Utilization of Endogenous and Exogenous Sources of Substrate for Cholesterol Biosynthesis by Isolated Hepatocytes

By GEOFFREY F. GIBBONS and CLIVE R. PULLINGER

Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, London W12 0HS, U.K.

(Received 1 June 1978)

The rates of cholesterol biosynthesis in isolated rat hepatocytes were determined by using a method based on measurement of the rate of formation of desmosterol (cholesta-5,24-dien-3 β -ol), which accumulates during inhibition of cholesterologenesis by the drug triparanol. Incubation of cells from normal or 24 h-starved animals in a medium containing albumin, glucose, amino acids and acetate as the only organic constituents led to an accelerating rate of sterol formation during the earlier stages of a 6 h incubation period. The contribution of exogenously added acetate (initial concentration 3.34 mM) to sterol synthesis in both types of cells reached an early maximum and then continually declined. Exogenously added pyruvate and lactate were more efficient sources of sterol carbon than was acetate. Exogenous glucose even at relatively high concentrations (11.1 mM) was incapable of providing more than 6% of the total sterol carbon. Although the proportion of total sterol carbon supplied from exogenous acetate increased with increasing concentrations of the extracellular substrate, the rates of total sterol synthesis in both types of cell remained unchanged. Similar observations were made when lactate or pyruvate was the cholesterologenic precursor in normal cells. These studies suggest that, although exogenous substrates were capable of expanding an intracellular pool of cholesterol precursor, the normal supply of intermediary metabolites was not rate-limiting for cholesterologenesis.

It is generally thought that an increase in the activity of the enzyme hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) measured *in vitro* gives rise to, and reflects, a true increase in the rate of cellular cholesterol synthesis *in vivo* (Rodwell *et al.*, 1976). The existence of such a relationship assumes, of course, that the cell contains or is capable of swiftly generating sufficient quantities of substrate hydroxymethylglutaryl-CoA to effect a real increase in cholesterologenesis, and in some instances this has been found not to be the case (Dugan *et al.*, 1972; Nervi *et al.*, 1976). For reasons of cellular economy, it would appear unlikely that the cell maintains a large and mainly redundant pool of hydroxymethylglutaryl-CoA waiting to be called into service as the demand for more cholesterol arises. A more plausible and economic means of meeting this demand would involve an increase in the rate of provision of more primary metabolites such as acetyl-CoA. This, ultimately, would require the establishment of a new cellular equilibrium in which more of the products of intermediary metabolism were made available for cholesterol synthesis. This aspect of the control of cholesterologenesis has received little attention. We have developed a chromatographic method that allows us to measure directly the mass of newly synthesized sterol in

isolated hepatocytes (Gibbons & Pullinger, 1977, 1977a). With this technique it is possible to differentiate between the contribution to cholesterol synthesis of endogenous substrate and of exogenously added labelled cholesterol precursors. In this way we have been able to determine the capability of various intermediary metabolites, and of acetate, to produce the amount of carbon necessary to maintain the observed rates of cholesterol synthesis under conditions where this varied widely. Comparison of the rates of cholesterol synthesized obtained with the chromatographic method and the calculated rates based on incorporation of ^3H from $^3\text{H}_2\text{O}$ (Brunen-graber *et al.*, 1972; Barth *et al.*, 1972; Fears & Morgan, 1976; Lakshmanan & Veech, 1977) also provided some information as to the intracellular source of substrate for cholesterologenesis.

Experimental

Methods

Animals, preparation of hepatocytes and incubation procedures. The housing and treatment of animals, preparation of isolated hepatocyte suspensions and incubation procedures have been described previously (Gibbons & Pullinger, 1977a). All surgical

operations were started at 09:30h. The only additions or modifications to the above procedures were as follows. For experiments on hepatocytes from starved animals, food was removed at 09:30h on the day before the experiment. Amino acids of the type and concentration recommended by East *et al.* (1973) were added to the cell suspension before incubation. The standard incubation medium therefore contained glucose (11.1mM), amino acids and fatty acid-free dialysed albumin (1.1%) in Krebs-Henseleit (1932) bicarbonate buffer (pH 7.4). In most experiments triparanol (added as a solution in propane-1,2-diol) was present at a concentration of 2 μ M. Incubations were conducted in silane-treated glass flasks with stoppers, and a stream of O₂+CO₂ (19:1) was passed slowly into the atmosphere of each flask for approx. 10s every hour. The flasks were incubated at 37°C with shaking at 70 oscillations/min. Usually under these conditions we were able to maintain high cell viabilities (>80%) for the duration of the longest incubation periods studied (6h).

Conversion of sterols into stenones and their isolation from the incubation mixture

At the end of the incubation period, enzymic activity was destroyed by heating the cell suspension at 90°C for 2 min. To the inactivated suspension was added a solution of Triton X-100 (0.125%) in potassium phosphate buffer, pH 7.5, followed by cholesterol oxidase (0.25 unit) and cholesterol esterase (0.20 unit). The mixture was incubated at 37°C for 20 min. This converted the newly synthesized desmosterol (formed in the presence of triparanol) and the much larger quantity of cellular endogenous cholesterol into their 4-en-3-one derivatives cholesta-4,24-dien-3-one (desmostenone) and cholest-4-en-3-one (cholestenone) respectively (Gibbons & Pullinger, 1977b). In the latter study, a preliminary chromatography on plates of silica gel of the ether-extractable lipid obtained after this procedure was necessary to remove certain (presumed) polyunsaturated triacylglycerols that had the same polarity as desmostenone in the argentation system required for desmostenone purification. This preliminary t.l.c. has been eliminated in the present work, since triacylglycerols were removed by direct alkaline hydrolysis of the enzyme-treated incubation mixtures. This was done by the addition of 2 vol. of ethanolic KOH to give a final alkali concentration of 890mM (5%) followed by heating at 60°C for 0.5h. The non-saponifiable lipid containing the stenones was then extracted with ether in the normal way (Gibbons & Pullinger, 1977a). Triton X-100, which was also extracted, and which interfered with the argentation chromatography, was removed by passing an ether solution of the non-saponifiable lipid through a small column (about 3g) of alumina (grade III) and eluting

with ether (30ml). After evaporation of the ether, the residue was chromatographed on a plate of silver nitrate-impregnated alumina (Gibbons & Pullinger, 1977a), which separated the small quantity of desmostenone derived from the newly synthesized desmosterol from the much larger mass of cellular endogenous cholesterol-derived cholestenone. Each purified fraction was eluted from the plate with ether. Protein was measured by the method of Lowry *et al.* (1951). For each experiment an additional flask was prepared containing all the ingredients of the experimental samples. After addition of the substrate, enzyme activity was destroyed as above and the stenones extracted in the normal way. After forming derivatives of the desmostenone fraction (see below) the quantity (always very small or non-existent) of material with the same chromatographic properties as desmostenone heptafluorobutyrate was subtracted from the values obtained for the incubated samples.

Determination of the weight of sterol synthesized. To the purified desmostenone was added small quantities (usually 25–500 ng, depending on the type of experiment) of stigmasta-4,22-dien-3-one as internal standard, and the stenones were converted into their heptafluorobutyryl enol esters (cholesta-4,24-dien-3-yl heptafluorobutyrate and stigmasta-4,22-dien-3-yl heptafluorobutyrate respectively) by dissolving in benzene (50 μ l) followed by addition of heptafluorobutyric anhydride (50 μ l). The mixture was heated to 60°C for 20 min, and the solvents evaporated. Portions of the mixture were removed for assay of mass by g.l.c. with electron-capture detection (Gibbons & Pullinger, 1977a). The weight of cholestenone was measured directly by g.l.c. with flame-ionization detection. The radioactivity of the desmostenone derivative and of the cholestenone were then determined by liquid-scintillation counting. Corrections for quenching were made by the external-standard-ratio method. To account for manipulative losses of sterols and their derivatives during the purification procedure, all observed values for mass and radioactivity were normalized in terms of the amounts produced per 100 μ g of endogenous cellular cholesterol. The small weight of cholesterol formed during the incubation (usually 10–20% of the desmosterol) was calculated from the radioactivity of the purified cholestenone and the observed specific radioactivity (d.p.m./nmol) of the derivative of the newly synthesized desmosterol. The formula for calculating the normalized total weight (nmol) of sterol synthesized during a particular time period is therefore:

$$\frac{\text{Cholestenone radioactivity (d.p.m.)}}{\text{Desmostenone specific radioactivity (d.p.m./nmol)}} + \frac{\text{Weight of desmostenone (nmol)} \times 100}{\text{Weight of cholestenone } (\mu\text{g})}$$

(in nmol of total sterol synthesized per 100 μ g of cellular cholesterol).

Determination of the contribution of labelled precursors to cholesterol biosynthesis. The contributions of the exogenous ^{14}C -labelled precursors were determined from their specific radioactivities, the observed radioactivities of the purified sterone fractions and the known patterns of precursor incorporation into sterols. The calculated amounts (nmol) were then normalized to give the weights per 100 μ g of endogenous cholesterol. For instance, the contribution due to $[2-^{14}\text{C}]\text{acetate}$ is given by the expression:

$$\frac{\text{Cholestenone+desmostenone radioactivity (d.p.m.)}}{15 \times \text{Acetate sp. radioactivity (d.p.m./nmol)}} \times \frac{100}{\text{Weight of cholestenone } (\mu\text{g})}$$

(in nmol/100 μ g of endogenous cholesterol).

The contributions due to $[\text{U-}^{14}\text{C}]\text{pyruvate}$ and $[\text{U-}^{14}\text{C}]\text{lactate}$ per 100 μ g of endogenous cholesterol are given by the expression:

$$\frac{\text{Cholestenone+desmostenone radioactivity (d.p.m.)}}{9 \times \text{Precursor sp. radioactivity (d.p.m./nmol)}} \times \frac{100}{\text{Weight of cholestenone } (\mu\text{g})}$$

With $[\text{U-}^{14}\text{C}]\text{glucose}$ the expression is similar to that above, except that the precursor specific radioactivity must be multiplied by a factor of 4.5 rather than 9.0. With $[1,5-^{14}\text{C}_2]\text{citrate}$, the situation is more complex, owing to the randomization of label before entry into the sterol pathway. For convenience, it has been assumed that in the cytosol one molecule of citrate gives rise to one molecule of acetyl-CoA (labelled at C-1) having a molar specific radioactivity only 50% that of the original citrate. The expression for calculation of the normalized citrate contribution is the same as that above, except that the precursor specific radioactivity is multiplied by a factor of 7.5. Even if the label is completely randomized before incorporation, this would not affect the main conclusion regarding the contribution of citrate to cholesterol biosynthesis (see Table 3 below). In those incubations containing ^3H , the formula of Brunengraber *et al.* (1972):

i.e. Sterol synthesized (nmol) =

$$\frac{1.31 \times \text{Cholestenone+desmostenone radioactivity (d.p.m.)}}{13.5 \times ^3\text{H}_2\text{O sp. radioactivity (d.p.m./nmol)}}$$

or of Lakshmanan & Veech (1977):

$$\frac{\text{Cholestenone+desmostenone radioactivity (d.p.m.)}}{14.5 \times ^3\text{H sp. radioactivity (d.p.m./ng-atom)}}$$

were used to calculate the relationship between ^3H incorporation and sterol synthesis. These values were again normalized as described above.

Materials

The synthesis of non-radioactive cholest-4-en-3-one and cholesta-4,24-dien-3-one, the preparation of fatty acid-free bovine serum albumin, the purification of propane-1,2-diol and the sources of triparanol, collagenase, Nembutal, heparin and buffer constituents were described previously (Gibbons & Pullinger, 1977a). Stigmasta-4,22-dien-3-one was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A., and the material was twice recrystallized from methanol to give crystals of m.p. 129–131°C, $[\alpha]_D +56^\circ\text{C}$; reported m.p. 124°C, $[\alpha]_D +62^\circ\text{C}$ (Brooks, 1970).

Amino acids and the sodium salts of pyruvic acid and L(+)-lactic acid were also obtained from the same sources. Citric acid (monohydrate) was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., and was of analytical quality. Cholesterol oxidase (25 units/ml) and cholesterol esterase (20 units/ml) were supplied by Boehringer, Lewes, East Sussex, U.K. Triton X-100 was obtained from Nuclear Enterprises, Edinburgh 11, Scotland, U.K.

As most batches of commercial heptafluorobutyric anhydride from several sources were received in various states of impurity and reactivity, the crude liquid was fractionally distilled and the fraction boiling at 109–110°C was collected. This procedure was twice repeated and the resulting pure reagent was stored in a foil-covered glass tube at -20°C surrounded by desiccant. Peroxides, which led to desmostenone degradation during the isolation and purification procedure, were removed by distillation of the diethyl ether over reduced iron by using a water bath at 60°C as the source of heat. Immediately before use the distillate was passed through a foil-covered alumina column (activity grade I; M. Woelm, Darmstadt, Germany) into a foil-covered glass vessel. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Effect of time on the rate of sterol synthesis

Liver cells from normal or 24h-starved animals were incubated for various periods of time in the presence of $[2-^{14}\text{C}]\text{acetate}$ (3.34 mM) as the extracellular source of sterol carbon. At the end of each time period the cells were assayed for total sterol synthesis and for the contribution made by the exogenous acetate. The results are presented graphically in Fig. 1, which shows that, with cells from normal animals, the total weight of sterol synthesized during the first hour was considerably lower than

Fig. 1. Effect of time on the rate of sterol biosynthesis in liver cell suspensions

Liver cell suspensions from (a) normal (4.12×10^6 cells) or (b) 24h-starved animals (4.32×10^6 cells) were incubated in the presence of Krebs-Henseleit (1932) buffer, pH 7.4, containing albumin (1.1%), amino acids (East *et al.*, 1973), glucose (11.1 mM), [$2\text{-}^{14}\text{C}$]acetate (3.34 mM, 30 μCi) and a solution of triparanol in propane-1,2-diol (24 μl) such as to give an effective drug concentration of 2 μM . The final volume was 12.0 ml contained in a 100 ml stoppered silane-treated conical flask. The mixture was incubated at 37°C with gentle shaking (70 oscillations/min). Portions of cell suspension were removed at the times indicated and assayed for total sterol synthesis and [^{14}C]acetate incorporation into sterols. The original liver suspension from the normal animals contained 4.9 mg of protein/ml and that from the 24h-starved animals contained 3.5 mg of protein/ml. ●, Total sterol synthesized; ▲, amount of sterol due to extracellular acetate.

that observed during subsequent hourly intervals. The total weight of sterol produced in the cells from 24h-starved rats was much lower than in normal cells, but again there was a marked stimulation of sterol synthesis at later time intervals. The ability of extracellular acetate to provide carbon for sterogenesis varied during the 6 h incubation period. The contribution of [$2\text{-}^{14}\text{C}$]acetate (as reflected by the specific radioactivity of the newly synthesized sterol) reached a maximum in the normal cells after 1–2 h and in the starved cells after 2–3 h. However, despite the fact that during a part of the remaining period acetate contributed a constant or increasing absolute increment, this represented a gradually declining proportion of the newly synthesized sterol (Table 1). The length and steepness of the accelerating phase of cholesterol synthesis varied considerably between cells from similarly treated animals but prepared on different occasions. The continuous gradual acceleration observed in the present case with the normal cells should therefore not be regarded as significant when compared with the steep but short-lived accelerating phase observed in this particular cell preparation from starved animals. On every occasion, however, both an accelerating phase and a pattern of acetate incorporation similar to that described above have been observed. For instance, in other experiments, there was a significant difference between the contribution of acetate to sterol synthesis during the first 3 h and the last 3 h of incubation. In the normal animal, the average contribution during the first, second and third hour was 33% (± 3.39 s.e.m.), whereas the average contribution during the fourth, fifth and sixth hour was 13% (± 1.43 s.e.m.). This difference is significant at the $P < 0.05$ level. The corresponding values for cells from starved animals were 42% (± 2.54 s.e.m.) and 21% (± 5.10 s.e.m.). Again this is a significant difference ($P < 0.05$).

Effect of extracellular acetate concentration on the rate of sterol biosynthesis

Liver cell suspensions from normal or 24h-starved animals were incubated with different concentrations of [$2\text{-}^{14}\text{C}$]acetate (Table 2). In each case the total weight of sterol synthesized during a 1 h incubation was determined. The amount of sterol formed exclusively from the extracellular acetate was also calculated in each case and the results are shown in Table 2. Irrespective of whether the cells originated from normal or starved animals, exogenous acetate concentration had no effect on the total rate of sterol synthesis, although the proportion of sterol contributed by the acetate increased with its extracellular concentration. Although the contribution of acetate to total sterol production varied from preparation to preparation, so far we have never observed a situation in which the sterol carbon was derived exclusively

Table 1. *Time-dependence of [2-¹⁴C]acetate contribution to sterol synthesis in liver cells of normal and 24h-starved rats*
Incubations were conducted with cells from normal and 24h-starved rats for various periods of time and both the weight and [¹⁴C]radioactivity of the newly synthesized sterol were assayed at each time interval. For other details see the legend to Fig. 1.

Time (h)	Normal		24h-starved	
	Sp. radioactivity of sterol (d.p.m./nmol)	Contribution (%) of acetate to total sterol synthesized	Sp. radioactivity of sterol (d.p.m./nmol)	Contribution (%) of acetate to total sterol synthesized
0.25	6629	26.7	3445	13.0
0.50	11968	48.2	7927	32.2
1.00	14279	57.6	14007	56.7
2.00	14441	58.3	13573	54.9
3.00	12936	52.2	14765	59.6
4.00	10885	44.0	12929	52.2
5.00	8747	35.3	12276	49.6
6.00	7830	31.9	12119	49.0

Table 2. *Effect of extracellular acetate concentration on sterol synthesis*

Portions of liver cell suspension from normal (9.30×10^5 cells) or 24h-starved animals (1.41×10^6 cells) were preincubated for 1 h at 37°C in the presence of Krebs-Henseleit (1932) bicarbonate buffer, pH 7.4, containing albumin (1.1%), amino acids (East *et al.*, 1973) and glucose (11.1 mM) in a total volume of 2.7 ml per 25 ml silane-treated stoppered conical flask. The flasks were shaken gently at 70 oscillations/min. A solution of triparanol (to give an effective concentration of 2 μ M) in propane-1,2-diol (6 μ l) was added to each flask simultaneously with solutions of [2-¹⁴C]-acetate to give initial concentrations ranging from 5.75×10^{-3} mM to 10 mM. The cell suspensions (total volume 3.0 ml) were incubated under the same conditions for a further 1 h, whereupon the cells were inactivated by heating and assayed for total sterol synthesis and [2-¹⁴C]acetate incorporation.

	[Extracellular acetate] (mM)	Total sterols synthesized (nmol)*	Weight of sterol due to acetate (nmol)*	Percentage of total sterol due to acetate
Normal cells	5.75×10^{-3}	7.08	0.30	4.2
	3.34	8.44	1.96	23.2
	6.67	7.50	1.95	26.0
	10.00	7.84	2.12	27.0
Cells from 24h-starved animals	5.75×10^{-3}	0.82	0.01	0.2
	0.34	0.88	0.06	7.0
	0.67	0.64	0.09	14.5
	1.67	0.78	0.14	18.6
	3.34	0.81	0.19	22.9
	6.67	0.73	0.20	27.0
	10.00	0.68	0.22	32.5

* Per 100 μ g of endogenous cellular cholesterol.

from acetate. No significant difference between relative contributions of acetate to sterol biosynthesis in normal or starved animals has been observed.

Contribution of glycolytic and tricarboxylic acid-cycle intermediates to sterol biosynthesis

As a result of its early success in helping to unravel the biosynthetic mechanism of cholesterol synthesis, acetate has long been a popular substrate for investigating the regulatory aspects of this process. Its physiological relevance as a natural cholesterol precursor in non-ruminant animals, however, is questionable (Rosmos & Leveille, 1974). Thus the acetyl-CoA required for cholesterol synthesis is most

probably produced as a result of carbohydrate metabolism and/or β -oxidation of fatty acids rather than by direct activation of acetate. Because of this, and in view of the demonstrated inability of acetate to provide more than a fraction of the carbon required for cholesterol synthesis, we have studied the capacity of other, perhaps more relevant, metabolites to supply the necessary quantity of cytosolic acetyl-CoA. In the following experiments, therefore, we have compared the total weight of sterol synthesized with that produced exclusively from glucose, citrate, pyruvate or lactate, the last three being present at various initial extracellular concentrations. The results are shown in Table 3, from which several points are clear. Firstly, increasing the extracellular

Table 3. *Effect of intermediary metabolite concentrations on sterol synthesis*

Portions of liver cell suspension (5.5×10^5 cells) were preincubated for 1 h (see Table 2 legend for other details) after which a solution of triparanol (to give an effective concentration of $2 \mu\text{M}$) in propane-1,2-diol ($6 \mu\text{l}$) was added. Simultaneously, a solution of [^{14}C]pyruvate, [^{14}C]lactate or [^{14}C]citrate was added to give initial concentrations of each ranging from 0.1 to 3.0 mM. [^{14}C]Glucose was also added to two of the preincubated suspensions to label the bulk of material (11.1 mM initially) already present. The incubations (total volume 3.0 ml) were continued for a further 1 h, after which enzymic activity was destroyed by heating. The weight and ^{14}C radioactivity of the newly synthesized sterol were determined in each case. The contributions of the extracellular labelled metabolites to sterol synthesis were determined by application of the formulae shown in the Experimental section.

Extracellular substrate	Concentration (mM)	Total sterol synthesized (nmol)*	Weight of sterol due to extracellular substrate (nmol)*	Percentage of total sterol due to extracellular substrate
Lactate	0.1	4.083	0.116	2.8
	1.0	3.924	1.086	27.6
	3.0	4.440	3.062	69.0
Pyruvate	0.1	4.420	0.217	4.9
	1.0	4.456	1.562	35.0
	3.0	4.579	4.797	104.7
Citrate	0.1	2.969	0.009	0.3
	1.0	3.330	0.065	1.9
	3.0	4.169	0.246	5.9
Glucose†	11.1	3.506	0.186	5.6

* Per $100 \mu\text{g}$ of endogenous cellular cholesterol.

† Performed in duplicate; reported values are an average of the two results.

concentration of the first three metabolites did not result in an increase in the rate of sterogenesis, although at higher concentrations each made a greater contribution to the total sterol carbon. This pattern therefore resembles that observed with acetate as the extracellular precursor. Secondly, glucose at 11.1 mM (which is close to the physiological circulating concentration) was incapable of providing more than 5–6% of the carbon required for sterol synthesis. Citrate was also a poor precursor, its low contribution probably resulting from the impermeability of the plasma membrane to trianions of this type (Krebs *et al.*, 1973). Thirdly, lactate, and especially pyruvate, contributed to sterol synthesis to a far greater extent; the latter at an initial concentration of 3 mM was capable of providing all the carbon required. This probably results from a large dilution of the endogenously produced acetyl-CoA by that produced from the extracellular pyruvate, in which case the size of the acetyl-CoA pool available for cholesterol synthesis must have been considerably increased.

Effect of extracellular substrate on the time-dependent increase in the rate of cholesterol synthesis

Experimental conditions under which cellular cholesterol synthesis occurs at an accelerating rate have been described above (Fig. 1). In all these experiments, the cells were supplemented with an extra source of metabolizable carbon in the form of acetate

(3.34 mM). It is probable that the acetate, temporarily at least, expands the intracellular pool of substrates (e.g. acetyl-CoA) available for cholesterol synthesis. Conceivably, this could lead to activation of the rate-limiting step for cholesterol synthesis, perhaps in a manner similar to that by which substrate, or substrate precursors, are known to activate pyruvate dehydrogenase (Patzelt *et al.*, 1973) or acetyl-CoA carboxylase (Lane *et al.*, 1971; Goodridge, 1973). Accordingly, cell suspensions were incubated for periods of 1 or 2 h in the standard incubation medium containing triparanol ($2 \mu\text{M}$ added as a solution in propane-1,2-diol) and only trace quantities ($16.7 \mu\text{M}$) of [^{14}C]acetate. This was added simply as a means of locating the steroids on the thin-layer chromatogram by radioautography, and its contribution to sterol synthesis at such low concentrations is negligible (Table 2). Sterol synthesis in these cases is maintained almost exclusively from endogenous sources and the normal size of the cholesterol precursor pool is virtually unchanged. Similar incubations were conducted in which the acetate was replaced by much larger (5 mM) concentrations of [^{14}C]pyruvate that had already been shown (see above) to be capable of greatly expanding the size of the cholesterol precursor pool. At the end of the allotted time period, enzymic activity was destroyed by heating, and both the total sterol synthesized, and, where applicable, that formed exclusively from the exogenously added [^{14}C]pyruvate, were measured. The results are presented in Table 4,

Table 4. *Effect of substrate availability on the time-dependent increase in the rate of sterol biosynthesis*

Portions (8.50×10^5 cells) of a liver cell suspension from normal rats were incubated for 1 h or 2 h periods in the standard incubation medium containing triparanol ($2.0 \mu\text{M}$) and propane-1,2-diol ($6 \mu\text{l}$). In addition, the flasks contained either $[2\text{-}^{14}\text{C}]\text{acetate}$ or $[\text{U-}^{14}\text{C}]\text{pyruvate}$ at the concentrations shown. The total volume of each incubation mixture was 3.0 ml. At the end of the allotted incubation period, enzyme activity was destroyed by heating and the weight and (where applicable) the ^{14}C radioactivity of the newly synthesized sterol were determined in each case. The contribution of $[\text{U-}^{14}\text{C}]\text{pyruvate}$ to sterol synthesis was determined by application of the formula shown in the Experimental section. For other details, the legend to Table 2 should be consulted. Each incubation was conducted in duplicate and the reported values are an average of the two.

Extracellular substrate and concentration	Weight of sterol formed during the first hour (nmol/100 μg of endogenous cholesterol)		Weight of sterol formed during the second hour (nmol/100 μg of endogenous cholesterol)		Increase in total rate during the second hour (%)
	Total	From exogenous source	Total	From exogenous source	
Pyruvate (5 mM)	4.15	3.75	8.71	3.77	109.8
Acetate (16.7 μM)	3.77	—	7.67	—	103.4

from which it is clear that increasing the availability of sterol precursors, over and above that which is normal, from exogenous sources (pyruvate) during the first hour had very little further effect on the rate of cholesterol synthesis as measured during the second hour. Again, the absolute rates of sterol synthesis were almost the same irrespective of the presence or virtual absence of an extracellular cholesterol precursor. The relative decrease in the contribution of pyruvate to sterol synthesis during the second hour probably reflects the rapid mitochondrial oxidation of this substrate (Portenhauser & Wieland, 1972), so that its effective concentration, and therefore its availability for sterol synthesis, is gradually diminished.

Incorporation of ^3H from $^3\text{H}_2\text{O}$ during sterol synthesis

The known specific radioactivity of $^3\text{H}_2\text{O}$ in incubations or perfusions designed to measure rates of cholesterol synthesis eliminates the problem of dilution of other radioactive substrates by endogenous sources to give a precursor pool the effective specific radioactivity of which is unknown. However, the number of ^3H atoms incorporated per atom of newly synthesized sterol carbon may differ according to the metabolic source of the sterol precursor (Gibbons & Pullinger, 1977a) or of the necessary reducing equivalents (Jungas, 1968; Lakshmanan & Veech, 1977). Accordingly, we have compared the rates of sterol synthesis as measured directly with the chromatographic method described above and elsewhere (Gibbons & Pullinger, 1977a,b) with those rates calculated on the basis of ^3H incorporation from $^3\text{H}_2\text{O}$ by using the relationships derived by two independent groups of workers (see the Experimental section). In these experiments, the weight and ^3H content of desmosterol (isolated as desmostenone) formed in incubations with $^3\text{H}_2\text{O}$ were determined.

Table 5 shows the calculated weights of sterol synthesized as percentages of the actual observed values at various time intervals during a 6 h incubation of cells from normal or 24 h-starved animals. The most important point is not that the calculated rates generally overestimate or underestimate the true rates (depending on the formula adopted), but that the difference between the real and calculated rates is not constant over the time period studied. In a series of experiments with liver cells from normal animals, the average calculated rate of synthesis based on

Table 5. *Comparison of the rates of sterol synthesized measured by using the chromatographic method and ^3H incorporation from $^3\text{H}_2\text{O}$*

Liver cell suspensions from normal (3.89×10^6 cells) or 24 h-starved (5.88×10^6 cells) animals were incubated in the standard incubation medium containing a solution of triparanol (to give an effective concentration of $2.0 \mu\text{M}$) in propane-1,2-diol ($24 \mu\text{l}$) and $^3\text{H}_2\text{O}$ (80 mCi) in a total volume of 12.0 ml. At hourly intervals a portion of the suspension was removed and the weight and ^3H content of the newly synthesized desmosterol were measured. The ^3H incorporation was converted into the weight of sterol synthesized by using the formulae derived either by Brunengraber *et al.* (1972) (Method B) or by Lakshmanan & Veech (1977) (Method L), and in each case the calculated results are presented as percentages of the observed weight of newly synthesized desmosterol. For other details see the legend to Fig. 1.

Time (h)	Normal cells		24 h-starved cells	
	Method B	Method L	Method B	Method L
1	61.3	86.3	65.9	93.3
2	83.4	117.5	61.7	87.3
3	86.6	122.3	70.0	98.9
4	78.4	110.5	80.5	113.8
5	86.9	122.5	99.7	142.1
6	87.7	123.8	111.4	157.6

^3H incorporation during the first hour of incubation was 61% (± 3.21 S.E.M.; $n=14$) of the observed rates. During the second hour, however, this value increased to 76% (± 2.11 S.E.M.; $n=14$). This difference is significant at the $P<0.02$ level.

Discussion

The inability of acetate, even at high concentrations, to provide more than a small fraction of the total carbon required for sterol synthesis reflects either the impermeability of the cell membrane to this material or, more likely, a relatively low activity of some enzyme responsible for its conversion into sterol. In view of the observation that acetyl-CoA produced from pyruvate or lactate (Table 3) is much more efficiently transformed into sterol, the conversion of acetate must be restricted by the activity of acetyl-CoA synthetase (EC 6.2.1.1). Thus the assumption that the entire sterol carbon may be derived from acetate provided that it is present in sufficiently high concentration is not necessarily valid. The popular belief that the extent of acetate incorporation into sterol reflects the rate of sterol synthesis is also discredited by the present observations (Table 2). This may be true (but not necessarily) in mitochondria-free cell extracts, but it appears that, in the intact cells, sufficient endogenous acetyl-CoA is produced by catabolic processes to realize the full sterol-synthesizing capacity of the cell under a particular set of conditions. Hence, increasing the concentration of extracellular acetate results merely in an increasing dilution of the pre-existing stream or pool of endogenously produced acetyl-CoA and this is reflected in an increased contribution of acetate to sterol synthesis (Table 2). A wide variation in acetate incorporation is thus observed, although the true rate of sterol synthesis remains unchanged. A similar situation has been described during cholesterol synthesis in perfused rat liver (Barth *et al.*, 1972).

In view of the large difference between the total weights of sterol synthesized and those which acetate is capable of providing, the question arose as to the natural source of the endogenously produced acetyl-CoA used for cholesterol synthesis. Intermediary metabolites such as lactate, which are capable of generating pyruvate, provide a substantial quantity of the acetyl-CoA required for fatty acid synthesis in perfused mouse liver (Salmon *et al.*, 1974). In the present work, lactate, and especially pyruvate, at the relatively low concentration of 3 mM were capable of providing all, or nearly all the carbon required for cholesterol synthesis. Again, although increasing concentrations of these substrates led to their increasing contribution to sterol synthesis, the total amount of sterol synthesized remained unchanged, thereby confirming the conclusion reached in the previous experiments in which acetate was

used. These results strongly suggest that, at least within the short term, the rate of provision of primary metabolites such as acetyl-CoA from endogenous sources is not rate-limiting for hepatic cholesterol synthesis *in vivo*.

If metabolites such as pyruvate and lactate are indeed the natural precursors of the acetyl-CoA used for sterol synthesis, it is unlikely that they are derived from circulating glucose, since, even at a concentration similar to that found in peripheral blood of fed rats (Brunengraber *et al.*, 1973), glucose is capable of providing only 5–6% of the total sterol carbon (Table 3). The inability of glucose to provide substrate for lipogenesis has also been recognized in the perfused livers of mouse and rat (Salmon *et al.*, 1974; Hems, 1977; Brunengraber *et al.*, 1973). Alternative sources of pyruvate, and thus of cholesterologenic acetyl-CoA, are amino acids such as alanine, and glycogen. In the latter instance concentrations of liver glycogen have been correlated with the rates of lipogenesis in perfused mouse liver (Salmon *et al.*, 1974).

If glycogen is indeed the ultimate source of a large proportion of the sterol carbon, it is able to maintain sterol synthesis at an accelerating rate for up to 5 h (Fig. 1). The cause of this apparent stimulation or de-repression of cholesterol synthesis is obscure, but a similar situation with respect to labelled acetate incorporation occurs in various cultured cell lines (Bailey, 1977) incubated in the absence of cholesterol-containing material. In isolated liver cell suspensions similar to those used in the present work, the increased rate of cholesterol synthesis has been ascribed to an increase in the activity of the enzyme hydroxymethylglutaryl-CoA reductase (Edwards, 1975a), which is brought about by a continuous efflux of cellular cholesterol into the medium (Edwards, 1975b; Edwards *et al.*, 1976). This process is said to be enhanced by the extracellular presence of a phospholipid-protein complex (Edwards *et al.*, 1976). In the present work, although extracellular cholesterol concentrations were not measured, if cholesterol outflow is indeed responsible for increased cholesterol synthesis the presence of such an extracellular complex was not obligatory in effecting the efflux.

It is possible, however, that the continuous secretion of very-low-density lipoprotein, of which non-esterified cholesterol is an essential structural constituent, from the cell (Jeejeebhoy *et al.*, 1975) represents a net loss of cellular cholesterol that is compensated for by an increase in the rate of cholesterol synthesis. Such a relationship between the rates of very-low-density-lipoprotein secretion and cholesterol synthesis has been demonstrated in perfused rat liver (Goh & Heimberg, 1977). It is possible therefore that factors that increase the rate of fatty acid synthesis and secretion in the form of very-low-density lipoprotein may, after a certain time, increase the

rate of cholesterologenesis. In any case, the initial relatively low rates of cholesterol synthesis were clearly not due to substrate deficit (Harris, 1975; Lakshmanan *et al.*, 1977), as increasing the rate of substrate supply during this period did not result in an increased rate of synthesis (Tables 2, 3 and 4).

The relatively small contribution of exogenous acetate to sterol synthesis during the early part of the incubation period may have been due to a preferential utilization of pools of endogenous partially formed cholesterol precursors. These pools may include both water-soluble and triterpenoid cholesterol precursors, and, in the latter case, the contribution of these pools to cholesterol synthesis in cell-free homogenates of liver has been demonstrated (Gibbons, 1977). As these pools become increasingly labelled from newly synthesized material, so the specific radioactivity of the newly synthesized sterol increases (Table 1). The relatively low incorporation of ^3H from $^3\text{H}_2\text{O}$ during these early stages (Table 5) may also be explained in these terms, since fewer new C-H bonds are formed during sterol synthesis from these partially formed precursors (Nilsson, 1975; Lakshmanan & Veech, 1977). If this method is used to compare rates of sterol synthesis, therefore, one must ensure that the sources of sterol precursors are the same.

The declining contribution of acetate to sterol synthesis during the later stages of the incubation could be due to a decline in the rate of formation of acetyl-CoA from exogenous labelled acetate, an increase in the rate at which non-radioactive acetyl-CoA is formed from endogenous sources, or a combination of both. During this period, however, measurement of the incorporation of label from acetate in no way reflects changes in the true rate of sterol synthesis.

Although the rate of sterol synthesis increases with long incubation periods, increasing the availability of sterol precursors within the early stages does not lead to any subsequent further increase in the synthetic rate. The rate-determining enzyme of cholesterol synthesis therefore appears to differ from other key lipogenic enzymes such as pyruvate dehydrogenase and acetyl-CoA carboxylase, both of which can be activated within a very short period by the presence of higher concentrations of their substrate or substrate precursors.

References

- Bailey, J. M. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 2, pp. 323-352, Plenum Press, London and New York
- Barth, C., Liersch, M., Hackenschmidt, J., Ullman, H. & Decker, K. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1085-1093
- Brooks, C. J. W. (1970) in *Rodd's Chemistry of Carbon Compounds* (Coffey, S., ed.), vol. 2, part D, pp. 1-193, Elsevier, Amsterdam, London and New York
- Brunengraber, H., Sabine, J. R., Boutry, M. & Lowenstein, J. M. (1972) *Arch. Biochem. Biophys.* **150**, 392-396
- Brunengraber, H., Boutry, M. & Lowenstein, J. M. (1973) *J. Biol. Chem.* **248**, 2656-2669
- Dugan, R. E., Slakey, L. L., Briedis, A. V. & Porter, J. W. (1972) *Arch. Biochem. Biophys.* **152**, 21-27
- East, A. G., Louis, L. N. & Hoffenberg, R. (1973) *Exp. Cell Res.* **76**, 41-46
- Edwards, P. A. (1975a) *Arch. Biochem. Biophys.* **170**, 188-203
- Edwards, P. A. (1975b) *Biochim. Biophys. Acta* **409**, 39-50
- Edwards, P. A., Fogelman, A. M. & Popják, G. (1976) *Biochem. Biophys. Res. Commun.* **68**, 64-69
- Fears, R. & Morgan, B. (1976) *Biochem. J.* **158**, 53-60
- Gibbons, G. F. (1977) *Biochem. Biophys. Res. Commun.* **75**, 995-1003
- Gibbons, G. F. & Pullinger, C. R. (1977a) *Biochem. J.* **162**, 321-330
- Gibbons, G. F. & Pullinger, C. R. (1977b) *Biochem. Soc. Trans.* **5**, 1003-1004
- Goh, E. H. & Heimberg, M. (1977) *J. Biol. Chem.* **252**, 2822-2826
- Goodridge, A. G. (1973) *J. Biol. Chem.* **248**, 4318-4326
- Harris, R. A. (1975) *Arch. Biochem. Biophys.* **169**, 168-180
- Hems, D. A. (1977) *Biochem. Soc. Trans.* **5**, 886-890
- Jeejeebhoy, K. N., Ho, J., Breckenridge, C., Bruce-Robertson, A., Steiner, G. & Jeejeebhoy, J. (1975) *Biochem. Biophys. Res. Commun.* **66**, 1147-1153
- Jungas, R. L. (1968) *Biochemistry* **7**, 3708-3717
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33-66
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1973) *Proc. Alfred Benzon Symp.* **6**, 726-795
- Lakshmanan, M. R. & Veech, R. L. (1977) *J. Biol. Chem.* **252**, 4667-4673
- Lakshmanan, M. R., Muesing, R. A., Cook, G. A. & Veech, R. L. (1977) *J. Biol. Chem.* **252**, 6581-6584
- Lane, M. D., Moss, J., Ryder, E. & Stoll, E. (1971) *Adv. Enzyme Regul.* **9**, 237-251
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Nervi, F. O., Carrella, M. & Dietschy, J. M. (1976) *J. Biol. Chem.* **251**, 3831-3833
- Nilsson, Å. (1975) *Eur. J. Biochem.* **51**, 337-342
- Patzelt, C., Löffler, G. & Wieland, O. H. (1973) *Eur. J. Biochem.* **33**, 117-122
- Portenhauser, R. & Wieland, O. (1972) *Eur. J. Biochem.* **31**, 308-314
- Rodwell, V. W., Nordstrom, J. L. & Mitschelen, J. J. (1976) *Adv. Lipid Res.* **14**, 1-74
- Rosmos, D. R. & Leveille, G. A. (1974) *Adv. Lipid Res.* **12**, 97-146
- Salmon, D. M. W., Bowen, N. L. & Hems, D. A. (1974) *Biochem. J.* **142**, 611-618