EXISTENCE OF METABOLIC RESERVOIRS IN THE LATER STAGES OF THE CHOLESTEROL BIOSYNTHETIC PATHWAY

By

GEOFFREY F. GIBBONS

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

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SUMMARY

Incubation of a rat-liver cell-free system in the presence of a high concentration of mevalonic acid resulted in a high rate of cholesterol biosynthesis in which a large proportion of the newly biosynthesized cholesterol was produced from endogenous sources. Endogenous sources made no contribution when cholesterol biosynthesis proceeded at a lower rate in the presence of acetate or low concentrations of mevalonic acid. It is suggested that these endogenous sources are triterpenoid in nature and that they contribute to the precursor pool only when cholesterol synthesis is driven at a high rate. This was confirmed by comparing the contribution of the endogenous lipid-soluble precursors *in vitro* under conditions in which the *in vivo* rate of cholesterol biosynthesis varied considerably.

The presence of "non-metabolically active" pools of cholesterol precursors has been demonstrated in the past (1, 2). The physiological importance of these slowly equilibrating pools is at present obscure, and the present work has been carried out in order to compare the contribution of these compartments to cholesterol biosynthesis during periods in which the rate of cholesterol production varies considerably. This has been achieved in cell-free systems of rat liver by measurement of the specific radioactivity of the penultimate cholesterol precursor, 5α-cholest-7-en-3β-ol, which accumulated (4) during various periods of incubation in which cholesterol was biosynthesized in the presence of labelled substrates such as [2-^{14}C]acetate, [2-^{14}C] mevalonic acid (MVA) and [^{14}C]lanosterol. Comparison of the observed specific radioactivity of 5α-cholest-7-en-3β-ol with that of the exogenous labelled precursors provides a measure of the contribution by endogenous sources to cholesterol biosynthesis.

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MATERIALS AND METHODS

Male rats of the Wistar strain weighing betweem 125 g and 150 g were used. The animals were housed in a windowless room at 23° which was artificially lighted only between the hours of 9.00 a.m. and 7.00 p.m. Food and water were given ad libitum. The preparation of sub-cellular fractions and their incubation with labelled precursors in the presence of cofactors was carried out in a manner similar to that described previously (2, 3), except that air was used as the gas phase during incubation. Under these incubation conditions, 5α-cholest-7-en-3β-ol accumulates in quantities large enough for measurement of its mass by GC. Procedures for the preparation of [^{14}C]lanosterol (2), isolation of the total lipid fraction (2) or of the non-saponifiable lipid fraction (5) and isolation of the radioactive cholesterol and 5α-cholest-7-en-3β-ol as the acetate derivatives (4) have been described previously. However, in the present work, carrier 5α-cholest-7-en-3β-ol was not added to the mixture after incubation. To account for losses of sterols during the purification procedure, a known quantity of [1α,2α-^{3}H]cholesterol was added immediately after the incubations had been terminated.

After removal of a portion of the labelled 5α-cholest-7-en-3β-ol fraction for radioassay, the remainder was transferred to a small conical tube and dissolved in ethyl acetate (25 μl). Portions (1.0 μl) were removed for mass measurement by GC using flame ionisation detection. As this sterol is the penultimate cholesterol precursor (6) and since in non-incubated control samples no endogenous 5α-cholest-7-en-3β-ol could be detected, the specific radioactivity of this precursor must be equal to that of the newly synthesized cholesterol. This was used throughout the present work for determining the specific radioactivity of the cholesterol synthesized during an incubation.

RESULTS AND DISCUSSION

Effect of incubation time on the rate of incorporation of label from [2-^{14}C] acetate and [2-^{14}C]mevalonic acid into sterols

S_{10} fractions of a rat-liver homogenate were incubated with different concentrations of either [2-^{14}C]acetate or [2-^{14}C]mevalonic acid for various periods of time ranging from 10 min to 120 min. Labelled cholesterol and 5α-cholest-7-en-3β-ol were isolated as the acetate derivatives from each incubation and aliquots of each fraction were removed for radioassay. The results are presented in Fig.1. When [2-^{14}C]acetate (0.017 mM) was the cholesterol precursor, there was a lag period of 20-30 min during which time little radioactivity appeared in the sterol fractions. After this time, the sterols became labelled at a linear or slightly accelerating rate. A similar effect has been described previously (7) and was ascribed to a specific activation of enzymes in the earlier stages of the biosynthetic pathway. If this is the case, then the effect may be mediated by a progressive removal of lipoprotein cholesterol from the membrane of the endoplasmic reticulum into the soluble fract-

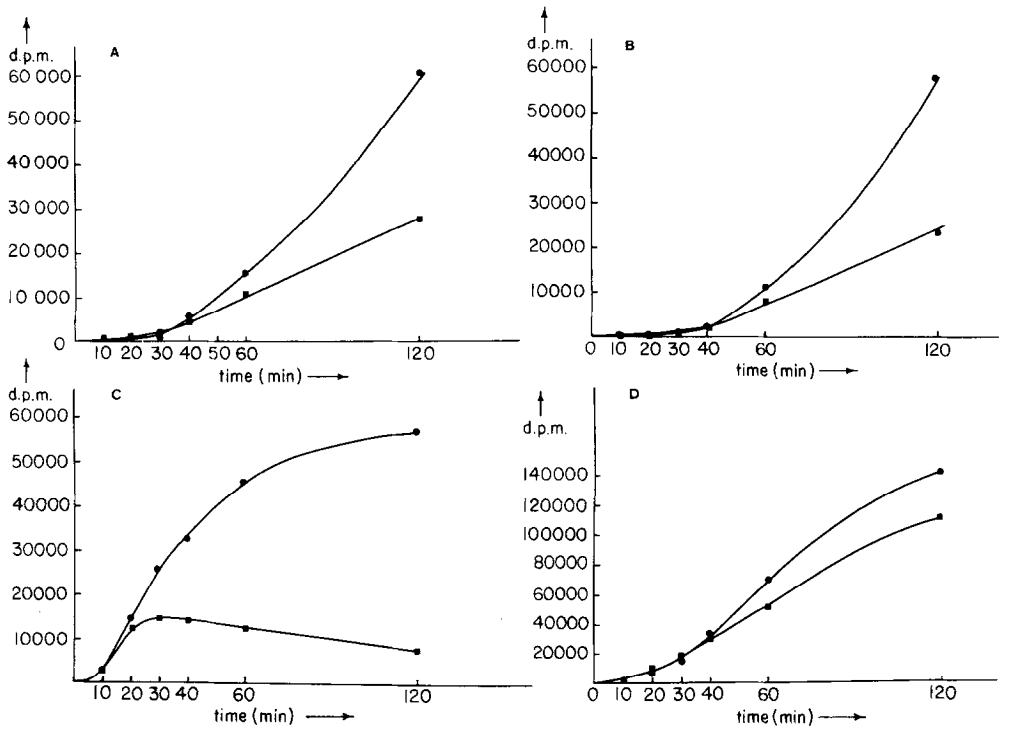

Fig.1. Incorporation of radioactive precursors into sterols. S_{10} (1.0ml) fractions of a rat-liver homogenate were incubated in the presence of either [2-^{14}C]acetate (2μCi, 0.017 mM (A)); [2-^{14}C]-acetate, (20 μCi, 4.14 mM (B)); 3RS-[2-^{14}C]mevalonic acid, (0.2μCi, 0.006 mM (C)); or 3RS- [2- ^{14}C]mevalonic acid, (2 μCi, 3.10 mM (D)) for various periods of time in the presence of cofactors (3, 4). Cholesterol and 5α-cholest-7-en-3β-ol were isolated as the acetate derivatives ●—● , Incorporation into cholesterol; ■—■ , incorporation into 5α-cholest-7-en-3β-ol.

ion (8). This would change the fluidity of the membrane (9, 10, 11,12) resulting in an increase in the activity of certain membrane-bound enzymes (12, 13, 14) such as HMG-CoA reductase (15), an important regulatory enzyme in cholesterol biosynthesis. However, this latent period could also be explained by a preferential utilization of unlabelled endogenous substrates during the early stages of the incubation, a situation which would not be abolished in the presence of higher concentrations of [2-^{14}C]acetate (Fig.1), since, regardless of its concentration, exogenous acetate contributes only a small fraction of the cholesterol substrate supply (16, 17).

TABLE 1

Contribution of endogenous precursors to the synthesis of 5α-cholest-7-en-3β-ol as a function of incubation time in the presence of [2-^{14}C]mevalonic acid (3.1mM).

Time (min)	Specific radioactivity of 5α-cholest-7-en-3β-ol (d.p.m./nmole)	Actual weight of 5α-cholest-7-en-3β-ol produced (nmoles)	Calculated weight[1] of 5α-cholest-7-en-3β-ol produced (nmoles)	% Contribution by endogenous sources[2]
10	1045	1.87	0.27	85.3
20	2475	3.63	1.26	65.2
30	3292	5.28	2.44	53.7
40	4137	7.12	4.14	41.9
60	6073	8.32	7.10	14.6
120	6717	16.07	15.17	5.6

S_{10} fractions of rat liver (1.0 ml, 16.3 mg protein) were incubated in the presence of [2-^{14}C]mevalonic acid (2.0 μCi, 3.10 μmoles) for the periods of time shown above. 5α-cholest-7-en-3β-ol was isolated as the acetate derivative. After removal of an aliquot for radioassay, its mass was determined by GC.

[1]The biosynthesis of one nmole of 5α-cholest-7-en-3β-ol requires 6 nmoles of [2-^{14}C]mevalonic acid (specific radioactivity = 1429 d.p.m./nmole). One of the labelled carbon atoms of the mevalonic acid is removed as the 4α-methyl group during C_{27} sterol synthesis. If all the 5α-cholest-7-en-3β-ol had been synthesized from the exogenous [2-^{14}C] mevalonic acid, therefore, its specific radioactivity would be 1429 x 6 x 5/6 = 7145 d.p.m./nmole. Division of the radioactivity of the 5α-cholest-7-en-3β-ol by this value gives the amount of this material which would have been produced if the exogenous mevalonic acid had not been diluted by endogenous sources.

[2]The difference between the observed weight and the calculated weight of 5α-cholest-7-en-3β-ol reflects that proportion which has been synthesized from endogenous sources.

Although in the presence of trace quantities of [2-^{14}C]mevalonic acid there was a latent phase of only 10 min during which time relatively little radioactivity appeared in the sterol fractions, when the concentration of mevalonic acid was increased to 3.1 mM, the rate at which radioactivity appeared in the sterol fraction continued to increase for 30-40 min, a situation which, although less pronounced, was similar to that which occurred when [2-^{14}C]ace-

tate was the precursor. That the reason for the accelerating incorporation of radioactivity from [2-^{14}C]mevalonic acid was due to an initial preferential utilisation of endogenous substrate was demonstrated by measurement of the specific radioactivity of 5α-cholest-7-en-3β-ol (and thus of the newly biosynthesized cholesterol) produced in the presence of [2-^{14}C]mevalonic acid (3.1 mM) and comparing this with the expected specific radioactivity if the exogenously added mevalonic acid had been utilized exclusively as the cholesterol precursor (Table 1). During short incubation periods, considerable quantities of endogenous precursor were utilized for cholesterol synthesis. It was to be expected, therefore, that this amount represented the normal contribution of endogenous precursors to cholesterol biosynthesis and that, under the same conditions, this absolute contribution would be maintained regardless of the nature or concentration of the exogenous precursors added in vitro. However, the quantity of newly synthesized cholesterol (as measured by 5α-cholest-7-en-3β-ol production) produced during the other incubations (A, B and C, Fig.1) were too small to be detected and were certainly much lower than the weight of 5α-cholest-7-en-3β-ol produced from endogenous sources in the presence of high concentrations of mevalonic acid.

It appeared, therefore, that when cholesterol biosynthesis was driven at a high rate by using a high concentration of a water-soluble precursor which by-passed the primary rate-limiting step, there was a significant contribution from endogenous substrate contained in a compartment which was metabolically isolated during periods of lower biosynthetic activity.

An explanation of this effect may be found in the existence of the "metabolically inactive" or slow equilibrating pools of cholesterol precursors such as squalene (1) and lanosterol (2). Under normal conditions, these compartments do not contribute to cholesterol biosynthesis and are metabolically isolated. However, when cholesterol biosynthetic activity is high, the active intermediate pool may be expanded so that it spills into its corresponding isolated pool. This originally isolated pool now becomes connected with the

mainstream of the cholesterol biosynthetic pathway. If the active pool is labelled, connection with the isolated pool will result in a large dilution of radioactivity by the non-labelled endogenous material and any metabolic product of the combined pool will, during the early stages of the incubation, have a low specific radioactivity (Table 1). Gradually, the specific radioactivity of the combined pool (and of its products, 5α-cholest-7-en-3β-ol and cholesterol) will increase as unlabelled molecules are used up and replaced by labelled ones. Eventually, therefore, the unlabelled molecules will have been replaced and the whole of the combined pool (and of the newly biosynthesized cholesterol) will have been produced exclusively from the exogenous MVA (Table 1).

Contribution of Endogenous sources to cholesterol biosynthesis in the presence of [^{14}C]lanosterol at different times of the day.

It is probable that these endogenous contributions arise from precursors which occur at a stage after mevalonic acid in the biosynthetic sequence since their effect only becomes apparent after the primary rate-limiting step has been by-passed. In view of the known accumulation in liver of the triterpenoid precursors squalene and lanosterol, it seems likely that one or both of these pools are at least partly responsible for the effects described above. To determine whether this was the case during physiologically high rates of cholesterol biosynthesis, we determined the specific radioactivity of the newly synthesized cholesterol produced in the presence of [^{14}C]lanosterol at a time five hours after the onset of the dark phase, when the rate of cholesterol synthesis is high, and during the light phase, when synthesis is lower. The microsomal fraction of the cell was chosen as the source of enzymes since this would eliminate any variation in the contribution to cholesterol synthesis of endogenous water-soluble precursors since these intermediates require soluble enzymes for their metabolism. The use of microsomes therefore ensured that changes in the specific radioactivity of the newly biosynthesized chol-

TABLE 2

Effect of time of day on the contribution of endogenous triterpenoid precursors to cholesterol biosynthesis in the presence of [^{14}C] lanosterol

Time of day	Specific radioactivity of 5α-cholest-7-en-3β-ol (d.p.m./nmole)	Actual weight of 5α-cholest-7-en-3β-ol produced (nmoles)	Calculated weight[1] of 5α-cholest-7-en-3β-ol produced (nmoles)	% Contribution by endogenous sources[2]
12.00	931 ± 93	5.49 ± 0.54	5.94 ± 0.17	0
24.00	399 ± 32	7.92 ± 0.48	3.81 ± 0.57	52

At each time of day, six rats were separated into three groups of two. Microsomal fractions were prepared from the livers of each group and suspended in potassium phosphate buffer (0.1 M, pH 7.4) so that 1.0 ml of suspension contained the microsomes derived from 1.0 g of liver. The microsomal fractions (0.6 ml) from each group were incubated independently for 45 min in the presence of cofactors and [^{14}C]lanosterol (8.71×10^4 d.p.m., 0.094 μ mole). Labelled cholesterol and 5α-cholest-7-en-3β-ol were isolated as the acetate derivatives. Aliquots of the 5α-cholest-7-en-3β-ol fraction were removed for measurement of radioactivity and for assay of mass by GC.

[1]This is the amount of sterol which would have been synthesized if the exogenous [^{14}C]lanosterol (927 d.p.m./nmole) had been used exclusively as the sterol precursor, i.e. assuming no contribution from endogenous sources.

[2]The difference between the observed weight of 5α-cholest-7-en-3β-ol and the calculated weight is a measure of the contribution by endogenous sources.

The values given above represent the standard error of the mean. Each value obtained at 12.00 h was significantly different ($P < 0.05$) from the corresponding value obtained at 24.00 h.

esterol arose solely as a result of any change in the contribution of endogenous water-insoluble triterpenoid cholesterol precursors. The absence of the soluble enzymes also limited the choice of substrate. [^{14}C]Lanosterol, which is metabolised efficiently by microsomal enzymes, was therefore utilized in this investigation.

At each time of day rat-liver microsomes were incubated with [^{14}C]lanosterol, and the radioactive cholesterol and 5α-cholest-7-en-3β-ol fractions

were isolated. The results are presented in Table 2, which shows that the specific radioactivity of the newly biosynthesized cholesterol (measured by that of 5α-cholest-7-en-3β-ol) was significantly lower at midnight than at midday. The proportion of newly synthesized cholesterol which was due to endogenous triterpenoid precursors was therefore greater at midnight (when the *in vivo* rate of cholesterol biosynthesis was high), and at midday, the newly biosynthesized cholesterol was produced exclusively from the exogenously added [^{14}C]lanosterol.

These observations provide evidence for the existence of pools of triterpenoid cholesterol precursors which are either isolated from, or connected to, the major biosynthetic pathway, depending upon the overall rate of cholesterol production. If, during periods of lower cholesterol biosynthetic activity, the stream of "active" intermediates is so delicately balanced that a small initial increase in the capacity of the primary rate-determining step causes the active pool to spill over and bring the isolated pools into the biosynthetic pathway, this would provide a mechanism for a large and almost instantaneous increase in the total rate of supply of triterpenoid precursors in response to a small change in the rate of flow of the "active" metabolites. It is proposed, therefore, that these metabolic reservoirs satisfy the need for a change in the rate of cholesterol synthesis more immediate than can be provided for by a longer-term change in the capacity of the primary rate-limiting step.

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