The relationship between the rate of hepatic sterol synthesis and the incorporation of [^{3}H]water

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Abstract The true rate of sterol synthesis in liver cells was determined by measurement of the weight of desmosterol produced over a given time period during incubations in the presence of triparanol. The simultaneous presence of tritiated water (^3H_2O) during the incubations permitted a direct observation of the weight of tritium incorporated into a given mass of newly synthesized sterol. The incorporation of tritium per atom of sterol carbon (H/C ratio) was lower than some previously reported values and suggests that a sizeable proportion of the reducing equivalents (NADPH) required for sterol synthesis arises via the pentose phosphate pathway. The H/C ratio changed significantly with length of the incubation period. The value of the ratio was also dependent upon whether the acetyl-CoA units utilized for sterol synthesis were derived predominantly from a carbohydrate or a fatty acid source.—**Pullinger, C. R., and G. F. Gibbons.** The relationship between the rate of hepatic sterol synthesis and the incorporation of [^{3}H]water. *J. Lipid Res.* 1983. **24:** 1321–1328.

Supplementary key words glycogenolysis • β-oxidation • NADPH • pentose phosphate • acetyl-CoA • precursor pools • hepatocytes

There are several major drawbacks to the use of labeled acetate in measuring the rate of sterol biosynthesis and these have been well documented (1–4). One of the most serious defects of the method is the unknown extent to which the labeled acetyl-CoA produced from the exogenous material is diluted by that produced from endogenous sources. The use of tritiated water (^3H_2O) was originally introduced to overcome this difficulty in the measurement of fatty acid synthesis (5–10) and has more recently been applied to the measurement of rates of cholesterogenesis in vitro and in vivo (2, 4, 11–15). In these cases the specific radioactivity of the cholesterol precursor is known with certainty, being that of the water in the incubation medium. However, despite several investigations (2, 4, 11, 13, 15), there is no general agreement as to the exact number of ^{3}H atoms incorporated per molecule of cholesterol synthesized. Another disadvantage of this technique is that in some cases, particularly with cells in tissue culture dishes, the large quantities of radioactivity required present considerable technical difficulties in order to avoid atmospheric contamination with ^3H_2O.

The effective specific radioactivity of the acetyl-CoA pool used for cholesterol biosynthesis has also been estimated by labeling it from [^{14}C]acetyl-CoA which is produced in such large quantities from exogenous [^{14}C]octanoate that further dilution from endogenous sources is considered negligible (1, 4, 16). A drawback to this approach is that octanoate is not a natural metabolite and bypasses the normal metabolic control mechanisms by which acetyl-CoA is made available for cholesterol synthesis. This may give rise to an abnormally high availability of substrate which may not accurately reflect the normal physiological situation.

The present report describes a chromatographic method by which the mass of newly synthesized sterol is measured directly. Simultaneous measurement of tritium incorporation during sterol synthesis in the presence of ^3H_2O permits, for the first time, a direct measurement of the number of tritium atoms incorporated per molecule of sterol synthesized. In rat hepatocytes, this ratio varies somewhat according to the experimental conditions employed. Application of these ratios in strictly controlled in vitro and in vivo studies should permit a more accurate assessment of the rate of sterol production than has hitherto been possible.

MATERIALS AND METHODS

Materials

Alumina G (containing 10% gypsum by weight) was obtained from M. Woelm (Eschwage, Germany). 'Analar' grade silver nitrate was obtained from BDH Chemicals Ltd. (Poole, Dorset, England). Solvents were of the highest purity grade available and were purchased from BDH Chemicals Ltd. and also from May and Baker Ltd. (Dagenham, Essex, England).

Abbreviations: GLC, gas–liquid chromatography; HFBA, heptafluorobutyric anhydride.

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All solvents were distilled before use. In particular, diethyl ether (nominally peroxide-free) was distilled over reduced iron powder using a water bath at 60°C. Any remaining peroxides were removed by passing the distillate through a column of ethanol-washed Alumina grade I (M. Woelm) immediately before use. Triparanol [2-(4-chlorophenyl)-1-(4-diethylaminoethoxyphenyl)-1-*p*-tolylethanol] was a gift from Dr. B. Morgan (Beecham Research Division, Epsom, Surrey, England) and was dissolved in redistilled propane-1,2-diol (BDH Chemicals Ltd.) to give a concentration of 0.44 mg/ml. This solution was added to hepatocyte suspensions (2 μl/ml) to give a triparanol concentration of 2 μM in the incubation mixture. Desmosterol was obtained from various commercial sources and most batches were very impure when received in the laboratory. Pure desmosterol was obtained by chromatography of the impure material on silica gel H using chloroform as the developing solvent (System 1). After elution, the resulting sterol mixture was acetylated and chromatographed on thin-layer plates of silica gel H impregnated with silver nitrate (17) using toluene–hexane 2:1 (v/v) as the developing solvent mixture. After elution of the band (R_f 0.31) containing desmosteryl acetate, pure demosterol was obtained after hydrolysis followed by rechromatography on silver nitrate-impregnated plates of silica gel H developed with toluene–ethyl acetate 7:3 (v/v). After recrystallization from methanol–chloroform 6:1 (v/v), the crystals melted at 122–124°C [reported mp 121°C (18)] and showed only one peak on several GLC stationary phases. [26,27-^{14}C]Desmosterol (53.0 mCi/mmol) and [4-^{14}C]cholesterol (58.4 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., England). The source and preparation of all other materials were as described previously (3, 19).

Treatment of animals, preparation of liver cells, and incubation procedures

Rats were allowed unrestricted access to a commercial pellet diet (Formula PRD, Labsure, Poole, Dorset, England). All other procedures were carried out as described previously (3, 19), except that during the cell preparation EGTA (0.5 mM) was included in the perfusion mixture before addition of collagenase (20). The isolated cells were suspended in 16 volumes of Krebs-Henseleit bicarbonate buffer containing Ca^{2+} (2.6 mM) and fatty acid-free bovine serum albumin (1.1%, w/v). Before incubation, glucose (11.1 mM) and amino acids (21) were added. This was the standard incubation medium and contained 4–6 × 10^6 cells/ml. Triparanol (to give a concentration of 2 μM) and ^3H_2O were added immediately before incubation.

Confirmation of the identity of desmosterol biosynthesized in the presence of triparanol

Preliminary experiments have been carried out to determine the quantitative and qualitative effects of triparanol on sterol biosynthesis in hepatocytes (3). Thus the major sterol labeled from [^{14}C]acetate in the presence of triparanol behaved in a manner identical to desmosterol in three separate thin-layer chromatographic systems and on three separate stationary phases (XE-60, QF-1, and OV-225) during GLC. Further evidence was obtained from the constancy of the specific radioactivity during successive recrystallizations and from the similarity of the mass spectrum of the acetate derivative with a sample of authentic desmosteryl acetate (3).

Purification and estimation of biosynthetic desmosterol

At the end of incubations in the presence of triparanol, an aliquot of the cell suspension (1.0 ml) was transferred to an ice-cold conical tube fitted with a ground-glass stopper. The cells were isolated by centrifugation at 80 *g* for 1.5 min and the supernatant was discarded. The cell pellet was washed by resuspension in albumin-free Krebs-Henseleit buffer followed by recentrifugation at 80 *g*. The washed pellet was resuspended in a solution of Triton X-100 (0.125%) in potassium phosphate buffer, pH 7.5 (1.5 ml). A solution of [26,27-^{14}C]desmosterol (12,200 dpm) in ethanol (40 μl) was added as internal standard and the solution was heated to 90°C for 1 min. After cooling, cholesterol oxidase (0.25 units) and cholesterol esterase (0.20 units) were added and the mixture was incubated at 37°C for 0.5 hr. Under these conditions, the relatively small quantity of newly synthesized steryl esters was completely hydrolyzed and the resulting desmosterol, together with the original newly synthesized nonesterified desmosterol, was converted into desmostenone (cholesta-4,24-dien-3-one, **Fig. 1**). Simultaneously, endogenous cellular cholesterol and cholesteryl ester were converted into cholestenone (cholest-4-en-3-one). To remove di- and triacylglycerols, which interfere with the assay, the enzyme-treated mixture was saponified by addition of 3.0 ml of a solution consisting of potassium hydroxide (7.5 g) dissolved in water (7.5 ml) and made up to 100 ml with ethanol. The mixture was heated to 60°C for 0.25 hr. The hydrolysate was diluted with water (10.0 ml) and the solution was saturated with sodium sulfate. The nonsaponifiable lipid fraction (containing desmostenone and cholestenone) was extracted with two 10-ml portions of hexane. The combined hexane extracts were washed with three 10-ml portions of distilled water and evaporated to dryness. The nonsaponifiable lipid mix-

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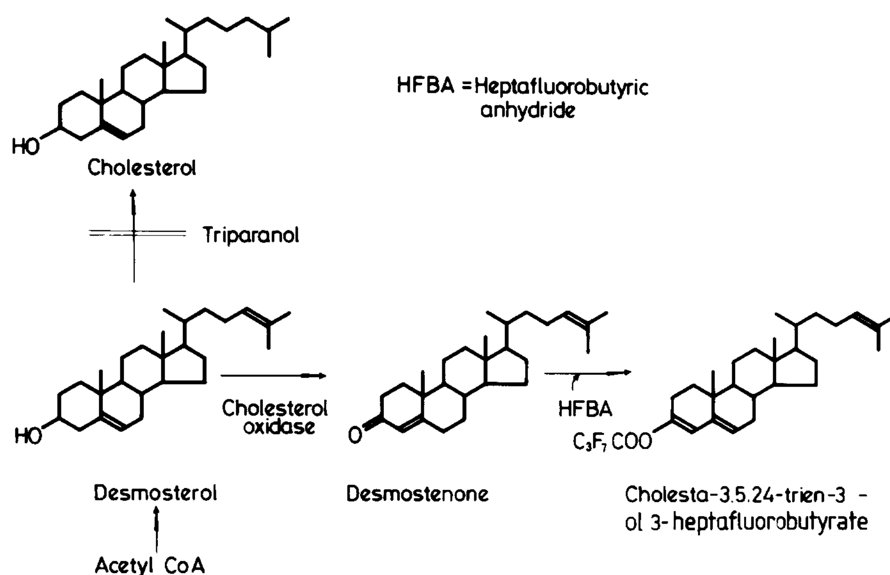

Fig. 1. Formation of an electron-capturing derivative of desmosterol.

ture was chromatographed on thin-layer plates of alumina impregnated with silver nitrate (17) developed with toluene–hexane 4:1 (v/v) at 4°C. Each plate contained five samples. This procedure separated the small quantities of desmostenone (R_f 0.20) from the much larger quantities of cholestenone (R_f 0.32) derived from the endogenous cellular cholesterol. This procedure failed to completely separate desmostenone from cholesta-4,7-dien-3-one which was produced during enzymic oxidation of cholesta-5,7-dien-3β-ol (provitamin D_3) and which was present in rat liver at a concentration of approximately 2.8 nmol/mg protein.[1] Since this compound has a similar retention time to the desmostenone derivative on GLC, it was removed by irradiation (see below). The desmostenone-containing band was eluted with peroxide-free ether (30 ml) into a conical tube. After evaporation of the ether the heptafluorobutyryl enol ester (Fig. 1) was prepared as follows. The residue was dissolved in toluene (1.0 ml). An aliquot of the solution was transferred to a small transparent glass vial (0.8 ml, Anchor Glass Co. Ltd., London, England) containing suitable quantities (25–2000 ng) of stigmastadienone as an internal standard. After evaporation of the solvent the residue was dissolved in toluene (50 μl) and heptafluorobutyric anhydride (HFBA; 50 μl) was added. The tubes were flushed with nitrogen, capped, and heated at 60°C for 0.25 hr. Excess reagent was evaporated under nitrogen at room temperature. Under these conditions toluene and HFBA form two phases and it was important, during solvent removal, to ensure that at least some toluene was always present while the HFBA was being removed. This was done by constant addition of small quantities of toluene during the evaporation process. The residue was dissolved in a suitable volume of heptane (100–600 μl). This solution also contained cholesta-3,5,7-trien-3-ol 3-heptafluorobutyrate obtained by esterification of endogenous provitamin D_3 (see above). The triene was removed by exposing the heptane solution to direct sunlight or to ultraviolet irradiation (210 nm) for 2 hr. This procedure converted the triene into the 9,10-seco-derivative.

The weight of the fluorinated desmosterol derivative (cholesta-3,5,24-trien-3-ol 3-heptafluorobutyrate; Fig. 1) was determined by GLC using a Pye 204 gas chromatograph fitted with a pulsed ^{63}Ni electron capture detector and a glass column of dimensions 1.5 m $\times$ 3 mm (i.d.). The stationary phase was 3% OV-225 coated on Chromosorb WHP (80–100 mesh). The operating conditions were: argon flow rate, 65 ml/min; column temperature, 213°C; injector and detector temperatures, 250°C. Under these conditions the relative retention times (cholesta-3,5-dien-3-ol 3-heptafluorobutyrate = 1) of the fluorinated desmostenone and stigmastadienone derivatives were 1.26 and 1.45, respectively. The weight of the desmosterol derivative present was determined from the ratio of the peak heights of the fluorinated desmostenone and stigmastadienone derivatives and from the known quantity of stigmastadienone added before esterification. Preparation of standard mixtures of known desmostenone: stigmastadienone ratios showed that, after esterifica-

[1] Pullinger, C. R., and G. F. Gibbons. Unpublished observation.

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tion, the peak height ratio was linearly related to the mass ratio over a wide range (**Fig. 2**). In most cases samples were injected automatically using a Pye-Unicam S8 Autojector. After determination of the mass of desmostenone derivative present, the heptane solution was transferred to a scintillation vial. The recovery of the ^{14}C-labeled desmostenone was then determined by scintillation counting and used to determine losses during the extraction and purification procedure.

The above procedure was also carried out with non-incubated liver cell suspensions (zero-time controls). The very small quantity of material with the same relative retention time as the desmosterol derivative (usually <10 ng) was subtracted from the values obtained for the incubated cells.

Measurement of rates of fatty acid synthesis

The incorporation of ^3H from ^3H_2O into cellular fatty acids was determined as follows. To the aqueous phase after extraction of the nonsaponifiable lipid was added [1-^{14}C]oleate (17,500 dpm) as internal standard and the solution was adjusted to pH 1.0 by the addition of concentrated hydrochloric acid. The fatty acid fraction was extracted using 10 ml of hexane which was then washed with four 10-ml portions of distilled water. Aliquots of the hexane extract were transferred to scintillation vials for measurement of ^{14}C and ^3H radioactivity. Losses of ^3H during extraction were accounted for by the known recovery of [^{14}C]oleate.

RESULTS AND DISCUSSION

Effect of triparanol on sterol and fatty acid synthesis

To determine whether triparanol at the concentration used (2 μM) had any effect on cholesterol synthesis other than to cause an accumulation of desmosterol, and to determine its effects on fatty acid synthesis, hepatocyte suspensions were incubated for various periods of time in the presence of ^3H_2O and either a solution of triparanol in propanediol or propanediol alone. The sterol fractions were extracted and purified as described above and their ^3H radioactivities were determined. The results are presented in **Fig. 3** which shows that triparanol had no effect on the incorporation of ^3H_2O into the total biosynthetic sterol. In several similar experiments after 3 hr incubation, the incorporation of label into the total biosynthetic sterol in the presence of triparanol was 91.8% ± 5.6 (SEM, n = 5) of that observed in its absence. However, in the presence of triparanol, most of this label (>90%) was associated with desmosterol. At higher concentrations of triparanol (30

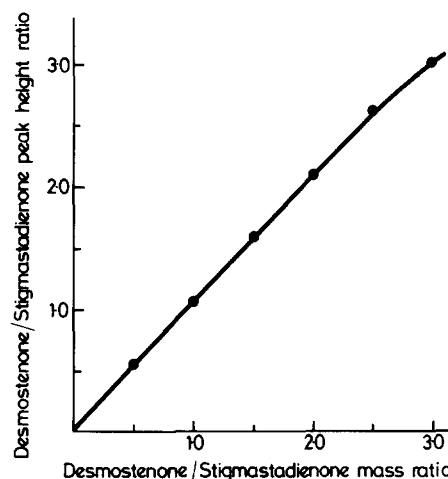

Fig. 2. Response of the ^{63}Ni detector to mixtures of the heptafluorobutyryl enol ester derivatives of desmostenone and stigmastadienone. Desmostenone and stigmastadienone were mixed to give ratios varying from 0.5 to 3.0. The components of each mixture were converted into their heptafluorobutyryl enol ester derivatives and aliquots were assayed by gas chromatography.

μM), it has been reported that squalene-2,3-oxide accumulates in rat liver cells (22). At the much lower concentrations (2 μM) used in the present work, the close similarity in the incorporation of label into total C_{27} sterols in the presence and absence of triparanol (Fig. 3) suggested that this was not the case. The rates of incorporation of ^3H_2O into fatty acids were also determined in the same incubations. **Fig. 4** shows that triparanol had no significant effect on the rate of lipogenesis as measured by ^3H_2O incorporation.

In view of the fact that triparanol at a concentration of 2 μM did not completely inhibit cholesterol synthesis (Fig. 3), measurement of the weight of desmosterol alone underestimates the total weight of sterol produced by approximately 10%. This was the case irrespective of whether the cells alone or the cells plus the medium were analyzed for sterol (3), suggesting that there is no secretion of newly synthesized cholesterol into the medium in preference to newly synthesized desmosterol. This extra weight of sterol produced may be calculated by inclusion of a ^3H-labeled precursor (e.g., ^3H_2O (Fig. 3) or [^3H]mevalonic acid) in the incubation medium and dividing the radioactivity of the cholesterol (cholestenone) fraction by the specific radioactivity of the desmosterol. In practice, however, because the relative amount of cholesterol is constant and small, the weight of desmosterol alone was used as a measurement of the total rate of sterol synthesis.

Assessment of the accuracy of desmosterol determination

The accuracy of the method was determined by measuring the recovery of various known quantities of des-

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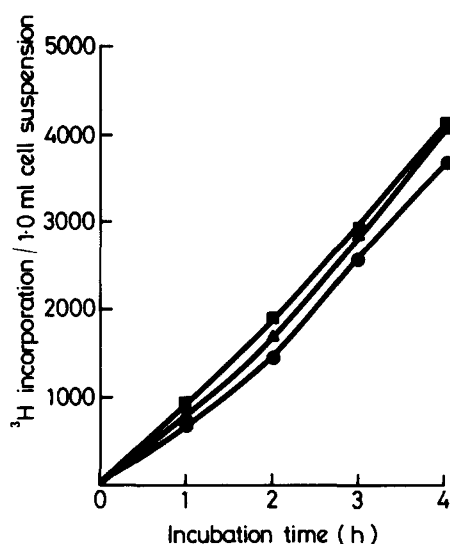

Fig. 3. Incorporation of ^3H_2O into sterols in the presence and absence of triparanol. Hepatocyte suspensions (15.0 ml, 4.56 × 10^6 cells/ml; 7.2 mg protein/ml) were incubated in the standard medium containing ^3H_2O (87.6 dpm/nmol). To one set of flasks was added a solution of triparanol in propanediol, to the other was added propanediol only. All flasks were incubated at 37°C for 4 hr. At hourly intervals 1.0-ml aliquots of the suspensions were removed and the incorporation of ^3H_2O into cholesterol and desmosterol (isolated as cholestenone and desmostenone, respectively as described in the Methods) was determined after the addition of [4-^{14}C]cholesterol (6640 dpm) and [26,27-^{14}C]desmosterol (7150 dpm) as internal standards. The value for each time point represents the average of duplicate incubations. ● —— ●, Desmosterol radioactivity (triparanol present); ▲ —— ▲, desmosterol + cholesterol radioactivity (triparanol present); ■ —— ■, cholesterol radioactivity (triparanol absent).

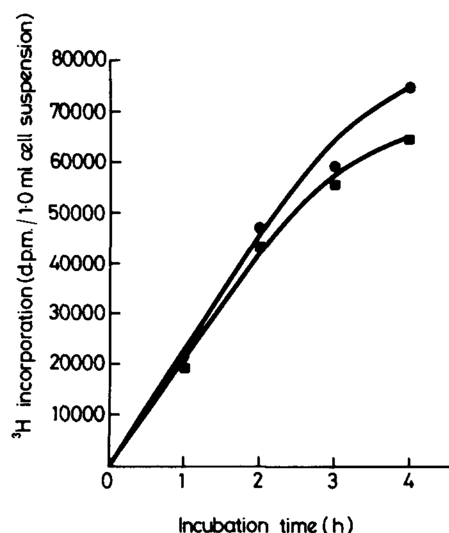

Fig. 4. Effect of triparanol on ^3H_2O incorporation into fatty acids. The incubation conditions were identical to those described in the legend to Fig. 3. The aqueous phase obtained after extraction of the nonsaponifiable lipid fraction was acidified to pH 1 and the fatty acids were extracted and assayed for ^{3}H radioactivity. ● —— ●, Triparanol present; ■ —— ■, triparanol absent.

mosterol added to liver cell preparations. Thus desmosterol (six samples ranging from 50 to 6000 ng) was added to aliquots of freshly prepared hepatocytes and after extraction and derivatization, the recoveries of desmosterol were determined as described above. The results are presented in **Table 1** which shows that the method is capable of accurately measuring quantities of desmosterol down to 50 ng.

Relationship between ^3H_2O incorporation into sterol and the weight of sterol synthesized

There have been several attempts to define the relationship between the incorporation of tritium and carbon into cholesterol during its biosynthesis in the presence of ^3H_2O (2, 4, 11, 13, 15). Although different approaches were used in these studies, in all cases estimation of the weight of sterol produced ultimately relied upon using the specific radioactivity of a ^{14}C-labeled sterol precursor to calculate the specific radioactivity of the carbon pool from which cholesterol was derived. These studies have not led to any general agreement as to the number of tritium atoms incorporated per atom of carbon utilized for cholesterol synthesis and estimates vary from values as low as 0.35 and 0.54 μg atom of tritium per μg atom of carbon (the H/C ratio) (13, 15) to those in which the H/C ratio is as high as 0.76 and 0.89 (11, 14). The approach used in the present work permits a straightforward determination of the number of g-atoms of tritium incorporated into a given quantity of newly synthesized sterol, the weight of which is measured directly. There is thus no need for the assumption inherent in calculations based upon the specific radioactivity of ^{14}C-labeled precursors.

Hepatocytes were incubated for various periods of time in the presence of ^3H_2O and at the end of each period the weight of sterol synthesized (weight of carbon incorporated) was compared to the incorporation

TABLE 1. Recovery of various quantities of desmosterol from rat hepatocytes

Desmosterol Added	Desmosterol Recovered	Average Error
ng	*ng*	%
50	45 ± 3.5[a]	−10.0
150	156 ± 13.4	+4.0
400	390 ± 7.1	−2.5
800	792 ± 31.8	−1.0
1600	1618 ± 61.7	+1.0
6000	5975 ± 207	−0.4

Various quantities of desmosterol were added to aliquots of freshly prepared hepatocytes. [26,27-^{14}C]Desmosterol (12,200 dpm) was added in each case and the recovery of demosterol was determined.
[a] Each value represents the average ± SEM of quadruplicate determinations.

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TABLE 2. Relationship between sterol synthesized and ^3H_2O incorporation

Incubation Time	nmol ^3H_2O/ nmol Desmosterol	ng-atom H/ng-atom C (Desmosterol)	ng-atom H/ng-atom C (Cholesterol)
hr			
2	6.235 ± 0.261	0.461 ± 0.019	0.562 ± 0.023
3	7.172 ± 0.219	0.531 ± 0.016	0.649 ± 0.020
4	7.900 ± 0.184	0.585 ± 0.014	0.715 ± 0.017
2 hr + Acetate (6 mM)	5.929 ± 0.240	0.439 ± 0.017	0.536 ± 0.021

Hepatocytes were incubated in the standard medium (15.0 ml) containing triparanol (2 μM) and ^3H_2O (33.1 mCi). At the times shown, aliquots of the suspensions were removed and the weight and ^3H radioactivity of the newly biosynthesized desmosterol were determined. These values were used to calculate the results shown in column 2. The values in the third column were derived by multiplying the values in column 2 by two to give ng-atom of ^3H incorporated and dividing the product by 27 to give the number of ng-atoms per ng-atom of carbon incorporated into desmosterol. These latter values represent only 18 of the 22 H atoms incorporated during cholesterol biosynthesis from acetyl-CoA. The remaining 4 H atoms correspond to the 2 H atoms incorporated during reduction of the Δ^{24} bond of desmosterol and 2 H atoms removed from carbons 3 and 4 during formation of the 4-en-3-one derivative (Fig. 1). The values in column 4 account for this and were derived by multiplying the values in column 3 by 22/18. All the above values are the mean ± SEM for determinations on hepatocytes from nine animals. Statistical analysis by a paired Student's *t*-test showed that there was a significant difference in the H/C ratio after the third hour ($P < 0.02$) and the fourth hour ($P < 0.001$) compared to that observed after the second hour. The difference between the third and fourth hours of incubation were also significant ($P < 0.02$). The presence of acetate (6 mM) during the first 2 hr had no significant effect on the H/C ratio.

of ^3H. **Table 2** shows that the H/C ratio was not constant over the whole of the incubation period and increased gradually but significantly with time. Tritium enters the sterol molecule during its reductive synthesis from acetyl-CoA either directly from ^3H_2O or from NADPH which becomes labeled during the course of the incubation. Lakshmanan and Veech (13) have pointed out that the source of the NADPH determines whether it is labeled or not; that arising from the pentose phosphate pathway being unlabeled whilst that derived from other pathways has equilibrated with ^3H_2O. It has been calculated (4) that if all the NADPH used for sterol synthesis is derived via the former pathway (i.e., unlabeled) then the H/C ratio would be 0.26; if derived exclusively from other pathways, then every one of the 22 H atoms incorporated between acetyl-CoA and cholesterol would be labeled and a ratio of 0.81 (i.e., 22/27) would result. The very large difference between these two extreme values highlights the importance of ensuring that the source of reducing equivalents remains constant when using ^3H_2O incorporation to compare the rates of sterol synthesis under different physiological conditions.

The results observed in the present work show that after 2 hr incubation in the presence of ^3H_2O, the average H/C ratio in desmostenone (derived from the biosynthetic desmosterol) was 0.461. Since this material contains only 18 H atoms incorporated per mol of sterol synthesized (13; see legend to Table 2), if all these H atoms, including those derived from NADPH, were labeled, then the H/C ratio would have been 0.667 (i.e., 18/27). The lower observed ratio suggests that during a 2-hr incubation, unlabeled NADPH produced via the pentose phosphate pathway contributes a substantial proportion of the total NADPH utilized for sterol synthesis. Andersen and Dietschy (4) report an H/C ratio of 0.89 for cholesterol, a value obtained after incubating liver slices in ^3H_2O for a similar period of time, and they conclude that all the NADPH used for sterol synthesized in the presence of ^3H_2O is labeled. This situation could arise only if either the pentose phosphate pathway was completely inactive, an unreasonable proposition in view of reports that about 50% of the NADPH utilized for fatty acid synthesis is derived from this source (13, 23, 24), or if the NADPH used for sterol synthesis is compartmentalized. The fact that the value reported by these authors (H/C = 0.89) is higher than the theoretical maximum (i.e., H/C = 0.81) suggests that a more plausible explanation for the high ratio observed in this study is that acetyl-CoA derived from the exogenous octanoate used as the cholesterol precursor was itself labeled owing to incorporation of ^3H_2O during the enoyl-CoA hydratase stage of the β-oxidation process. Evidence that this was the case was obtained in the present work in which the ratio was determined during sterol biosynthesis in hepatocytes incubated in the presence or absence of exogenous oleate. Previous studies have shown that, in vitro, most of the hepatic sterol carbon is probably derived from acetyl-CoA produced by glycogenolysis (19, 25) whilst in the presence of ex-

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TABLE 3. Effect of oleate on the H/C ratio of newly synthesized sterol in hepatocytes from normal and starved animals

Incubation Time	Cholesterol ng-atom H/ng-atom C			
	Normal Animals		24-hr-Starved Animals	
	Oleate Absent	Oleate Present	Oleate Absent	Oleate Present
hr				
2	0.645 ± 0.060	0.854 ± 0.048	0.629 ± 0.057	
3	0.657 ± 0.057	0.860 ± 0.055	0.697 ± 0.038	0.877 ± 0.054
4	0.701 ± 0.053	0.876 ± 0.045	0.733 ± 0.051	

Hepatocytes were derived from normal animals or from animals that had been starved for 24 hr. Incubations were conducted in the standard medium (15.0 ml) containing triparanol (2 μM) and ^3H_2O (50.3 mCi). The H/C ratio was determined as described in the legend to Table 2. The above values represent the mean ± SEM for determinations on hepatocytes from four animals. Statistical analysis by a paired Student's *t*-test showed that oleate produced a significant ($P < 0.05$) increase in the H/C ratio at all time intervals in hepatocytes from both the normal and 24-hr-starved animals. There was no significant difference in the H/C ratio of sterol synthesized by hepatocytes from starved animals compared to that observed in hepatocytes from normal animals.

ogenous fatty acid, acetyl-CoA for sterol production is derived predominantly via the β-oxidation pathway (26). **Table 3** shows that during each incubation period in the presence of oleate, the average value for the H/C ratio in the newly synthesized sterol was 25–30% higher than that observed in the absence of the fatty acid. This was the case irrespective of whether the liver cells were derived from normally fed or 24-hr-starved animals. After 3 hr in the presence and absence of oleate, liver cells from the starved animals had synthesized 0.30 ± 0.01 and 0.29 ± 0.03 nmol desmosterol per mg protein, respectively. The corresponding figures for the fed animals were 1.54 ± 0.35 and 2.02 ± 0.34 nmol/mg protein, respectively. Despite this difference in the rate of synthesis, there was no significant difference in the H/C ratio observed in sterol synthesized in liver cells from fed or starved animals incubated in the absence of oleate. The constancy of the $^3H/^{14}C$ ratio after rechromatography of the isolated desmostenone in a different TLC system (silica gel H, chloroform) provided evidence for the radiochemical purity of the tritium-labeled steroid (**Table 4**).

TABLE 4. Radiochemical purity of desmostenone labeled biosynthetically from ^3H_2O

Thin-layer Chromatography System	$^3H/^{14}C$ Ratio in Desmostenone	
	Oleate Absent	Oleate Present
Alumina/silver nitrate	1.483	1.539
Silica gel H	1.499	1.540

[26,27-^{14}C]Desmosterol (12,200 dpm) was added to hepatocytes after incubation in the standard medium containing triparanol (2 μM) and ^3H_2O (50.3 mCi). Where applicable, oleate was present at a concentration of 2 mM. The doubly labeled desmostenone was isolated by argentation chromatography as described in Materials and Methods and a portion was removed for determination of the $^3H/^{14}C$ ratio. The remainder was rechromatographed on a thin-layer plate of silica gel H developed with chloroform. After elution of the desmostenone-containing band, the $^3H/^{14}C$ ratio was again determined.

The increase in the H/C ratio observed with increasing incubation time (Table 2) may be explained by a gradual decrease in the contribution of NADPH derived from the pentose phosphate pathway. Alternatively, either there may be a relatively large pool of unlabeled NADPH initially present that takes some time to reach a constant specific radioactivity, or during the early stages of the incubation a part of the carbon incorporated into cholesterol is derived from a relatively unlabeled pool of post-MVA metabolites. The time-dependence of the sterol H/C ratio emphasizes the importance of strictly standardizing incubation conditions when using ^3H_2O incorporation as a measure of the rate of sterol synthesis in vitro and, probably, in vivo. Although this may make little difference when rates of sterol synthesis differ by an order of magnitude or more, it may affect conclusions drawn from results in which the true rates differ by smaller amounts.

In cells other than hepatocytes, triparanol may cause an accumulation of cholesterol precursors other than desmosterol. However, this need not necessarily preclude the use of the present method for determining the rate of sterol production. In these cases, in the steady state, the specific radioactivity of desmosterol biosynthesized in the presence of labeled substrate is identical to that of the other labeled precursor sterols. This value may be used to calculate the mass of non-desmosterol sterols. This approach has been used successfully to calculate the rate of sterol synthesis in cultured human macrophages.[2]

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[2] Knight, B. L. Personal communication.

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