Measurement of the Absolute Rates of Cholesterol Biosynthesis in Isolated Rat Liver Cells

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Triparanol [2-(4-chlorophenyl)-1-(4-diethylaminoethoxyphenyl)-1-*p*-tolylethanol] at a concentration of $2\mu\text{M}$ has no effect on the overall conversion of [2- ^{14}C]acetate into C_{27} sterols by isolated liver cells. In the presence of triparanol, however, the formation of radioactive cholesterol is inhibited by 85–90% and the balance of radioactivity appears in the C_{27} sterol desmosterol (cholesta-5,24-dien-3 β -ol). The very small weights of desmosterol which accumulate under these conditions were, as a routine, quantitatively converted into the heptafluorobutyrate 3-enol ester of cholesta-4,24-dien-3-one. This derivative has a high electron-capturing capability, a property that enables extremely small quantities (<0.25 pmol) of the material to be accurately measured by gas chromatography with electron-capture detection. Measurements of the mass and specific radioactivity of the newly biosynthesized desmosterol formed in the presence of triparanol provides an accurate assessment of the amount of cholesterol that would be synthesized by the liver cells in the absence of the drug.

It is widely recognized that the method of measuring the rates of cholesterol synthesis based on the incorporation of label from [^{14}C]acetate is subject to severe limitations (Dietschy & McGarry, 1974), the most important of which is the unknown extent to which [^{14}C]acetyl-CoA produced from the exogenously added material is diluted by that produced from endogenous sources. The inaccuracies inherent in this method have been demonstrated by Dietschy & Brown (1974), who attempted to overcome the unknown dilution factor by flooding the cytosolic compartment, the site of the early stages of cholesterol synthesis, with [^{14}C]acetyl-CoA produced intramitochondrially from [^{14}C]octanoate. This approach, although obviously superior to its predecessor, is not without drawbacks, the most important of which is the rate at which [^{14}C]acetyl-CoA enters the cytosol from the mitochondrial compartment. The use of $^3\text{H}_2\text{O}$ (Barth *et al.*, 1972; Brunen-graber *et al.*, 1972; Nilsson *et al.*, 1973) may also be open to criticism in some cases, since the number of ^3H atoms incorporated into the cholesterol molecule per atom of carbon depends to some extent on the metabolic source of acetyl-CoA units.

The present report describes a method in which the actual mass of newly synthesized sterol is measured directly. This was accomplished by conducting incubations with liver cells in the presence of triparanol [2-(4-chlorophenyl)-1-(4-diethylaminoethoxyphenyl)-1-*p*-tolylethanol], which blocks Δ^{24} -bond reduction during cholesterol biosynthesis and results in the accumulation of desmo-

sterol (cholesta-5,24-dien-3 β -ol). After preparation of a suitable derivative, the weight of desmosterol formed was then measured by g.l.c. with electron-capture detection.

Experimental

Animals

Male Wistar rats were obtained from Bantin and Kingman (Aldbrough, Hull, U.K.) at least 10 days before the start of the experiment. During this time they were given food (Gibbons & Mitropoulos, 1973) and water *ad libitum* and were housed in a windowless room which was artificially lighted only between 09:00h and 21:00h. The rats were used when they weighed between 250 and 300 g.

Preparation of liver cells and incubation procedures

Before the operation, the animal was anaesthetized by intraperitoneal injection of Nembutal (sodium pentobarbitone, 60mg/kg body weight), and then heparin (200 units) was administered via a saphenous vein. The liver was perfused, and the liver cells were isolated essentially by the method of Krebs *et al.* (1973), except that collagenase (30mg/100ml) was the only enzyme added to the perfusate and the isolated cells were washed and suspended in Krebs–Henseleit (1932) bicarbonate buffer (pH 7.4) containing fatty acid-free dialysed albumin (1.1%). The number of cells obtained was determined by counting a portion in a haemocytometer. Viability was assessed by the proportion of cells that were

permeable to the vital stain Trypan Blue (0.1%). Usually more than 90% of the cells were initially viable. Immediately after preparation the final cell suspension was stored at 0°C while the incubation flasks were prepared. The time lapse between the start of the surgical operation and the preparation of the final cell suspension was usually about 1 h. Incubations were conducted in 25 ml silane-treated glass conical flasks each of which contained the cell suspension (2.0 ml, containing 0.125 g fresh wt. of liver cells), glucose (11.1 mM) and albumin (1.1%) in a total volume of 3.0 ml of Krebs-Henseleit bicarbonate buffer (pH 7.4). Where appropriate, a solution of triparanol in propane-1,2-diol (6 μ l) was added. Control flasks contained propane-1,2-diol (6 μ l) only. Immediately before incubation, each flask was sealed with a SubaSeal rubber septum and was gassed for 30 s with O₂+CO₂ (95:5). Incubations were started by injection of [2-¹⁴C]acetate as a solution in water (100 μ l) and were conducted in a water bath at 37°C during which the vessels were shaken at a rate of 100 oscillations/min. At intervals of 30 min, flasks were regassed for a period of 30 s with the O₂+CO₂ gas mixture. This considerably increased the proportion of cells which remained viable after longer incubation periods. A flask containing all the components of a normal incubation except the [2-¹⁴C]acetate was also shaken under the same conditions. Portions of this cell suspension were removed periodically for measurement of cell viability. Usually, high viabilities (>75%) were maintained for up to 4 h of incubation, but after this time, in some cases, viability decreased considerably. The reasons for this are obscure, but may be related to the degree of physical agitation of the cells either before incubation (e.g. centrifugation and resuspension) or during incubation (e.g. vigorous shaking or gassing).

Extraction and oxidation of the non-saponifiable lipids

Each incubation was terminated by the addition of ethanol (10.0 ml), and the mixture was hydrolysed in the presence of KOH (final concentration 5%, w/v) at 60°C for 1 h. After addition of distilled water (50 ml) to the hydrolysate, the non-saponifiable fraction was extracted with 2 $\times$ 40 ml of ether and the extracts were combined and washed with 4 $\times$ 50 ml of water. After evaporation of the ether, the residue was dissolved in ethanol and a portion removed for assay of radioactivity.

Oppenauer oxidation of the non-saponifiable lipids converted radioactive C₂₇ sterols into their corresponding 4-en-3-one derivatives and was achieved by a micro-modification of the method of Shepherd *et al.* (1955) as follows. Toluene and cyclohexanone were dried by slowly percolating each through a column of activated neutral alumina.

This was repeated with fresh alumina in each case. The non-saponifiable lipids, contained at the base of the narrow cone of a 10 ml conical test tube, were desiccated thoroughly. Aluminium isopropoxide (150 mg) was dissolved in 4.6 ml of a mixture of dry cyclohexanone and toluene (1:3, v/v), 0.5 ml of this solution was added to the contents of the conical tube and the mixture was heated slowly to reflux temperature under an atmosphere of O₂-free N₂. The upper cooler part of the tube acted as an air condenser. The solution was refluxed for 0.5 h, the tube was cooled and the contents were transferred to ether (80 ml), which was then washed successively with 0.5 M-HCl (2 $\times$ 20 ml), satd. NaHCO₃ (2 $\times$ 40 ml) and distilled water (3 $\times$ 30 ml). After evaporation of the ether, most of the cyclohexanone was removed by azeotropic distillation with ethanol at 50–60°C under reduced pressure. The small amount of residual cyclohexanone was removed by chromatography of the oxidized non-saponifiable fraction on Rhodamine-impregnated t.l.c. plates of Kieselgel H developed with chloroform (System 1, Gibbons & Mitropoulos, 1973). In this system, cholestenone (cholest-4-en-3-one) had the same polarity (R_F 0.32) as desmostenone (cholesta-4,24-dien-3-one). Cholesterol had R_F 0.19. The radioactive C₂₇ stenone band was located by radioautography, eluted from the plate and a portion removed for assay of radioactivity. The radioactive cholestenone (R_F 0.38) was then well separated from radioactive desmostenone (R_F 0.26) by t.l.c. on AgNO₃-impregnated alumina (Gibbons *et al.*, 1973), developed with a mixture of toluene and hexane (3:1, v/v, System 3c) at 5°C. The radioactive fractions were again located by radioautography, eluted from the plate and assayed for radioactivity. Each stenone fraction was transferred to a conical test tube, and the heptafluorobutyrate derivatives of the desmostenone fractions were prepared by addition of dry benzene (50 μ l) and heptafluorobutyric anhydride (160 μ l). The mixture was heated at 60°C for 0.5 h and the solvent was evaporated under N₂.

Although all the experiments described in this work involved isolation of the radioactive desmostenone in the manner just described, we have recently been able to shorten the procedure considerably by direct chromatography of the oxidized non-saponifiable lipids in System 3c. This was only feasible if all the cyclohexanone was removed from the residue after evaporation of the ether from the organic extract. This was accomplished by azeotropic distillation of the residue with distilled water (not ethanol) at 80–90°C under reduced pressure. In all cases when storage of fractions containing desmostenone or its derivatives was necessary, the material was dissolved in benzene (Svoboda & Thompson, 1967) and maintained at a temperature slightly above the freezing point of the solution (5–6°C) to prevent radiolysis (Bayly & Evans, 1968).

Other chromatographic procedures

In some cases, the non-saponifiable lipids were not oxidized. In these experiments, the 4-demethyl sterols (cholesterol and other C_{27} sterols) were separated from the 4,4-dimethyl sterols (C_{30} and C_{29} sterols) and 4α -methyl sterols (C_{28} sterols) by t.l.c. in System 1. The labelled 4,4-dimethyl sterol mixture was then separated into its individual components by acetylation and by sequential 'argentation' t.l.c. as described previously (Gibbons *et al.*, 1973). The individual components of the 4-demethyl sterol fraction were separated as the acetate derivatives by use of a variety of systems involving $AgNO_3$ -impregnated silica gel with a mixture of toluene and hexane (1:1, v/v, System 2) and $AgNO_3$ -impregnated alumina with toluene/hexane (1:3, v/v, System 3a, or 1:1, v/v, System 3b) as the mobile phase (Table 1).

General procedures, such as acetylation of sterols, radioautography, recovery of steroids from t.l.c. plates, analytical g.l.c. and radioassay, have been described previously (Gibbons & Mitropoulos, 1972, 1973). Mass spectra were determined on an A.E.I. MS-12 mass spectrometer with a direct probe. The energy of the electrons was 70 eV. Protein was measured by the method of Cleland & Slater (1953).

Materials

Non-radioactive cholest-4-en-3-one was prepared from cholesterol by the method of Shepherd *et al.* (1955). The isolated material after t.l.c. (System 1) and recrystallization from 95% methanol had m.p. 82–83°C [reported, 80–81°C (Shoppee & Summers, 1950)] with $\lambda_{max.}$, 240 nm; ϵ_{240} , 14170 litre·mol⁻¹·cm⁻¹ (reported $\lambda_{max.}$, 241 nm; ϵ_{241} , 16600 litre·mol⁻¹·cm⁻¹, Fieser & Fieser, 1959). Non-radioactive cholesta-4,24-dien-3-one was prepared from desmosterol (Serva, Heidelberg, Germany) by the same method. After crystallization, the material had m.p. 89.5–90.5°C, with $\lambda_{max.}$, 240 nm; ϵ_{240} , 18300 litre·mol⁻¹·cm⁻¹. The reported physical constants for this material are: m.p. 83–85°C; $\lambda_{max.}$, 240 nm; ϵ_{240} , 17400 litre·mol⁻¹·cm⁻¹ (Scallen, 1965). Heptafluorobutyric anhydride was obtained in 1.0 ml sealed ampoules from Phase Separations, Queensferry, Flint., U.K. Fatty acid-free bovine serum albumin (fraction V powder; Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared by the method of Chen (1967). Contaminating acetic acid and ethanol were removed by dialysis (Krebs *et al.*, 1973). Propane-1,2-diol (BDH Chemicals, Poole, Dorset, U.K.) was shaken with Na_2CO_3 for 15 h, decanted and distilled under reduced pressure. Triparanol was the gift of Dr. B. Morgan, Beecham Research Laboratories, Tadworth, Surrey, U.K. Collagenase (type 1) was purchased from Sigma Chemical Co. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The inorganic

constituents of the Krebs–Henseleit buffer were of AnalaR grade (BDH Chemicals), as were glucose and sodium acetate. Aluminium isopropoxide (laboratory reagent) and cyclohexanone ('puriss') were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Nembutal was from Abbott Laboratories, Queenborough, Kent, U.K., and heparin was from Paynes and Byrne, Greenford, Middx., U.K. The source of solvents and other materials used in this work have been reported elsewhere (Gibbons & Mitropoulos, 1973; Gibbons *et al.*, 1973).

Results

Nature of the radioactive sterols produced from [2-¹⁴C]acetate by liver cells

Extensive studies in the late 1950's and early 1960's had clearly demonstrated that triparanol inhibits cholesterol synthesis in liver both *in vivo* (Blohm & McKenzie, 1959; Steinberg & Avigan, 1960; Avigan *et al.*, 1960; Schroeffer, 1961; Goodman *et al.*, 1963) and *in vitro* (Steinberg & Avigan, 1960; Dempsey, 1965), and that desmosterol accumulates. However, the drug is a general inhibitor of sterol Δ^{24} -bond reductase (Avigan *et al.*, 1963) and in some tissues other sterols besides desmosterol accumulate (Franz *et al.*, 1962; Clayton *et al.*, 1963). In addition, at higher concentrations of the drug, other enzymes of sterol biosynthesis may be affected (Dempsey, 1965). Before any quantitative work could be attempted, therefore, it was necessary to define precisely the qualitative changes in the radioactive-sterol composition of isolated liver cells that arose as a result of cholesterol biosynthesis from [2-¹⁴C]acetate in the presence of triparanol.

Liver cells were incubated in the presence or absence of triparanol with [2-¹⁴C]acetate as the labelled sterol precursor. In each case the labelled-sterol fractions were isolated from the non-saponifiable lipids by t.l.c. in System 1 and the individual labelled sterols were separated and identified by 'argentation' t.l.c. The results are presented in Table 1, which shows clearly that, regardless of the presence or absence of the drug, by far the most heavily labelled fraction was that containing the C_{27} sterols. Separation of the components of these fractions, however, showed that, in the presence of triparanol, the most heavily labelled sterol had the chromatographic properties of desmosterol (isolated as the acetate) and whose radiochemical identity was subsequently confirmed by addition of carrier material followed by crystallization to constant specific radioactivity. Only a small amount of labelled cholesterol was formed. In another experiment, the labelled fractions corresponding to desmosteryl acetate from a number of incubations conducted in the presence of triparanol were combined and

Table 1. *Effect of triparanol on the distribution of radioactivity among cholesterol and its precursors during sterol biosynthesis in the presence of [2-¹⁴C]acetate*

Isolated liver cells (0.125g) were incubated in Krebs-Henseleit bicarbonate buffer, pH 7.4 (3.0ml) containing albumin (1.1%), glucose (11.1mM), propane-1,2-diol (6μl) and, where appropriate, triparanol (2μM). The flasks were sealed with a rubber septum, and, after gassing with O₂+CO₂ (95:5), [2-¹⁴C]acetate (10μCi, 1μCi/μmol) was injected as a solution in 100μl of water. Incubations were conducted for 3 h at 37°C. After extraction of the non-saponifiable lipids, the crude sterol fractions were separated by t.l.c. in System 1. The dienic (desmosterol and companions) were separated from the monoenic (cholesterol and companions) C₂₇ sterols by acetylation and subsequent t.l.c. in System 2. The individual components of the dienic and of the monoenic C₂₇ sterol acetate fractions were isolated by t.l.c. in Systems 3b and 3a respectively. The components of the C₂₉+C₃₀ sterol fraction were isolated as described previously (Gibbons *et al.*, 1973). The amount of the radioactivity in each sterol fraction is given, followed by the percentage composition. N.D., Not detected.

Sterol fraction	Type of incubation	...	Normal	With triparanol
C ₂₉ and C ₃₀ sterols			7500 d.p.m.	8000 d.p.m.
Lanosterol			22.6%	16.8%
4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol			59.8%	83.2%
Dihydrolanosterol			N.D.	N.D.
4,4-Dimethyl-5α-cholest-8-en-3β-ol			17.6%	N.D.
C ₂₈ sterols			N.D.	N.D.
C ₂₇ sterols			326000 d.p.m.	396000 d.p.m.
Cholesterol			94.4%	12.0%
Cholest-7-enol			0.9%	N.D.
Desmosterol			N.D.	88.0%
Unknown 1			2.8%	N.D.
Unknown 2			1.9%	N.D.

analysed by mass spectrometry. There was no molecular ion indicating the presence of a Δ⁵ sterol acetate (Galli & Maroni, 1967). The base peak (*M*⁺-acetate) occurred at *m/e* 366, suggesting a doubly unsaturated steroid. Other diagnostically important peaks occurred at *m/e* 351, 253 and 213 (Galli & Maroni, 1967) and the spectrum was similar to that observed with an authentic specimen of desmosteryl acetate.

In the absence of triparanol, cholesterol was the major radioactive sterol. In this case, however, a minor radioactive band had the same *R_F* value as desmosteryl acetate after t.l.c. of the acetylated C₂₇ sterol mixture in System 2. This was resolved into two components in t.l.c. System 3b, neither of which showed the chromatographic behaviour of desmosteryl acetate. These are denoted 'Unknown 1' and 'Unknown 2' in Table 1. Cholest-7-enol (5α-

cholest-7-en-3β-ol) was detected in small amounts in the absence of triparanol. Its acetate derivative was less polar than that of cholesteryl acetate in t.l.c. System 3a and its radiochemical identity was confirmed after addition of carrier material and successive recrystallization with measurement of specific radioactivity. None of these materials was formed in incubations conducted in the presence of triparanol. The drug also had an effect on the distribution of radioactivity among the much less heavily labelled components of the 4,4-dimethyl sterol fraction (C₂₉+C₃₀ sterols). Although in both cases the most radioactive component was 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol, the side-chain saturated derivative 4,4-dimethyl-5α-cholest-8-en-3β-ol was not formed in the presence of triparanol. Relatively small amounts of lanosterol (5α-lanosta-8,24-dien-3β-ol) were formed in both types of incubation, but in neither case was dihydrolanosterol (5α-lanost-8-en-3β-ol) detected (Table 1).

Effect of triparanol on the rate of radioactive C₂₇-sterol synthesis from [2-¹⁴C]acetate

For the present study with triparanol to be of any quantitative value as a measure of the true rate of cholesterol synthesis, conditions had to be established under which, first, the drug exerted a truly specific effect, in that it inhibited no enzyme of cholesterol biosynthesis other than the desmosterol Δ²⁴-bond reductase. This is the crucial criterion which must be met if the weight of sterol produced in the presence of the drug is equal to that normally produced in its absence. Secondly, the accumulation of desmosterol should be maximal under these conditions. In establishing these optimal conditions, preliminary studies had shown that concentrations of triparanol greater than 2–5 μM resulted in virtually complete inhibition of radioactive cholesterol synthesis from [2-¹⁴C]acetate and that the yield of radioactive desmosterol was lower than expected. As the drug had no effect on the incorporation of label into the non-saponifiable lipids at these higher concentrations, we attributed this effect to the formation of other labile sterols such as cholesta-5,7,24-trienol (Dempsey, 1965). The optimal concentration of triparanol was found to be 2 μM, at which, during an incubation period of 1 h, the amount of labelled cholesterol formed from [2-¹⁴C]acetate was decreased by 85% and this decrease was exactly balanced by the amount of radioactivity associated with desmosterol. That this situation remained unchanged over different periods of time was shown by a time-course study of incorporation of label from [2-¹⁴C]acetate into the C₂₇ sterols in the presence or absence of triparanol. The results are presented in graphical form in Fig. 1, which shows that the

incorporation of radioactivity into the sterol fractions exhibits a sigmoidal variation with respect to time. The results also show that triparanol, at a concentration of $2\mu\text{M}$, gives rise only to a qualitative change in sterol biosynthesis.

Electron-capture detection of pmol quantities of biosynthetic desmosterol

Various electron-capturing derivatives of steroids have been prepared (Landowne & Lipsky, 1963; Brownie *et al.*, 1964; Nakagawa *et al.*, 1966). Of these, the heptafluorobutyrate appeared to be suitable for our purpose because of its reported high electron affinity (Clark & Wotiz, 1963; Nakagawa *et al.*, 1966; van der Molen *et al.*, 1967; Exley & Chamberlain, 1967) and because it is available commercially.

Fig. 1. Effect of triparanol on the rate of formation of radioactive C_{27} sterols from $[2\text{-}^{14}\text{C}]\text{acetate}$ by isolated rat liver cells

Isolated liver cells (0.125g, 21.2mg of protein, 265 μg of cholesterol) were incubated in Krebs-Henseleit bicarbonate buffer (3.0ml, pH 7.4) containing glucose (11.1mM), albumin (1.1%) and $[2\text{-}^{14}\text{C}]\text{acetate}$ (10.41 μCi , 1.06 $\mu\text{Ci}/\mu\text{mol}$) for various periods of time. Where appropriate, triparanol (final concentration $2\mu\text{M}$) was added as a solution in propane-1,2-diol (6 μl). Control flasks contained propane-1,2-diol (6 μl) only. Incubations were terminated by the addition of ethanol (10.0ml) and the incubation contents were saponified. The non-saponifiable fraction was chromatographed in t.l.c. System 1 and the isolated radioactive C_{27} sterol mixture was acetylated. The sterol acetates were chromatographed in t.l.c. System 2 and portions of the separated cholesteryl and desmosteryl acetate fractions were removed for measurement of radioactivity. Manipulative losses were accounted for by the addition of $[1\alpha,2\alpha(\text{n})\text{-}^3\text{H}]\text{cholesterol}$ (51400 d.p.m.) as an internal standard immediately after incubation. ▲, Cholesterol (no triparanol); ●, cholesterol plus desmosterol (triparanol); △, desmosterol (triparanol); ○, cholesterol (triparanol).

Our original aim, therefore, was to isolate and purify the demosterol as the heptafluorobutyrate derivative by t.l.c. followed by direct assay of mass by g.l.c. with electron-capture detection. This intention was frustrated, however, first, by the instability of the derivatives during elution after t.l.c., and, secondly, by the disappointingly low response to known quantities of desmosteryl heptafluorobutyrate by the electron-capture detector. The problem of overcoming the second of these difficulties was made easier by the report (Exley & Chamberlain, 1967) that the heptafluorobutyryl enol esters of steroid hormones containing a conjugated $\Delta^4\text{-}3\text{-one}$ grouping showed a much higher electron-capture capability than the esters of the corresponding $\Delta^5\text{-}3\beta\text{-ol}$ compounds.

Preparation of the enol ester of cholesta-4,24-dien-3-one (desmostenone) showed that this was also true in the present case; the sensitivity of the electron-capture detector to this compound was increased by a factor of approx. 60 compared with that of the ester of desmosterol itself (Table 2). We

Table 2. Gas-chromatographic behaviour of some steroids and their heptafluorobutyrate esters

For electron-capture detection a non-pulsed ^{63}Ni detector was used at a temperature of 246°C . The stationary phase was XE-60 (3%). The electrometer output was such that 0.4nA generated a recorder full-scale deflexion corresponding to 1mV. Other operating conditions were: N_2 gas flow, 70ml/min; injector temperature, 225°C ; column temperature, 219°C . The QF-1 (3%) column was fitted with a flame-ionization detector. Operating conditions were: N_2 gas flow, 50ml/min; injector temperature, 248°C ; column and detector temperatures as above. For each determination, at least three individual measurements were made, and the average value was calculated. The relative retention time was relative to cholesteryl heptafluorobutyrate taken as 1.00. Peak area is given in mm^2 , where $1\text{cm}^2 = 87\text{pA}\cdot\text{min}$. N.D., Not determined.

Steroid	Relative retention time		Response of electron-capture detector [peak area (mm^2)]
	XE-60 (3%)	QF-1 (3%)	
Cholesterol	N.D.	0.97	N.D.
Cholestenone	N.D.	3.10	N.D.
Desmostenone	N.D.	3.30	N.D.
Cholesteryl heptafluorobutyrate (10ng)	1.00	1.00	18.7
Desmosteryl heptafluorobutyrate (10ng)	1.21	1.08	18.0
Cholestenone heptafluorobutyrate (1ng)	0.99	0.94	121.5
Desmostenone heptafluorobutyrate (1ng)	1.22	1.06	115.9

therefore investigated the possibility of isolating, on a micro-scale, radioactive desmostenone prepared from the desmosterol synthesized experimentally by liver cells. If this could be achieved, then the heptafluorobutyrate derivative could be prepared after the pure compound had been isolated by t.l.c.

Our next task, therefore, was to find optimum conditions for the routine quantitative conversion of biosynthetic desmosterol into its Δ^4 -3-one derivative and its chromatographic isolation in a pure state. This was achieved by a micro-modification of the Oppenauer technique, as described in the Experimental section. The efficiency of the method was determined by subjecting trace amounts of [26(27)- 14 C]desmosterol to the Oppenauer procedure for various periods of time and isolation of the oxidation product by t.l.c. in System 1. The recoveries of [26(27)- 14 C]desmostenone are presented in Table 3. Under experimental conditions, however, it was necessary to separate the small quantities of desmostenone from the much larger mass of cholestenone (formed from endogenous cholesterol) which had the same polarity in this system. This was tested by Oppenauer oxidation of trace amounts of [26(27)- 14 C]desmosterol (44000 d.p.m.) in the presence of a larger weight of unlabelled cholesterol (1.0 mg) for 30 min. After t.l.c. of the oxidized extract in System 1, 41000 d.p.m. (93.4% of the original) was recovered in an area of the plate corresponding to stenones. Chromatography of this recovered stenone mixture in System 3c resulted in a recovery of 39900 d.p.m. (90.7% of original) in the area of desmostenone. These values represent the average of duplicate determinations and no correction has been made for manipulative losses. In practice, it was possible to subject the whole of the non-saponifiable

lipids to the modified Oppenauer procedure, after which the labelled stenones were isolated individually by the chromatographic techniques just described.

The purified biologically labelled desmostenone was converted into the heptafluorobutyrate enol ester. That the enol ester was formed in virtually quantitative yield under these conditions was shown by treating authentic desmostenone (0.5 mg) in the same way and analysing the products by g.l.c. by using conventional flame-ionization detection. The major peak had a retention time much shorter than that of the starting material (Table 2). A very small peak appeared with the retention time of desmostenone, and comparison with standards showed that this amounted to less than 5% of the total. We consider, therefore, that in practice the much smaller weights of desmostenone formed are, under these conditions, esterified by the vast excess of reagent to an extent greater than 95%.

The desmostenone isolated after chromatography in System 3c was usually contaminated with small quantities of cholestenone. As the stationary phase for g.l.c., therefore, we chose XE-60, as previous experience had shown that this was particularly efficient at separating pairs of sterols differing only by the presence or absence of a Δ^{24} bond. Table 2 illustrates this capacity and compares the chromatographic behaviour of various steroidal heptafluorobutyrate esters on the stationary phases XE-60 and QF-1. As the 63 Ni electron-capture detector gave a linear response over a limited range of derivative concentration, a standard curve was always prepared before any biological samples were assayed. Fig. 2 shows a typical response of the detector to various amounts of standard desmostenone heptafluorobutyrate enol ester applied to the column. The response was always linear within the range 0–1000 pg. The relative response of the electron-capture detector to a number of heptafluorobutyrate derivatives is given in Table 2. If the limit of sensitivity is arbitrarily selected as that at which the signal/noise ratio is 3, then it is possible to detect as little as 40 pg (0.105 pmol) of desmostenone in a sample injected on to the gas chromatograph. The standard solutions of desmostenone heptafluorobutyrate enol ester were prepared in hexane and stored at -20°C . The solutions were stable under these conditions for several months.

Use of the method in practice

Liver cells were incubated in the presence of triparanol for periods of time ranging from 0.25 to 4 h, with [2- 14 C]acetate as the labelled cholesterol precursor. Labelled desmostenone and cholestenone fractions were isolated from each incubation as described in the Experimental section, and portions of each were removed for assay of radioactivity.

Table 3. *Effect of time on the formation of [26(27)- 14 C]-cholesta-4,24-dien-3-one (desmostenone) from [26(27)- 14 C]-cholesta-5,24-dien-3 β -ol (desmosterol)*

[26(27)- 14 C]Desmosterol (44000 d.p.m., 53 mCi/mmol) was oxidized with the Oppenauer reagent (0.5 ml) for various periods of time. The mixture was cooled, and the oxidized material extracted and chromatographed in System 1. The radioactive bands were located by radioautography. Values in parentheses represent the proportion of that component as a percentage of the radioactivity of the starting material. The values are not corrected for losses during extraction and t.l.c.

Reaction time (h)	Radioactivity (d.p.m.) associated with:		
	Desmosterol	Desmostenone	Total
0.25	7033 (15.9%)	34390 (78.1%)	41423 (94.0%)
0.50	1454 (3.3%)	38134 (86.6%)	39588 (89.9%)
1.00	0	39566 (89.9%)	39566 (89.9%)

Fig. 2. Sensitivity of the ^{63}Ni electron-capture detector to the heptafluorobutyrate enol ester of cholesta-4,24-dien-3-one (desmostenone)

Two standard solutions of cholesta-4,24-dien-3-one heptafluorobutyrate enol ester (containing 1.0ng and 0.2ng respectively of original dienone per μl) were prepared in hexane. Various volumes of these solutions were injected into the gas chromatograph and a standard curve was prepared. For each weight of dienone three measurements were taken and the average value was calculated. The stationary phase was XE-60 (3%) on Varoport 30 (80-100 mesh) and the column temperature was 217°C . Other operating parameters were: injector temperature, 230°C ; detector temperature, 258°C ; N_2 flow rate, 65 ml/min. Under these conditions, the retention time of the enol ester was 6.25 min. The instrument electrometer setting was such that an output of 0.4 nA gave a recorder full-scale deflexion corresponding to 1 mV (23 cm).

In addition, the radiochemical purity of the biosynthetically labelled desmostenone was confirmed by addition of a larger amount of unlabelled desmostenone to a portion of one of the labelled dienone fractions. After recrystallization, the specific radioactivity of the crystals showed virtually no change (4350, 5020 and 4650 d.p.m./mg respectively) and did not differ significantly from the original value (5170 d.p.m./mg). The remainder of each stenone fraction was transferred to a stoppered conical tube. The desmostenone fraction was esterified with heptafluorobutyric anhydride, and after evaporation of the solvent the residue was dissolved in a suitable volume of hexane (usually 40-800 μl). Portions were removed for measurement of mass by quantitative g.l.c. The weight of cholesterol recovered at the end of the procedure

Fig. 3. Absolute rate of cholesterol production by isolated rat liver cells

Liver cells (0.125 g, 20.0 mg of protein) were incubated for various periods of time in Krebs-Henseleit bicarbonate buffer, pH 7.4 (3.0 ml), containing glucose (11.1 mM), albumin (1.1%), propane-1,2-diol (6 μl), triparanol (2 μM) and [$2\text{-}^{14}\text{C}$]acetate (10 μCi , 1.0 $\mu\text{Ci}/\mu\text{mol}$). Incubations were stopped by the addition of ethanol (10 ml), and the extracted non-saponifiable lipids were oxidized by the modified Oppenauer procedure. The oxidized extract was chromatographed in t.l.c. System 1 and the radioactive stenone mixture was separated into its individual components by t.l.c. in System 3c. After removal of a portion of each fraction for measurement of radioactivity, the mass of desmostenone was measured as the heptafluorobutyrate enol ester derivative by g.l.c. with electron-capture detection. ●, Desmosterol; ▲, desmosterol + cholesterol.

was also measured by g.l.c. on the stationary phase QF-1, with the use of flame-ionization detection, and was used to correct for non-specific losses of sterol derivatives during the work-up process. The results are shown in Fig. 3. The small amount of cholesterol biosynthesized during each time-interval was calculated from the radioactivity of the purified cholesterol and the observed specific radioactivity of the newly synthesized desmosterol at the end of that particular time-interval. This value was added to the observed weight of desmosterol formed to give the rate of production of total C_{27} sterols (cholesterol plus desmosterol). This is equivalent to the amount of cholesterol that would have been synthesized in the absence of triparanol and is thus an absolute measure of the rate of cholesterol synthesis. Fig. 3 shows that this rate is linear for 1 h

and then begins to accelerate between 2 and 4h of incubation.

Discussion

The drawbacks of using incorporation of radioactivity from labelled acetate as a measure of the rate of cholesterol synthesis have been well documented (Dietschy & McGarry, 1974; Dietschy & Brown, 1974). Despite their ingenuity, other techniques, which have again relied on the incorporation of label from a radioactive precursor, have not eliminated all of these pitfalls, and, unless some assumptions are made, the exact degree of dilution of the radioactive material with non-labelled precursors of cholesterol still remains unknown. For instance, with [^{14}C]-octanoate (Dietschy & McGarry, 1974) as the source of [^{14}C]acetyl-CoA units for cholesterol biosynthesis, one assumption, the validity of which has not been established, is that the specific radioactivity of new intramitochondrially synthesized ketone bodies is a measure, not only of the specific radioactivity of the intramitochondrially generated acetyl-CoA units from which they are derived, but of the specific radioactivity of the cytosolic acetyl-CoA from which cholesterol is produced. This may be true over the long term, during which label from both intra- and extra-mitochondrial acetyl-CoA has had time to reach equilibrium. However, in the short period, the specific radioactivity of cytosolic acetyl-CoA depends first on its initial concentration and secondly on the rate at which [^{14}C]acetyl-CoA produced from [^{14}C]octanoate in the mitochondrion is released into the cytosolic compartment, a factor that depends on the activity of the citrate-cleavage enzyme [ATP citrate lyase; EC 4.1.3.8 (Lowenstein, 1968)]. In other words, if the specific radioactivity of cytosolic acetyl-CoA changes slowly but appreciably during the incubation period, then the specific radioactivity of the ketone bodies cannot be used to adjust the specific radioactivity of the acetyl-CoA used for cholesterol synthesis. In addition, this method does not account for any endogenous accumulation of cholesterol precursors after acetyl-CoA in the biosynthetic pathway, which would dilute radioactivity from [^{14}C]acetyl-CoA, especially during the early stages of an incubation.

Although its specific radioactivity obviously does not change during the course of an incubation, the use of $^3\text{H}_2\text{O}$ incorporation as a measure of cholesterol synthesis may yield neither absolute nor even relative rates of cholesterol synthesis, since the number of ^3H atoms incorporated per carbon atom may change under different physiological conditions, depending on the metabolic source of the acetyl-CoA units, and may differ significantly from the ratio of 1.31 determined by Brunengraber *et al.*

(1972) in a study of cholesterol biosynthesis from [^{14}C]glucose in the presence of $^3\text{H}_2\text{O}$. For instance, if fatty acids are used predominantly as the metabolic source of acetyl-CoA units for cholesterol biosynthesis, then the numerous β -oxoacyl-CoA intermediates involved will non-enzymically incorporate substantial quantities of ^3H from $^3\text{H}_2\text{O}$ because of keto-enol tautomerism. The acetyl-CoA units so produced, therefore, will contain proportionately more ^3H than those produced, say, by glycolysis, a process which involves few tautomeric carbonyl-containing intermediates. Another drawback occurs during short-term incubations in which endogenous precursors of cholesterol, such as hydroxymethylglutaryl-CoA or squalene, may be preferentially utilized for cholesterol synthesis. Substrates such as these do not incorporate as much ^3H per atom of carbon as does acetyl-CoA (Nilsson, 1975).

The validity of the present technique relies partly on the demonstration that $2\mu\text{M}$ -triparanol has no effect on the rate of sterol synthesis. We believe that the results presented in Fig. 1 leave little doubt that triparanol gives rise only to a qualitative change in sterol synthesis. The only objection to this conclusion would be that triparanol changed the pool size of acetyl-CoA from which sterols are synthesized, without changing the rate of incorporation of radioactivity. We consider this unlikely in view of the similarity in the amount of label from [2- ^{14}C]-acetate incorporated into fatty acids in the presence and absence of triparanol. In addition, it would be expected that, with cells from the same animal, any change in the acetyl-CoA pool size would be reflected in the amount of ^3H incorporated during lipid synthesis in the presence of $^3\text{H}_2\text{O}$. Under these conditions, however, triparanol did not affect the amount of ^3H incorporated into either fatty acid or sterol derivatives (G. F. Gibbons, unpublished work). Also, early investigations showed that chronic treatment of rats with high concentrations of triparanol affected neither the specific radioactivity of $^{14}\text{CO}_2$ produced from labelled acetate (Blohm & McKenzie, 1959) nor the concentration of tissue sterols (Avigan *et al.*, 1960). These observations provide further evidence that the drug has no effect on the size of the acetyl-CoA pool and adds further support for the validity of the present technique.

We believe, therefore, that measurement of the actual weight of newly synthesized sterol as described in the present study eliminates all the problems associated with radioactive substrate dilution. In addition, although the present work was carried out with rat liver cells, the sensitivity of the method allows its application to studies in cells which synthesize cholesterol at a much lower rate. In these tissues, the low specific radioactivity of the $^3\text{H}_2\text{O}$ used as the incubation medium precludes its use as a measure of cholesterol synthesis, since the incorporation of ^3H

is very low and barely measurable with accuracy. With the present method, measurement of the specific radioactivity of the newly synthesized sterol also gives some indication of the extent to which [^{14}C]acetyl-CoA derived from exogenous [^{14}C]acetate is diluted during the course of an experiment. This provides information on the extent to which endogenous precursors are being utilized for cholesterol production, and is of some importance in studying the process of cholesterol biosynthesis, not in isolation, but as part of the overall metabolic capabilities of the cell in a given physiological state.

The preferential utilization of endogenous substrates beyond acetyl-CoA for cholesterol biosynthesis during the early stages of an incubation is illustrated in the present study by the lower rate of incorporation of label from [2- ^{14}C]acetate into C_{27} sterols at 0.25 h and 0.5 h (Fig. 1) than at longer time-intervals. This probably represents a greater dilution of radioactivity from [^{14}C]acetyl-CoA during the early stages, and this has also been observed during cholesterol biosynthesis from [2- ^{14}C]acetate and, to a lesser degree, from [2- ^{14}C]mevalonic acid in cell-free systems of rat liver homogenates (G. F. Gibbons, unpublished work). In the present study, further experiments, in which the weight of cholesterol synthesized was based on the actual weight of desmosterol produced in the presence of triparanol, the actual rate was linear for up to 1 h (Fig. 3).

In this investigation, it was observed that during cholesterol biosynthesis in the presence of [2- ^{14}C]acetate, of the small amount of label associated with the 4,4-dimethyl sterols (C_{30} and C_{29} sterols) only a small part was associated with lanosterol. Radioactive dihydrolanosterol was not detected. The largest proportion of the radioactivity was contributed by the 14-demethyl derivatives of lanosterol, namely 4,4-dimethylcholesta-8,24-dienol and its side-chain-saturated derivative 4,4-dimethylcholesta-8-enol. The situation is very similar to that which occurs in the cell-free systems of rat liver homogenate (Gibbons & Mitropoulos, 1973), and provides further evidence for our suggestion that, during cholesterol biosynthesis in liver, side-chain reduction occurs predominantly, if not exclusively, at the C_{29} sterol level. It follows, therefore, that desmosterol is not normally an intermediate in cholesterol biosynthesis, but results, in the presence of triparanol, from a rerouting of the normal pathway through side-chain-unsaturated intermediates. The rate at which this pathway operates, however, is equivalent to that by which cholesterol is normally synthesized (Fig. 1).

The effort involved in the overall assay would be considerably decreased if the Oppenauer oxidation procedure could be replaced by an enzymic method which produces steroidal Δ^4 -3-ones from the parent Δ^5 -3 β -ols (Richmond, 1973).

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