 × $10^5$ dpm/mg, and 8.1 × $10^6$ dpm/mg, respectively.

3α-$^{3}H$-5α-Cholest-7-en-3β-ol was prepared by reduction of 5α-cholest-7-en-3-one (13) with $NaB^3H_4$ in anhydrous methyl alcohol. Successive recrystallizations resulted in a product identical to authentic 5α-cholest-7-en-3β-ol when compared by gas–liquid chromatography and thin-layer chromatography.

All substrates were shown to be radiochemically pure by thin-layer chromatography using three different solvent systems. Authentic standards were chromatographed simultaneously as unlabeled carriers.

Results are expressed uniformly as nmoles of substrate converted. Deviations are reported where multiple analyses were made.

Coenzyme A was purchased from Calbiochem, Los Angeles, Calif. ATP, $NAD^+$, and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo. Triton WR-1339 was purchased from Ruger Chemical Co., Hillside, N.J. $^{14}C$-labeled methyl iodide was purchased from New England Nuclear Corp., Boston, Mass. Petroleum ether used in this work was designated 30–60°C (Mallinckrodt).

## RESULTS

### *Isolation and Characterization of a Sterol Ester*

30-$^{14}C$-4α-Methyl-5α-cholest-7-en-3β-ol (150 nmoles) was incubated with 4 ml of supernatant fraction from the 10,000 *g* centrifugation. Product(s) and substrate were isolated and chromatographed as described under Methods and Materials. Upon chromatography, the resulting elution pattern revealed $^{14}C$ radioactivity in two major fractions. More than 95% of the $^{14}C$

radioactivity applied to the column was recovered. The elution of sterol metabolite with petroleum ether–benzene and the elution of recovered substrate with benzene are shown in Fig. 1.

The contents of the similar fractions were combined and analyzed further by thin-layer chromatography on silica gel sheets. The species in the benzene fraction was shown to be homogeneous by chromatography in two solvent systems (benzene; and petroleum ether–diethyl ether–acetic acid 160:20:5). Furthermore, in both cases the mobility of the $^{14}C$-labeled species was coincident with authentic 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol.

The labeled compound(s) in the fraction eluted with the mixture of petroleum ether and benzene was found to be homogeneous by thin-layer chromatography in four different solvent systems (benzene; petroleum ether–diethyl ether–acetic acid 160:20:5; petroleum ether–diethyl ether 8:1; and petroleum ether–benzene–ethyl acetate 25:25:3). After demonstrating homogeneity, the identity of the component in the petroleum ether–benzene fraction was established by three separate approaches:

(*a*) The labeled compound was chromatographed with an authentic sterol ester, cholesteryl stearate, on silica gel thin-layer sheets. More than 75% of the $^{14}C$ radioactivity was recovered. The chromatographic mobility was identical to that of cholesterol stearate (Table 1).

(*b*) The labeled product was heated under reflux with 1 ml of 20% ethanolic KOH for 30 min. Following isolation in the usual manner, the resulting product was chromatographed on silica gel thin-layer sheets using three solvent systems. Most of the $^{14}C$ radioactivity was recovered. The mobility of the $^{14}C$-labeled product was identical to the mobility of authentic 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol that was added as carrier (Table 1).

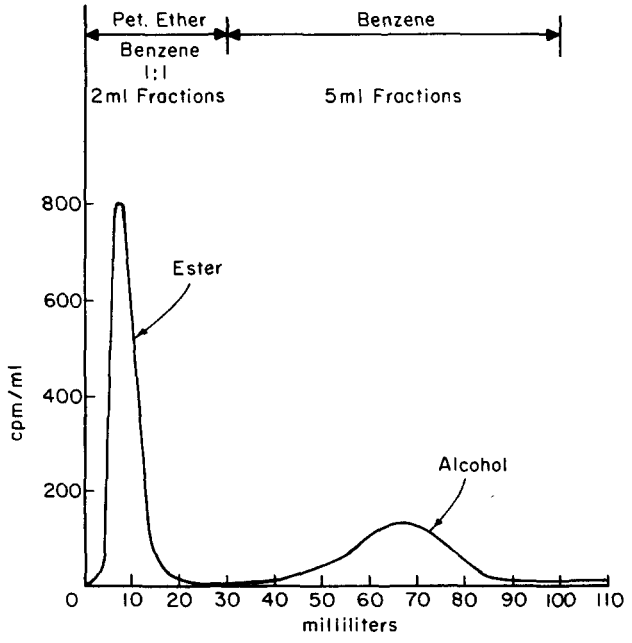


FIG. 1. Silicic acid column chromatography. Chromatography was carried out as described in Methods and Materials.

TABLE 1 THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION OF ESTER*

| Treatment | Solvent System | dpm Coincident with Sterol Ester | % Recovery from Treatment |
|---|---|---|---|
| Cochromatography with cholesteryl stearate | I | 2900 | 78 |
| | II | 3100 | 84 |
| | III | 3100 | 84 |
| | | dpm Coincident with 4$\alpha$-Methyl-5$\alpha$-cholest-7-en-3$\beta$-ol | |
| Saponification followed by chromatography | IV | 374 | 76 |
| | V | 416 | 85 |
| | VI | 367 | 75 |

* Chromatograms were developed with the following solvent systems: (I) benzene; (II) petroleum ether–diethyl ether–acetic acid 80:10:2; (III) petroleum ether–diethyl ether–pyridine 80:10:5; (IV) benzene–chloroform 1:1; (V) petroleum ether–diethyl ether–acetic acid 80:10:3; and (VI) petroleum ether–diethyl ether–pyridine 80:10:7.5.

(*c*) 30-$^{14}C$-4$\alpha$-Methyl-5$\alpha$-cholest-7-en-3$\beta$-ol (22,800 dpm; 150 nmoles) was incubated with 1 ml of suspension of microsomes under anaerobic conditions. Formation of the component isolated in the petroleum ether–benzene fraction was minimal (Table 2). Addition of either supernatant fraction or a mixture of ATP and coenzyme A to the microsomes resulted in essentially complete restoration of activity.

These three lines of evidence suggested that the component isolated in the petroleum ether–benzene fraction was an ester of 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol. First, the chromatographic behavior of the product was similar to an authentic sterol ester. Substrate was recovered when the product was saponified. Finally, cofactors required for ester formation (i.e., ATP and coenzyme A) could be substituted for the supernatant fraction recovered during preparation of microsomes.

A large quantity of labeled esters was prepared by incubating 16 ml of supernatant fraction from the 10,000 *g* centrifugation with 1200 nmoles of labeled 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol. The esters were collected and purified by column chromatography as described above. After thin-layer chromatography of the esters in two solvent systems (I and II of Table 1) and saponification, methyl esters of the recovered fatty acids were prepared by treatment with $BF_3$ in methanol. Gas–liquid chromatographic analysis revealed 8%

TABLE 2 RESTORATION OF ESTERIFICATION BY A MIXTURE OF ATP AND COENZYME A

| Treatment* | Conversion to Ester | Ester Formed |
|---|---|---|
| | % | *nmoles* |
| Microsomes† | 6.5 | 9.7 ± 0.2 |
| Microsomes + ATP + | | |
| 0.01 mM CoASH | 10.9 | 16.4 ± 0.2 |
| 0.02 mM CoASH | 11.8 | 17.7 ± 0.2 |
| 0.04 mM CoASH | 14.1 | 21.2 ± 1.9 |
| 0.08 mM CoASH | 14.1 | 21.2 ± 0.4 |
| 0.16 mM CoASH | 15.5 | 23.3 ± 1.0 |
| Supernatant fraction from 10,000 *g*‡ | 18.5 | 27.8 ± 2.3 |

* Microsomes (1 ml) were incubated anaerobically for 30 min with 150 nmoles of 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol, 8.2 $\mu$moles of ATP, and coenzyme A at the concentrations indicated above. Final volume was 6.2 ml. Supernatant fraction from centrifugation at 10,000 *g* (4 ml) was incubated anaerobically for 30 min with 150 nmoles of 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol in a final volume of 6.0 ml. The incubation flasks were depleted of oxygen by the addition of 60 mg of glucose and 4 mg of glucose oxidase (13 units/mg).

† For microsomes, each value represents the average of duplicate results from two separate experiments.

‡ For supernatant fraction from 10,000 *g* centrifugation, four separate experiments were performed.

stearic acid, 15% palmitic acid, 34% $C_{18}$ polyunsaturated acids, 8% oleic acid, an unidentified $C_{20}$ acid, and many minor fatty acids.

### *Competition between Esterification and Demethylation*

Methyl sterol demethylation requires oxygen (12). The rate of esterification was significant under aerobic conditions (Table 3). The amount of substrate esterified was equal to about 36% of the amount of $^{14}CO_2$ formed. However, if demethylation was limited by incubation under anaerobic conditions, esterification increased to a rate that was equal to the maximal rate of aerobic demethylation (Table 3). Thus, supernatant fraction from centrifugation at 10,000 *g* potentially may metabolize as much methyl sterol to ester as to demethyl sterol products.

TABLE 3 YIELD OF ESTER UNDER AEROBIC AND ANAEROBIC CONDITIONS*

| Product | Atmosphere | nmoles Formed | % |
|---|---|---|---|
| Ester | $O_2$ | 14.9 ± 2.0 | 9.9 |
| Carbon dioxide | $O_2$ | 41.7 ± 12.9 | 27.8 |
| Ester | $N_2$ | 27.8 ± 2.3 | 18.5 |

* Supernatant fraction from 10,000 *g* centrifugation (4 ml) was incubated both anaerobically (Table 2) and aerobically with 150 nmoles of 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol; $NAD^+$ (1.4 $\mu$moles) and NADPH (1.2 $\mu$moles) were added to some aerobic incubations without effect. Values are the averages of results from four separate experiments, except for those values for carbon dioxide release, which are the averages of results from 12 experiments.

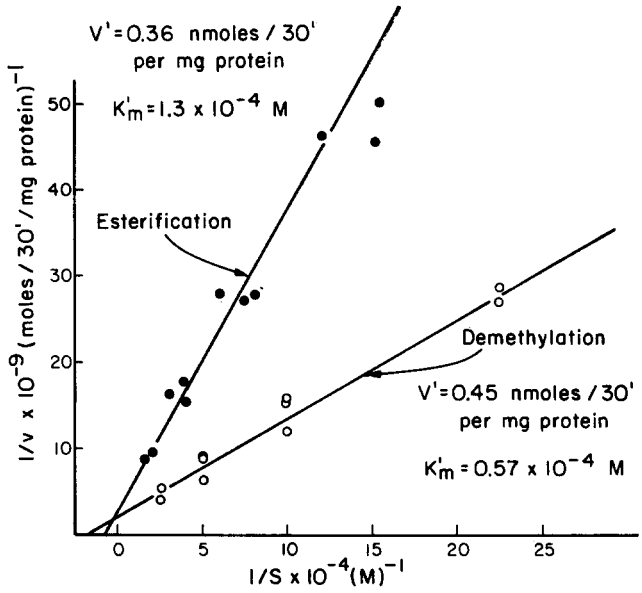


FIG. 2. Comparison of $K_m'$ and $V'_{max}$. Supernatant fraction from centrifugation at 10,000 *g* (4 ml) was incubated for 10 min with approximately 50–400 nmoles of 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol in a final volume of 6.0 ml. The velocity of reaction for both demethylation and esterification was found to be constant with time up to 10 min. The incubations were conducted aerobically for measurement of demethylation and anaerobically for measurement of esterification. For aerobic incubations, 1.4 $\mu$moles of $NAD^+$ was added. The values reported are individual observations from three separate experiments.

$V'_{max}$ and $K'_m$[1] for both demethylation and esterification were determined by conventional Lineweaver-Burk plots (Fig. 2). Maximal rates of esterification were determined under anaerobic conditions. $V'_{max}$ for esterification (0.36 nmole/30 min per mg of protein) was almost equal to $V'_{max}$ for demethylation (0.45 nmole/30 min per mg of protein). $K'_m$ for esterification ($1.3 \times 10^{-4}$ M) and demethylation ($0.57 \times 10^{-4}$ M) differed only slightly.

Whether or not esters of methyl sterols are demethylated has not been established. Accordingly, small amounts of the $^{14}$C-labeled ester were incubated with microsomes; the rates of demethylation of ester and alcohol were compared (Table 4). The ester was not metabolized to carbon dioxide. The inability of microsomes to demethylate sterol ester may not be ascribed to an inhibitor that might have been isolated with the ester, because a mixture of equal amounts of isolated $^{14}$C-labeled ester and 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol was demethylated as well as 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol that was incubated alone. Thus, esterification renders the methyl sterol inert for the demethylating enzyme system.

[1] Apparent ($V'_{max}$ and $K'_m$) values are indicated because the effective concentrations of substrate in solutions prepared with the aid of detergent are unknown.

### *Prevention of Ester Formation*

Investigation of demethylation would be less complicated if the competing esterification side reaction could be eliminated. Accordingly, the rates of demethylation and esterification were measured using either the supernatant fraction from the 10,000 *g* centrifugation or microsomes as the source of enzymes (Table 5). Under aerobic conditions, esterification was eliminated when microsomes were used as the source of enzymes. When the supernatant fraction resulting from centrifugation at 105,000 *g* was added to the microsomes (i.e., reconstituting Bucher homogenate [2]), restoration of esterification was observed. The supernatant fraction alone did not catalyze esterification. Thus, use of a microsomal preparation rather than Bucher homogenate for the study of demethylation completely eliminated the disadvantageous esterification.

### *Specificity of Sterol Substrate*

Other sterol precursors of cholesterol were tested as substrates for ester formation (Table 6). Rates of esterification of the $C_{28}$- and $C_{27}$-precursors were about equal, and these rates were 7–8 times faster than the rates of esterification of the di- and trimethylated sterols. The bulky gem-dimethyl group at position 4 of the sterol nucleus probably accounts for the smaller amount of di- and trimethyl sterol esters formed.

TABLE 4 METABOLISM OF RECOVERED ESTER*

| Substrate | $CO_2$ | Conversion |
|---|---|---|
| | *nmoles* | % |
| Recovered ester | 0 | 0 |
| 4α-Methyl-5α-cholest-7-en-3β-ol | 1.58 ± 0.04 | 20.3 |
| 4α-Methyl-5α-cholest-7-en-3β-ol + recovered ester | 1.31 ± 0.23 | 16.8 |

* Microsomes (2 ml) were incubated aerobically for 20 min with 7.8 nmoles of substrate and 2.8 μmoles of $NAD^+$ in a final volume of 2.1 ml. Values are the average of results from two separate experiments. The 30-$^{14}$C-4α-methyl-5α-cholest-7-en-3β-ol substrate used for this experiment was that isolated in the benzene fraction from the silicic acid column (Fig. 1). Further, the method of suspending the substrates was not that described in the Methods and Materials section, because cloudy suspensions result. Rather, the substrates in 0.1 ml of acetone containing 10 mg of Triton WR-1339 were added slowly to the suspension of microsomes, which was maintained at 37°C.

TABLE 5 DEPENDENCE OF ESTERIFICATION ON SUPERNATANT FRACTION FROM 105,000 *g* CENTRIFUGATION*

| Treatment | % Converted to Ester |
|---|---|
| Supernatant fraction from 10,000 *g* | 9.9 ± 1.3 |
| Microsomes alone | 0.2 ± 0.2 |
| Supernatant fraction from 105,000 *g* alone | 1.1 ± 0.9 |
| Microsomes + supernatant fraction from 105,000 *g* | 10.2 ± 1.7 |

* Either microsomes (1 ml) or 4 ml of supernatant fraction from centrifugation at 10,000 *g* or from centrifugation at 105,000 *g* were incubated aerobically with 150 nmoles of 30-$^{14}$C-4α-methyl-5α-cholest-7-en-3β-ol in a final volume of 6.0 ml. Values are the average of results from eight separate experiments.

### *Formation of Esters of Methyl Sterol in Rat Skin*

Unlike internal tissues that contain cholesterol as the only sterol of quantitative consequence, rodent skin contains a high percentage of 5α-cholest-7-en-3β-ol and methyl sterol precursors of cholesterol (4). In rat skin, these sterols occur as esters (6). Thus, the relative rates of esterification and demethylation of 30-$^{14}$C-4α-methyl-5α-cholest-7-en-3β-ol in broken-cell preparations of rat skin were compared (Fig. 3). The initial rate of ester formation was 10 times greater than the initial rate of demethylation. After a short lag period the rate of demethylation was constant, and the rate of ester formation exceeded the rate of demethylation by about 4- to 6-fold.

The markedly greater rates of esterification compared to demethylation in rat skin (Fig. 3) were compared further to rat liver (Fig. 2). Whereas in rat liver the ratio of the rate of esterification to the rate of demethylation was 0.36:1, the ratio was 6:1 for rat skin!

## DISCUSSION

Bucher homogenates and other cell-free homogenates of rat liver (2) have been used to elucidate many of the enzymic transformations that occur during hepatic cholesterol biosynthesis (1). There is, however, a particular disadvantage encountered in the use of such homogenates; namely, esterified methyl sterol side-products are formed (Fig. 1; Tables 1, 2, and 4). Three lines of evidence are consistent with the conclusion that the side-products are sterol esters: (*a*) chromatographic mobility was identical to mobility of a known sterol ester (Table 1); (*b*) saponification of the side-product yielded the original substrate (Table 1); and (*c*) through addition of a mixture of coenzyme A and ATP, esterification was restored to control levels in a microsomal

TABLE 6 SUBSTRATE SPECIFICITY FOR ESTER FORMATION*

| Substrate | % Esterified |
|---|---|
| 5α-Cholest-7-en-3β-ol | 14.2 ± 0.03 |
| 4α-Methyl-5α-cholest-7-en-3β-ol | 18.5 ± 1.5 |
| 4,4-Dimethyl-5α-cholest-7-en-3β-ol | 2.2 ± 0.03 |
| 4,4,14α-Trimethyl-5α-cholest-7-en-3β-ol | 1.9 ± 0.05 |

* Supernatant fraction from 10,000 *g* centrifugation (4 ml) was incubated anaerobically as described in Table 2 with either 150 nmoles of 30-$^{14}$C-4α-methyl-5α-cholest-7-en-3β-ol or 160 nmoles of one of the other substrates for 30 min. Values are the average of results from three separate experiments.

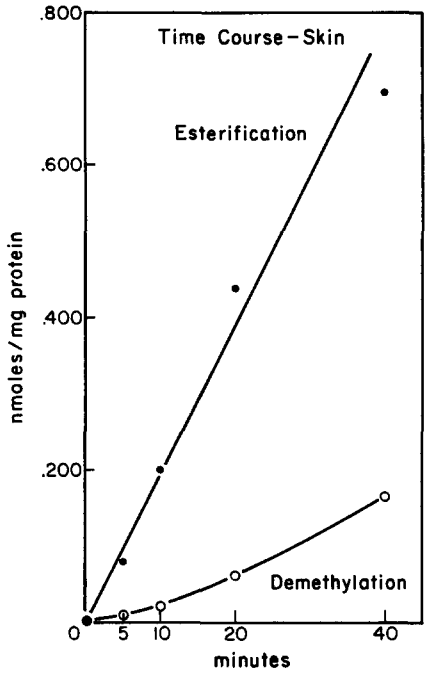


FIG. 3. Time course of esterification and demethylation in the cell-free preparation of skin. Supernatant fraction from centrifugation at 10,000 *g* (4 ml) was incubated aerobically with 150 nmoles of 30-$^{14}$C-4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol for the times indicated. Final volume was 6.0 ml. For measurement of esterification, 8.2 $\mu$moles of ATP and 1.2 $\mu$moles of coenzyme A were included in the incubation mixture. For measurement of demethylation, 4.2 $\mu$moles of $NAD^+$ was added to the incubation mixture. Results are the averages from three separate experiments.

system that did not catalyze the enzymic process in the absence of added cofactors (Table 2).

The rate of formation of esterified 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol was a significantly competing process when compared to the rate of demethylation (Table 3; Fig. 2). Under aerobic conditions the rate of esterification was slightly greater than one-third of the rate of demethylation. When demethylation was limited, i.e., incubation under anaerobic conditions, esterification rates were maximal and approximately equivalent to $V'_{max}$ values for demethylation. Significance of this competing process was further demonstrated by the lack of demethylation of esterified 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol (Table 4). Thus, the ester may not be further metabolized, i.e., demethylated, in the process of cholesterol formation. Although sterol esterases are known to occur in rat liver (14), they either did not catalyze hydrolysis of esterified 4$\alpha$-methyl-5$\alpha$-cholest-7-en-3$\beta$-ol or the rate was sufficiently slow under the conditions used that very little free sterol was released.

Microsomes recovered from Bucher homogenate did not contain significant esterifying activity when assayed under aerobic conditions that are optimal for demethylation (Table 5). The significant drop in esterifying activity when microsomes were used instead of supernatant fraction from centrifugation at 10,000 *g* and the restoration of esterification by addition of the mixture of ATP and coenzyme A suggest that esterification was eliminated by removing needed cofactor(s) (Table 2). Presumably, formation of esters of methyl sterol intermediates by Bucher homogenates has been undetected because most workers generally saponify flask contents following incubation.

The physiological role of methyl sterol esters is not known. Methyl sterols in both plant and animal tissues generally are esterified to a greater extent than is cholesterol (6, 15–17). Rat skin particularly contains an abundance of esterified methyl sterols (5, 6, 17). When Frantz, Dulit, and Davidson (6) noted the high degree of esterification, they suggested that accumulation of skin sterols other than cholesterol and esterification may be associated. There was a very high relative rate of esterification, more than six times the rate of demethylation, in rat skin (Figs. 2 and 3). Thus, these observations support the suggestion that methyl sterols may accumulate in rat skin and that the high rate of methyl sterol esterification may be metabolically significant. Presumably, the ester is not demethylated in rat skin, just as it is not demethylated in rat liver (Table 4). However, there is no direct evidence that rules out action of an esterase in skin that acts on fatty acyl esters of sterols.

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