notes on methodology

Synthesis of [24,25-^{3}H]cholesterol: a new substrate for determining the rate of cholesterol side chain oxidation

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Summary A procedure for the synthesis of [24,25-^{3}H]cholesterol from the nonradioactive precursor desmosterol is described. The intermediate, isodesmosterol, which was purified by column chromatography, was formed to protect the original double bond (Δ 5–6) from hydrogenation. Tritium was introduced into the side chain by catalytic reduction of the double bond (Δ 24–25) of the isodesmosterol in the presence of carrier-free tritium. After ring rearrangement of the iso-[24,25-^{3}H]cholesterol acetate, the acetate was hydrolyzed to form the free, labeled cholesterol. Hepatic oxidation of the [24,25-^{3}H]cholesterol side chain releases tritium into water which freely equilibrates with cell and body water pools. Thus, the rate of ^3H_2O appearance corresponds to the rate of cholesterol side chain oxidation. Applications of this method to in vivo, isolated perfused liver, and isolated hepatocyte preparations of the rat are discussed.

Supplementary key words isodesmosterol · hepatocytes · isolated perfused liver

Bile acids are the chief oxidative end products of cholesterol catabolism and, therefore, their formation, metabolism, and excretion are of fundamental importance to the problem of the relationship of cholesterol to arterial disease and gallstone formation. Although various pathways have been worked out by observing the conversion of cholesterol and cholesterol metabolites to bile acids, there is little understanding of what actually regulates a number of these distinctly different pathways.

The cholesterol molecule undergoes structural changes in the process of bile acid formation; these can be generalized as either alterations of the steroid nucleus, including hydroxylations, or oxidation of the side chain, resulting in partial cleavage. Concerning these two general groups of reactions, far more has been done and is known about the changes involving the steroid nucleus. For instance, the effects of hormones (1, 2), dietary fat (3), glucose (4), individual bile acids (5), alcohol (6), and phenobarbital (7) have been shown to influence either directly or indirectly the rate of hydroxylation reactions at the steroid nucleus.

In contrast, very little is known concerning what endogenous or exogenous factors might influence the rate of cholesterol side chain oxidation. In fact, investigators are still at odds over whether some of the reactions are primarily mediated by microsomal (8) or mitochondrial (9) enzymes.

After investigating the metabolism of 26-hydroxycholesterol, Danielsson (10), and later Javitt and Emerman (11) and Wachtel, Emerman, and Javitt (12) proposed a hypothesis which states that following initial side chain oxidation, 12α-hydroxylation of cholesterol may be restricted, thus affecting the catabolic pathways and ultimately changing the ratios of the individual bile acids.

Since the bile acid ratios in cholesterol gallstone patients are altered (13) and since it is generally assumed that changes in liver metabolism of cholesterol, bile acids, and phospholipids initiate the formation of cholesterol gallstones (14, 15), it becomes important to be able to study all aspects of cholesterol metabolism to bile acids.

For this reason, a tritium-labeled cholesterol was synthesized to facilitate the study of the effects of endogenous and exogenous factors upon the overall process of cholesterol side chain oxidation in several experimental systems routinely used in our laboratory. This report describes the synthesis and some initial experiments demonstrating the usefulness of this new assay system.

SYNTHESIS OF [24,25-^{3}H]CHOLESTEROL

The procedure described is similar to that published for the conversion of stigmasterol to β-stigmasterol (16) and consists of the following steps (**Fig. 1**).

Desmosterol tosylate

Desmosterol (100 mg, 0.260 mol) mp 121.5°C obtained from Applied Science, Inc. and *p*-toluenesulfonyl chloride (150 mg, 0.787 mol) freshly recrystallized from hexane were added to a reaction "miniactor" vessel (Applied Science, Inc.) and dissolved in 1 ml of pyridine that had been prepared by distillation in the presence of CaH_2. The Teflon-capped miniactor was tightly sealed and left overnight at room temperature in the dark. The entire reaction

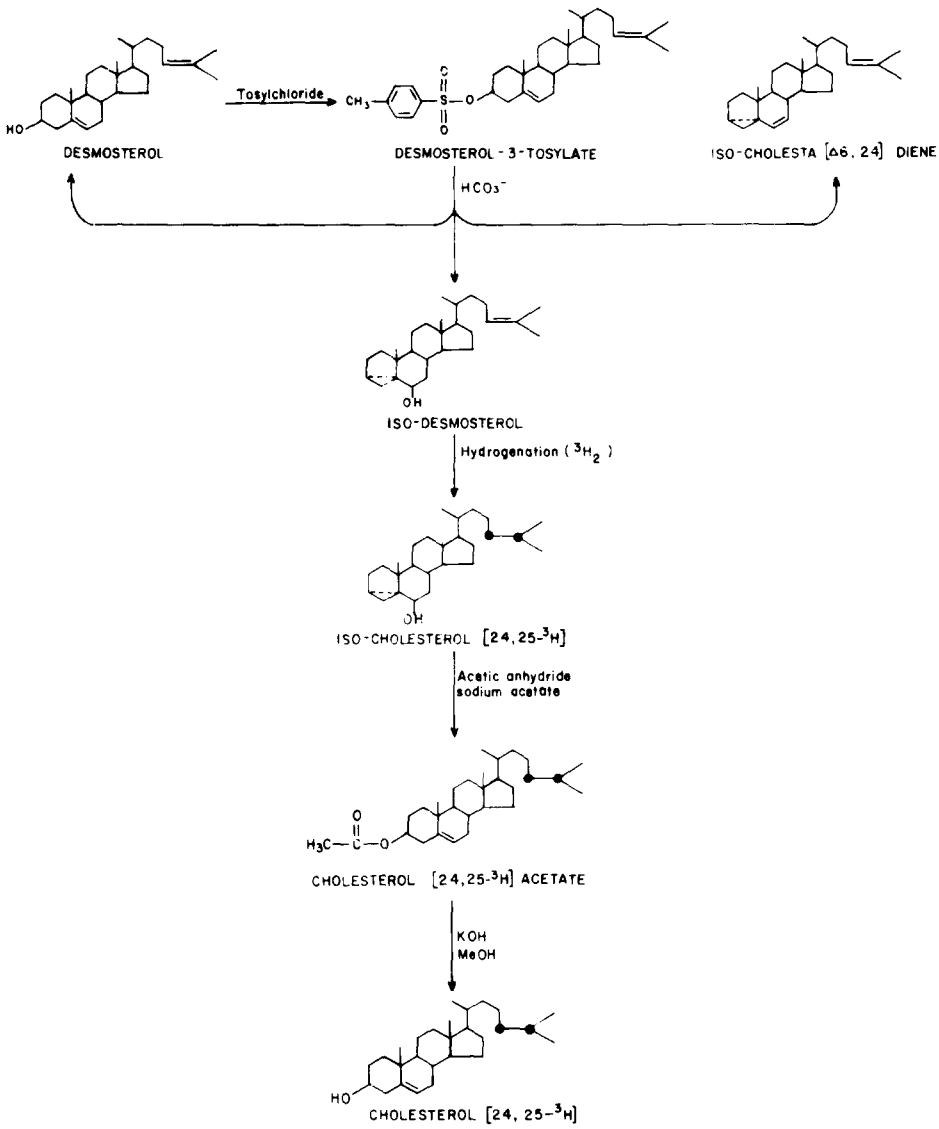

Fig. 1. Synthetic scheme for the preparation of [24,25-^{3}H]cholesterol from desmosterol. Filled circles indicate the position of tritium.

mixture was then poured into 25 ml of ice-cold saturated potassium bicarbonate solution (5%). The precipitated tosylate was then filtered and washed carefully with three consecutive portions of 2 ml of acetone–water 1:1 solution. A small portion of the reaction product was dissolved in ether and developed on a TLC plate (precoated 0.25 mm Silica Gel, E. Merck Ag-EM Laboratories, Inc.) with chloroform as the solvent to identify the products and determine the extent of tosylate formation. The reaction proceeded with nearly quantitative yield as shown by the addition of a trace amount of [26-^{14}C]desmosterol (New England Nuclear) to the reaction medium, resulting in the incorporation of 95% of the label into the spot identified as desmosterol tosylate (**Fig. 2**) (17). There was a small amount of contaminating material remaining at the origin which was not further identified. Recrystallization from acetone gave white crystals which melted at 108–110°C. The yield was 83%.

Isodesmosterol

An aliquot of the purified tosylate (100 mg, 5.46 mmol) was added to a solution composed of potassium bicarbonate (48 mg, 14.52 mmol) dissolved in 3 ml of distilled water and 30 ml of acetone. The solution was refluxed for 6 hr and then evaporated to one-third of the original volume. The remaining solution was diluted with water until no further precipitation was apparent and then extracted with 10 ml of ether three times. The combined ether extracts were washed with 5 ml of water and dried

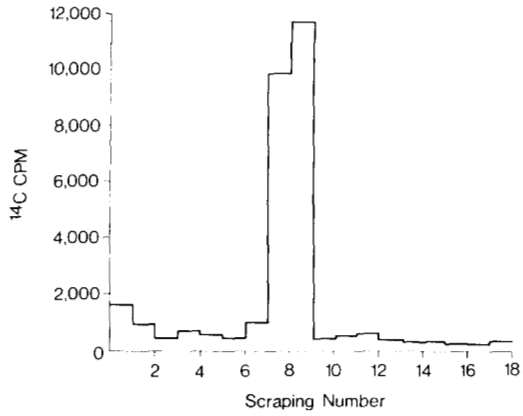

Fig. 2. Identification of the conversion of desmosterol to its tosylate. Ordinate in cm from origin.

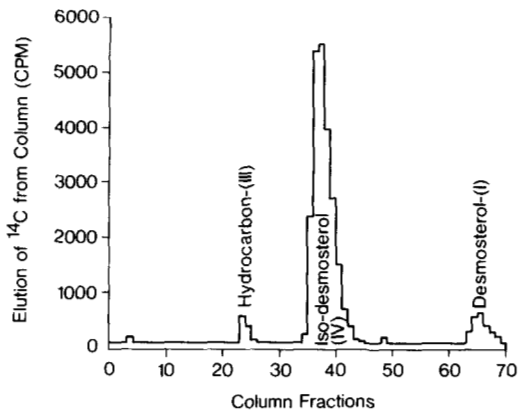

Fig. 4. Elution of [26-^{14}C]desmosterol tosylate solvolysis reaction products from a silicic acid column. Fraction: 1–20, hexane; 21–40, hexane–benzene 1:1; 41–60, hexane–benzene 2:1; 61–80, chloroform. Each fraction represents a 2-ml eluate collection from a 7.5-g silicic acid column.

over 1–2 g of anhydrous potassium carbonate. After decanting the ether, the potassium carbonate was washed two times with wet ether. The pooled portions of ether were then evaporated to dryness, yielding a faint yellow oil that was tentatively identified by TLC in accordance with the work of Steele and Mosettig (16) (**Fig. 3**) as a mixture of 5% desmosterol (I), 85% isodesmosterol (IV), and 10% hydrocarbon (III). The isodesmosterol (IV) was separated and purified by placing the yellow oil dissolved in hexane on a 7.5-g column of silicic acid (minus 325 mesh, Bio Rad Laboratories), and eluting the column as follows: 40 ml of hexane, fractions 1–20; 40 ml of hexane–benzene 1:1, fractions 21–40; 40 ml of hexane–benzene 2:3, fractions 41–60; 40 ml of chloroform, fractions 61–80. **Fig. 4** depicts the elution of the

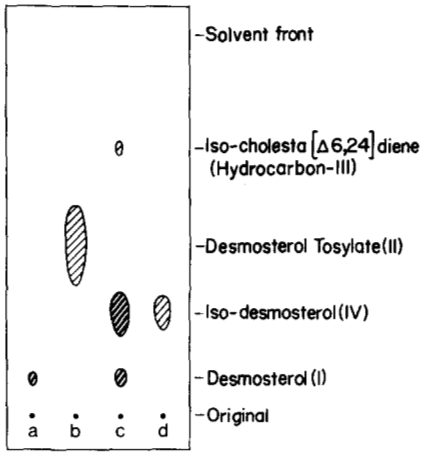

Fig. 3. Thin-layer chromatographic determination and separation of the various intermediate reaction products: *a*) desmosterol, initial starting material; *b*) reaction mixture of desmosterol and *p*-toluene sulfonyl chloride; *c*) solvolysis products of desmosterol tosylate; *d*) eluate fraction from silicic acid column containing isodesmosterol separated from the other solvolysis products. The precoated Silica Gel 60 I TLC plates (0.25 mm) were developed in chloroform.

solvolysis products from the silicic acid column when following the procedure with a tracer portion of [26-^{14}C]desmosterol (New England Nuclear).

An important modification of the Steele and Mosettig (16) procedure was the use of silicic acid instead of Florisil columns. These authors used Florisil, a very inactive absorbent, to prevent the dehydration of isostigmasterol to the hydrocarbon (III). We found that by preconditioning the silicic acid column with successive washings using respective 100-ml portions of water, methanol, and hexane, the separation of the solvolysis products was significantly better than on Florisil and resulted in a minimum of hydrocarbon formation. Further characterization of this hydrocarbon having a melting point 73°C has been carried out by Riegal, Hager, and Zenitz (18). The fractions containing the isodesmosterol were pooled and evaporated to dryness, leaving a colorless oil which on slow crystallization in 10 ml of methanol plus several drops of acetone gave 35 mg of colorless crystals with a melting point of 52–56°C.

[24,25-^{3}H]Isocholesterol

A special 1.5-ml reaction vessel with silicon rubber septum, side arm, and magnetic stirring bar was sent to New England Nuclear (Boston) to be filled with carrier-free tritium gas and then sealed. Upon return to the laboratory, a suspension of 0.5 ml of ethyl acetate and 5% palladium on charcoal (Matheson Coleman and Bell) was injected into the vial. The recrystallized isodesmosterol (IV), after being dissolved in 0.5 ml of ethyl acetate, was also injected into the reaction vessel and allowed to stir overnight. The reaction was then completed by pressurizing the vessel with $^{2}H_2$ followed by further stirring for 5 hr at room temperature. The entire

liquid content was then withdrawn from the vessel, the vial was washed with several portions of ethyl acetate, and this was followed by removal of the catalyst by centrifugation and evaporation of the supernatant to dryness. A small portion of the white residue was taken up in ether and mixed with material that was obtained in a previous run in which hydrogen was used instead of the tritium. For this material we had determined a melting point of 74–75°C after recrystallization from ethanol, with a 60% yield. This compound was first designated as isocholesterol by Wallis, Fernholz, and Gephart (19) and has been further studied (20, 21). A portion of this material was then plotted on silica TLC plates impregnated with $AgNO_3$ (Silica Gel H-ADN, 20% impregnation, Applied Science Laboratories, Inc.). When the plates were developed with chloroform, a single spot was shown to be different from desmosterol, isodesmosterol, cholesterol, or the hydrocarbon. Relative to a desmosterol standard (1.00), the migration values were cholesterol 1.31, isodesmosterol 1.7, isocholesterol 2.10, and hydrocarbon 3.20. A total of 93% of the radioactivity was found in the spot corresponding to [24,25-^{3}H]isocholesterol.

[24,25-^{3}H]Cholesterol acetate

The reaction product, about 25 mg, from the previous step was refluxed with magnetic stirring for 6 hr in 2 ml of acetic anhydride, 0.3 ml of acetic acid, and 45 mg of potassium acetate. The mixture was diluted with 20 ml of water, cooled to 0°C, and approximately 20 mg of [24,25-^{3}H]cholesterol acetate (VI) in the form of white crystals was filtered out. Analogous material obtained in a synthesis in which ^{2}H was used instead of ^{3}H yielded white crystals with a melting point of 115–116°C. This material showed no melting point depression when mixed with commercially available cholesterol acetate and exhibited identical migration in three different TLC solvent systems. To ensure stability of the label and purity of the compound, an equal portion (in mg) of nonlabled cholesterol acetate (99+% purity, Sigma Chemical Co.) was added followed by three consecutive recrystallizations from 5–10 ml of methanol.

[24,25-^{3}H]Cholesterol

Hydrolysis of cholesterol acetate (VI) (30 mg, 7.1 mmol) in 10 ml of 5% methanolic potassium hydroxide yielded 22 mg of [24,25-^{3}H]cholesterol (VII) with a melting point of 148–150°C and a specific activity of 1.41 mCi/mmol. This product was not altered by further recrystallization from methanol.

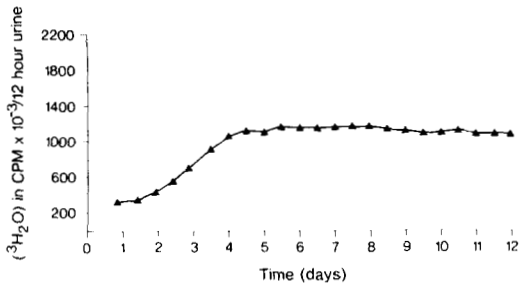

Fig. 5. The in vivo rate of ^3H_2O appearance in the urine of two rats.

BIOLOGICAL EXPERIMENTATION

All animals used were male Sprague-Dawley rats weighing between 250 and 300 g.

In vivo experiments

Two rats were administered the [24,25-^{3}H]cholesterol, 11.15 μCi per 100 g body weight, via the tail vein. After administration of the compound the animals were placed in metabolic cages (Econo Metabolism Unit, Scientific Products) for the entire experimental period, thus allowing collection of all urine and separation from feces. Out of each 12-hr urine sample, 1 ml was taken and washed with an additional ml of water through a mixed-bed resin column of Dowex I (hydroxyl form, dry mesh 200–400, Sigma Chemical Co.) and Dowex 50 W (hydrogen form, dry mesh 200–400, Sigma Chemical Co.) to ensure that all residual counts observed were indeed ^3H_2O separated from radiolabeled cholesterol, and secondly, to remove the urinary salts and color for more efficient liquid scintillation counting. Initial experiments had shown that cholesterol was retained in nonspecific absorption on these columns. In addition, the radioactivity eluted from the columns could be totally distilled in a microdistillation apparatus, giving further proof of cholesterol being retained by the columns.

Fig. 5 shows the average rate of appearance of ^3H_2O in urine over the experimental period for the two rats, the recovery being 31% of the injected dose.

Isolated perfused liver experiments

Livers were removed from rats maintained under light ether anesthesia. The livers were perfused according to the method described by Gonzalez de Goldeano, Bressler, and Brendel (22) with minor modifications. The first perfusion was done solely to demonstrate the rate of uptake of 0.2 μCi of [24,25-^{3}H]cholesterol complexed to fatty acid-free

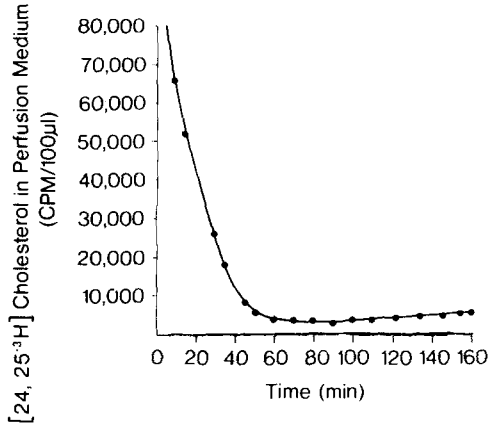

Fig. 6. Uptake of [24,25-^{3}H]cholesterol by the isolated perfused rat liver.

albumin (Sigma Chemical Company) by the liver (**Fig. 6**). Total counts were plotted over time. In subsequent liver perfusions, 1 ml of perfusion medium was collected at 15–30 min intervals, washed through a mixed Dowex column, as described above, and counted for ^3H_2O. The columns were tested for their ability to retain cholesterol bound to albumin as it appears in this system. The columns were found to totally absorb the labeled cholesterol at zero time as well as after reexcretion by the liver following buffer exchange. **Fig. 7** shows the rate of appearance of ^3H_2O in the perfusate for a 4-hr perfusion. The viability of isolated perfused livers for the period of time used in these experiments and the ability to take up and catabolize cholesterol to bile acids is well documented (23, 24).

Isolated suspended rat liver cell experiments

Isolated liver cells were obtained by the method of Hayes and Brendel (25). To each vial of suspended liver cells 1.8 µCi of labeled cholesterol was added at zero incubation time. At hourly intervals, 0.5 ml

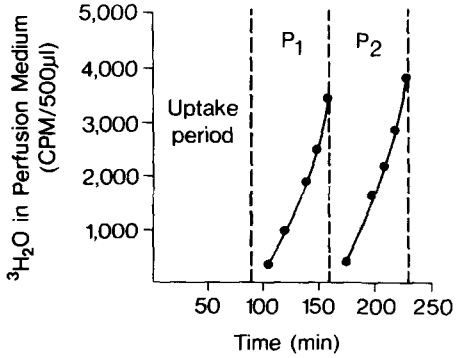

Fig. 7. The production of ^3H_2O from [24,25-^{3}H]cholesterol by the isolated perfused liver. Perfusion period 1 (P_1) and perfusion period 2 (P_2) were separated by a buffer washout (300 ml).

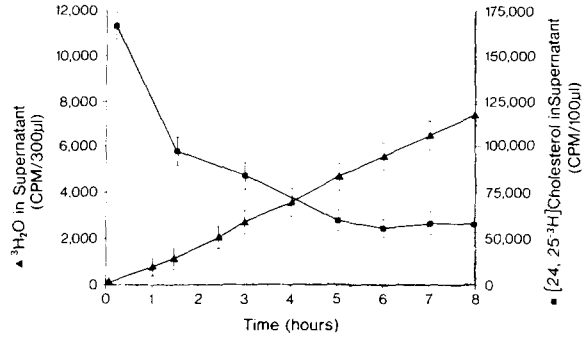

Fig. 8. The rate of [24,25-^{3}H]cholesterol uptake and ^3H_2O appearance in isolated suspended hepatocytes.

of suspended cells were centrifuged gently for 15 sec in a Beckman Microfuge to form a pellet, after which 300 µl of the supernatant was placed on a charcoal column and washed with 2 ml of water. This constitutes an alternative way of separating cholesterol from ^3H_2O in cases where there are relatively high levels of protein-bound radiolabeled cholesterol in the incubation medium. Samples from the columns were periodically injected into the microdistillation unit to ensure the efficiency of the charcoal in removing the radiolabeled cholesterol from produced ^3H_2O present in the incubation medium. The uptake of [24,25-^{3}H]cholesterol by the cells and appearance of tritiated water over an 8-hr incubation period are shown in **Fig. 8.**

[4-^{14}C]- and [24,25-^{3}H]Cholesterol metabolism

To establish whether identical distribution and metabolism of ^{14}C-ring-labeled cholesterol and the 24,25-^{3}H-side chain-labeled cholesterol occurred, an experiment in isolated liver cells was performed. A solution of 0.5 µCi of [4-^{14}C]cholesterol bound to albumin was added to a suspension of 10^8 hepatocytes in 25 ml of incubation buffer. A sonicated sample of this solution was adjusted to pH 10 and repeatedly extracted with ether. The ether extracts contained more than 99% of the radioactivity and the ^{14}C/^{3}H ratio was established at this time to be near 0.8. After 4 hr of incubation, another sample was treated in identical fashion and shown to have the same ^{14}C/^{3}H ratio in the ether extract as at zero time. In addition, the alkaline aqueous phase now contained approximately 4% of the overall radioactivity in the form of cholesterol metabolites, that is, ^3H_2O and 4-^{14}C-labeled bile acids. The ^{14}C/^{3}H ratio in the aqueous phase was identical to that in the organic extracts, which shows that the two cholesterol tracers are metabolized at the same rate and in the same manner.

DISCUSSION

The synthesis of [24,25-^{3}H]cholesterol has provided a unique and simplified method for determining the rate of cholesterol side chain oxidation in a variety of experimental systems. The intact rat, isolated perfused rat liver, and isolated suspended hepatocytes have been employed to test the usefulness of this method.

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