Studies on the Response of Cholesterol Biogenesis to Feeding in Rats

EVIDENCE AGAINST THE EXISTENCE OF DIURNAL RHYTHMS

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1. The biosynthesis of cholesterol was studied, by using various precursors, in rats subjected to several dietary regimes. 2. The use of $^3\text{H}_2\text{O}$ as a substrate to demonstrate differences in cholesterologenesis under various conditions was validated by using rats fed on cholesterol or cholestyramine. Cholesterol feeding resulted in decreased cholesterologenesis, whereas cholestyramine caused an increase. 3. With acetate as precursor, the biosynthesis of both digitonin-precipitable sterol and fatty acids was increased *in vitro* in response to a meal. 4. In rats fed *ad libitum*, hepatic cholesterologenesis was increased at midnight relative to mid-morning as measured by using acetate precursor *in vitro*. However, no such difference was found by using $^3\text{H}_2\text{O}$ *in vivo*. 5. The lipogenic response was measured in meal-fed rats by using $^3\text{H}_2\text{O}$ or octanoate *in vivo*. In contrast with findings with acetate *in vitro*, no postprandial stimulation of cholesterologenesis was seen with either $^3\text{H}_2\text{O}$ or octanoate as precursor, whereas fatty acid biosynthesis from either substrate was increased. 6. These findings are discussed with respect to current theories about the circadian rhythm of cholesterologenesis. Such theories are based on experiments using isolated enzyme measurements or non-physiological precursors such as acetate. 7. It is considered that results obtained with $^3\text{H}_2\text{O}$ give an accurate representation of cholesterologenesis under various conditions, and it is therefore suggested that hepatic cholesterologenesis in rats is not subjected to the same degree of diurnal rhythm as has previously been believed.

Following the experiments of Kandutsch & Saucier (1969) with mice, a number of workers have purported to show a diurnal rhythm in the biosynthesis of cholesterol in the rat. The experiments have often involved the measurement of the activity of β -hydroxy- β -methylglutaryl-CoA reductase (EC 1.1.1.34), which is believed to be the rate-limiting enzyme in the pathway of cholesterologenesis. Similar experiments have been performed by using the incorporation of acetate into cholesterol in various liver preparations, the measurements commonly being made *in vitro* (Bortz, 1973). Recent evidence has indicated, however, that hydroxymethylglutaryl-CoA reductase may not always be rate-limiting (Dugan *et al.*, 1972), and moreover that acetate is a substrate of doubtful physiological relevance. Therefore experiments on lipogenesis utilizing substrates such as $^3\text{H}_2\text{O}$ (Lowenstein *et al.*, 1975) and sodium octanoate (Dietschy & McGarry, 1974) are now considered to give more meaningful values than studies using acetate. In the present paper we report experiments using $^3\text{H}_2\text{O}$ and labelled sodium octanoate for the measurement of the synthesis of cholesterol and fatty acids under various conditions. We compare the results obtained with these

precursors with measurements made using labelled sodium acetate and present evidence to suggest that cholesterologenesis is not subject to diurnal rhythms.

Materials and Methods

Animals

Male CFY rats were obtained from Carworth (Europe) Ltd., Alconbury, Hunts., U.K. The rats were fed on a stock pelleted diet (Oxoid Breeding Diet obtained from Lillico and Sons, Wonham Mill, Betchworth, Surrey, U.K.).

Methods

Lipogenesis *in vitro*. The synthesis of digitonin-precipitable sterols from sodium [$1\text{-}^{14}\text{C}$]acetate was measured by the method of Atkin *et al.* (1972).

After extraction of digitonin-precipitable sterols, the aqueous phase was acidified to pH 2 with 50% (v/v) H_2SO_4 and was extracted three times with 20 ml of light petroleum (b.p. 40–60°C) to remove fatty acids. After concentration under reduced pressure, a portion was taken for measurement of radioactivity.

Lipogenesis in rings of small intestine (taken as distal 20 cm of ileum) (approx. 500 mg per flask) was

measured in a similar manner, except that substrate was added at $0.2\mu\text{mol}$ ($10\mu\text{Ci}$) per flask and the buffer used was Krebs-Ringer bicarbonate, pH 7.4 (Krebs & Henseleit, 1932).

Lipogenesis in vivo. Rats were each given an intraperitoneal injection of 0.1ml of 0.9% NaCl/ 100g body wt., containing $^3\text{H}_2\text{O}$ ($1\text{mCi}/100\text{g}$ body wt.), or $^3\text{H}_2\text{O}$ (1mCi) plus sodium [$1\text{-}^{14}\text{C}$]octanoate ($31.8\text{mCi}/\text{mmol}$, $5\mu\text{Ci}/100\text{g}$ body wt.). Exactly 1 h later, the rats were killed by cervical dislocation and their livers were quickly removed, rinsed in ice-cold 0.9% NaCl, blotted dry and weighed. A portion of liver was homogenized with 0.9% NaCl and extracted for precipitable sterols and fatty acids, after saponification, as described above. Preliminary experiments showed that the specific radioactivity of $^3\text{H}_2\text{O}$ in serum and liver remained constant over the 1 h duration of the experiment.

Serum lipids. Total cholesterol in serum was measured by using the Technicon AutoAnalyzer method N24A (Technicon Instruments Corp., 1965), as modified by Siegel & Bowdoin (1971). Total triacylglycerol in serum was measured by using the Technicon AutoAnalyzer method N78.

Acetyl-CoA in liver. Acetyl-CoA concentration was measured fluorimetrically on an Aminco-Bowman fluorimeter (4-8202) by the method of Williamson & Corkey (1969).

Liver moisture. Liver samples were dried in an oven at 110°C until attainment of constant weight (about 24 h).

$^3\text{H}_2\text{O}$ in tissues. A portion ($10\mu\text{l}$) of serum was counted directly for its ^3H content. Liver lipids were extracted by the method of Folch *et al.* (1957) as modified by Bligh & Dyer (1959) and a portion of the aqueous phase was counted for its ^3H content.

Calculation of rates of lipogenesis from $^3\text{H}_2\text{O}$ in vivo. Lipid synthesized can be calculated from the expression:

$$\frac{[^3\text{H} \text{ content/lipid fraction per g}] \times 111 \times \text{mol. wt. of lipid}}{[^3\text{H} \text{ content/ml of body water}] \times 'I'}$$

where 1ml of body water contains 111m-atoms of hydrogen, of which ' I ' m-atoms are incorporated for every mmol of lipid synthesized (Haft, 1973). For fatty acids, ' I ' is established at between 13 and 14 (Windmueller & Spaeth, 1966; Jungas, 1968). For cholesterol, ' I ' has been reported as 9 (Barth *et al.*, 1972), 19 (Salmon *et al.*, 1974) or 21 (Brunengraber *et al.*, 1972), depending on experimental design.

Preliminary observations (R. Fears, unpublished work) have indicated that in experiments similar to those reported here, for cholesterol ' I ' = 18, and this value is used in all calculations.

Radioactivity counting procedures. These were carried out as described by Atkin *et al.* (1972).

Chemicals

Sodium [$1\text{-}^{14}\text{C}$]acetate, sodium [$1\text{-}^{14}\text{C}$]octanoate and $^3\text{H}_2\text{O}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NAD $^+$, L-malate and acetyl-CoA were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Citrate synthase and malate dehydrogenase were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Cholestyramine, a quarternary ammonium anion-exchange resin, was obtained from Bristol Laboratories, Langley, Berks., U.K.

Experimental and Results

Effect of meal feeding on biosynthesis of cholesterol and fatty acid in vitro

Before consumption of a 2 h meal, activities synthesizing both precipitable sterols and fatty acid were low as measured by acetate incorporation (Fig. 1). Both activities increased rapidly during and after the meal, to reach a peak some 8–12 h later. The zenith of fatty acid synthesis appeared later than that of cholesterol synthesis but, at all times, except immediately preprandially, more acetate was incorporated into fatty acids than into precipitable sterols.

Comparison between cholesterol synthesis, in vivo and in vitro, at midnight and mid-morning

When rats were allowed to consume food *ad libitum*, cholesterol synthesis from sodium [$1\text{-}^{14}\text{C}$]acetate was increased during the feeding period, that

Fig. 1. Response of sterol (○) and fatty acid (●) synthesis from [$1\text{-}^{14}\text{C}$]acetate to a meal

Animals were adapted to meal-feeding for 21 days with a period of light 16:00 h–04:00 h. For the measurement of lipogenesis, approx. 250mg of liver slices was incubated with $10\mu\text{mol}$ ($2\mu\text{Ci}$) of sodium [$1\text{-}^{14}\text{C}$]acetate at 37°C for 1 h. Each point is the mean of five analyses. Each analysis was of tissue from a single rat.

is at midnight relative to mid-morning. The results are given in Table 1. These results conform with those obtained in the meal-feeding experiment. However, in contrast, no such difference was observed when $^3\text{H}_2\text{O}$ was used to measure the total synthesis of cholesterol

at the two times. The concentration of cholesterol in the serum was also unchanged. The rate of synthesis *in vivo* was approx. 10 times that *in vitro*.

Cholesterol synthesis in vivo in response to meal-feeding

To circumvent metabolic variations resulting from differences in time of consumption of food given *ad libitum*, cholesterol synthesis in response to a meal was measured *in vivo* by using both $^3\text{H}_2\text{O}$ and sodium $[1-^{14}\text{C}]$ octanoate simultaneously. The meal was given between 10:00h and 12:00h. Rats were killed at 10:00h and 14:00h, between which times a large difference in lipogenesis would be expected, on the basis of the first experiment (see Fig. 1). The experiment was performed twice, substantially the same measurements being made on each occasion.

In contrast with the experiment *in vitro* using acetate, there was no postprandial stimulation of cholesterol synthesis from either precursor *in vivo*, when expressed per unit weight of either liver or small intestine (Table 2).

There was a statistically significant increase in the incorporation of sodium $[1-^{14}\text{C}]$ octanoate into cholesterol when expressed per whole liver, but this

Table 1. *Sterol synthesis at mid-day and midnight*

Rats were maintained on either a daylight-dependent lighting cycle or a reversed-light cycle (light period 16:00h–04:00h). After 21 days on the appropriate cycle with food and water *ad libitum*, rats were killed at 10:00h. Cholesterol synthesis was measured *in vivo* with $^3\text{H}_2\text{O}$ and, in the same livers, *in vitro* with sodium $[1-^{14}\text{C}]$ acetate. Incubations with acetate were begun within 45 min of death. Results are the means $\pm$ S.E.M. of eight analyses; each analysis was of tissue from a single rat. Significant difference ($P < 0.05$) is indicated by *.

	Mid-day	Midnight
Gain in body wt. (g)	182 $\pm$ 9.2	179 $\pm$ 6.0
Serum cholesterol (mg/100ml)	73 $\pm$ 3.5	69 $\pm$ 3.6
Sterol synthesis from $[1-^{14}\text{C}]$ -acetate ($\mu\text{g/h}$ per g of liver)	8.5 $\pm$ 1.2	20.4 $\pm$ 3.3*
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	200 $\pm$ 65	149 $\pm$ 18

Table 2. *Sterol and fatty acid synthesis in vivo before and after a meal*

Rats were maintained for 21 days on a reversed-light cycle, light period 16:00h–04:00h, with a single daily meal 10:00h–12:00h. Water was allowed *ad libitum*. Animals were killed at 10:00h and 14:00h. Each rat was given $^3\text{H}_2\text{O}$ and sodium $[1-^{14}\text{C}]$ octanoate by intraperitoneal injection 1 h before it was killed. Results are the means $\pm$ S.E.M. of eight analyses; each analysis was of tissue from a single rat. Significant difference ($P < 0.05$) is indicated by *.

	Expt. 1		Expt. 2	
	Preprandial	Postprandial	Preprandial	Postprandial
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	200 $\pm$ 77	215 $\pm$ 69	93 $\pm$ 15	95 $\pm$ 18
Sterol synthesis from $[1-^{14}\text{C}]$ octanoate (d.p.m./h per g of liver)	3240 $\pm$ 674	4874 $\pm$ 733	2929 $\pm$ 384	3931 $\pm$ 704
Fatty acid synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	36 $\pm$ 6	381 $\pm$ 41*	22 $\pm$ 2	263 $\pm$ 25*
Fatty acid synthesis from $[1-^{14}\text{C}]$ -octanoate (d.p.m./h per g of liver)	6398 $\pm$ 916	47846 $\pm$ 6178*	344 $\pm$ 44	7964 $\pm$ 1028*
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of ileum)			46 $\pm$ 6	41 $\pm$ 7
Sterol synthesis from $[1-^{14}\text{C}]$ octanoate (d.p.m./h per g of ileum)			572 $\pm$ 69	676 $\pm$ 126
Fatty acid synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of ileum)			58 $\pm$ 8	163 $\pm$ 2*
Fatty acid synthesis from $[1-^{14}\text{C}]$ -octanoate (d.p.m./h per g of ileum)			391 $\pm$ 49	2140 $\pm$ 472*
^3H d.p.m./ml of liver water	0.61 $\pm$ 0.07	1.07 $\pm$ 0.06*	0.74 $\pm$ 0.03	0.89 $\pm$ 0.06*
^3H d.p.m./ml of serum				
Liver moisture (%)			69.9 $\pm$ 0.3	69.2 $\pm$ 0.4
Acetyl-CoA (nmol/g of liver)	94.7 $\pm$ 8.1	64.8 $\pm$ 8.8*		
Liver wet wt. (g)	4.07 $\pm$ 0.20	6.01 $\pm$ 0.22*	5.54 $\pm$ 0.16	7.56 $\pm$ 0.30*
Serum cholesterol (mg/100ml)	73 $\pm$ 3.9	73 $\pm$ 3.5	81 $\pm$ 3.2	84 $\pm$ 3.6
Serum triacylglycerol (mg/100ml)	32 $\pm$ 1.4	66 $\pm$ 1.7*	21 $\pm$ 1.9	61 $\pm$ 3.1*

Table 3. *Effect of dietary cholesterol plus cholic acid on synthesis of sterol and fatty acid*

Each rat was given $^3\text{H}_2\text{O}$ by intraperitoneal injection 1 h before it was killed. Measurements *in vitro* were made as described in Fig. 1. Results are the means $\pm$ S.E.M. of eight analyses; each analysis was of tissue from a single rat. Significant difference ($P < 0.05$) is indicated by *.

	Control	Cholesterol (1%) + cholic acid (0.2%) for 7 days
Gain in body wt. (g)	59.8 $\pm$ 2.3	58.4 $\pm$ 2.1
Wt. gain	0.39	0.37
Food eaten		
Serum cholesterol (mg/100ml)	83 $\pm$ 3.2	326 $\pm$ 20*
Sterol synthesis from $[1-^{14}\text{C}]$ acetate ($\mu\text{g/h}$ per g of liver)	12.0 $\pm$ 2.2	1.5 $\pm$ 0.8*
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	64 $\pm$ 5.3	12 $\pm$ 1.1*
Fatty acid synthesis from $[1-^{14}\text{C}]$ acetate ($\mu\text{g/h}$ per g of liver)	70 $\pm$ 4.2	61 $\pm$ 8.9
Fatty acid synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	179 $\pm$ 26	357 $\pm$ 79
Sterol synthesis from $[1-^{14}\text{C}]$ acetate ($\mu\text{g/h}$ per g of ileum)	0.30 $\pm$ 0.04	0.14 $\pm$ 0.02*
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of ileum)	41 $\pm$ 0.6	14 $\pm$ 0.2*
Fatty acid synthesis from $[1-^{14}\text{C}]$ acetate ($\mu\text{g/h}$ per g of ileum)	0.90 $\pm$ 0.01	0.81 $\pm$ 0.01
Fatty acid synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of ileum)	36 $\pm$ 8.2	35 $\pm$ 4.0

Table 4. *Effect of dietary cholestyramine on synthesis of sterol and fatty acid*

Each rat was given $^3\text{H}_2\text{O}$ by intraperitoneal injection 1 h before it was killed. Measurements *in vitro* were made as described in Fig. 1. Results are the means $\pm$ S.E.M. of eight analyses; each analysis was of tissue from a single rat. Significant difference ($P < 0.05$) is indicated by *.

	Control	Cholestyramine (2%) for 5 days
Sterol synthesis from $[1-^{14}\text{C}]$ -acetate ($\mu\text{g/h}$ per g of liver)	7.1 $\pm$ 1.8	80.1 $\pm$ 1.7*
Sterol synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	237 $\pm$ 86	516 $\pm$ 59*
Fatty acid synthesis from $[1-^{14}\text{C}]$ acetate ($\mu\text{g/h}$ per g of liver)	38.1 $\pm$ 9.8	82.0 $\pm$ 12.1*
Fatty acid synthesis from $^3\text{H}_2\text{O}$ ($\mu\text{g/h}$ per g of liver)	254 $\pm$ 37	400 $\pm$ 47*

can be attributed to the significant postprandial increase in liver weight.

In contrast with the results for cholesterol, but in agreement with those obtained *in vitro*, fatty acid synthesis was increased postprandially both in the liver and in the small intestine. There was a significant decrease in the concentration of acetyl-CoA in the liver and after the meal.

Postprandially, there was approximate equilibrium in the distribution of ^3H as $^3\text{H}_2\text{O}$ between the serum and the aqueous phase of the liver, whereas there was no such equilibrium preprandially. This finding is without apparent explanation, as the content of moisture in the liver was identical in the two groups of animals. This observation, however, emphasizes the importance of expressing results in terms of the specific

radioactivity of the precursors at the actual site of synthesis, that is the liver rather than the serum. Further, in experiments involving the incorporation of a precursor into a metabolic product, expression of results in terms of the specific radioactivity of the precursor, rather than merely as incorporation of radioactivity into product, gives a more meaningful indication of the true rate of synthesis and lessens complications resulting from fluctuating precursor pool sizes. However, in the experiment described above, it is necessary for the results relating to octanoate to be expressed as incorporation of radioactivity, as it is not known if the concentration of total hepatic acetyl-CoA parallels the concentration at the site of lipogenesis (the cytoplasm).

Serum cholesterol concentration did not change after the consumption of food, whereas serum triacylglycerol was significantly increased.

Effect of cholesterol or cholestyramine in the diet on lipogenesis

To validate the suitability of $^3\text{H}_2\text{O}$ as a substrate for demonstrating differences in cholesterogenesis under various conditions, the effect of cholesterol or cholestyramine was examined. The results are given in Table 3 (cholesterol feeding) and Table 4 (cholestyramine feeding). When cholesterol (1%) and cholic acid (0.2%) are included in the diet for 7 days, growth and efficiency of foodstuff utilization are not affected. Thus the changes observed in lipogenesis can be attributed to the specific stimulus of cholesterol and are unlikely to be a result of general disturbances in energy intake and metabolism.

Cholesterol synthesis in the liver is decreased by cholesterol feeding, whether measured *in vitro* with sodium $[1-^{14}\text{C}]$ acetate or *in vivo* with $^3\text{H}_2\text{O}$. Similarly, cholesterol synthesis in the ileum is also decreased,

although not to the same extent as in the liver. Fatty acid synthesis was essentially unchanged in either tissue with either substrate.

It may be noted that, although the ileum is a good site for lipogenesis as measured *in vivo*, it supports very little synthesis or precipitable sterols or fatty acids *in vitro*. It may be concluded that the experimental conditions used for the measurement of lipogenesis in the small intestine *in vitro* are far from optimal.

When cholestyramine (2%) is included in the diet for 5 days, the synthesis of both precipitable sterols and fatty acid is stimulated, whether measured *in vivo* with $^3\text{H}_2\text{O}$ or *in vitro* with sodium $[1\text{-}^{14}\text{C}]$ -acetate.

Discussion

The results obtained in our experiment *in vitro* by using sodium $[1\text{-}^{14}\text{C}]$ acetate show a diurnal variation in the synthesis of cholesterol that is entirely in agreement with the results of other workers (Back *et al.*, 1969; Hickman *et al.*, 1972; Bortz & Steele, 1973). It is generally accepted that the principal stimulus for the increase in cholesterologenesis at night, in the rat, is the consumption of food (Horton *et al.*, 1970; Edwards *et al.*, 1972).

However, there are certain problems associated with the measurement of lipogenesis *in vitro*, especially when acetate is used. First, it is commonly observed that the rate of synthesis supported *in vitro* is very much less than that possible *in vivo*, particularly when liver slices are used. Secondly, the status of acetate as physiological substrate for lipogenesis in the rat is debatable (Barth *et al.*, 1971; Knowles *et al.*, 1974). The relative activities of acetate thiokinase and acetyl-CoA hydrolase vary in different tissues of the rat and the equilibrium acetate $\rightleftharpoons$ acetyl-CoA may lie to the left in many physiological situations. In general, the origin of cytoplasmic acetate in the rat is not clear (Romsos & Leveille, 1974).

Thus the use of acetate as a precursor in the liver may measure changes in the activity of acetate thiokinase, which is rate-limiting when compared in activity with the enzymes catalysing the remainder of the reactions in the pathway of cholesterol biosynthesis (Murthy & Steiner, 1972, 1973). Therefore the apparent diurnal variation of cholesterologenesis *in vitro* may merely result from an actual diurnal variation in acetate thiokinase activity. As acetate may not be a precursor of physiological importance, then the changes observed could be of little consequence.

A diurnal rhythm in cholesterologenesis has also been observed in a study made with acetate *in vivo* (Edwards *et al.*, 1972). However, the same criticisms apply if acetate makes little significant contribution

to net rates of lipogenesis under normal conditions. Further, the expression of results as radioactivity incorporated is of little value unless some estimate is made of the specific radioactivity of the substrate. This has not been done in published studies. It is perhaps indicative that one of the better designed experiments using acetate, namely a liver-perfusion study, showed only a very moderate increase in cholesterol synthesis at night (Barth *et al.*, 1972).

Besides data on acetate incorporation, the evidence for diurnal changes in the rate of cholesterol synthesis relies mainly on the reported variations in hydroxymethylglutaryl-CoA reductase, the enzyme whose activity is lowest of those enzymes acting between acetyl-CoA and cholesterol. A number of authors have reported a diurnal rhythm in the activity of hydroxymethylglutaryl-CoA reductase in the rat, again with a peak at midnight, both in the liver (Shapiro & Rodwell, 1971; Edwards & Gould, 1974) and in the small intestine (Shefer *et al.*, 1973). Again, most of the increase in activity is attributed to the onset of feeding (Edwards *et al.*, 1972; Dugan *et al.*, 1972).

Although the diurnal variation in the activity of hydroxymethylglutaryl-CoA reductase appears to be correlated with changes in the incorporation of acetate into cholesterol, the physiological significance of this observation remains to be established in the absence of definitive data on the actual quantity of cholesterol produced. During the feeding period of the rat there is a general increase in hepatic protein synthesis (Lebouton & Handler, 1970) and the response by a particular enzyme depends on its turnover time (Schimke, 1970). Hydroxymethylglutaryl-CoA reductase has a short half-life of between 1.5 and 4 h (Edwards & Gould, 1974; Dugan *et al.*, 1972; Hickman *et al.*, 1972) and might respond rapidly to any general stimulus. Similarly, during starvation there is a general decrease in protein synthesis and the inhibition of hydroxymethylglutaryl-CoA reductase activity may not be specific (Horton *et al.*, 1970).

Further, although in some situations the reductase activity may be well controlled, a number of occasions have been identified where the enzyme does not appear to be rate-limiting for cholesterol synthesis, as measured in other ways. For example, after adrenalectomy, the rhythm of hydroxymethylglutaryl-CoA reductase activity was abolished and the activity remained low, whereas in the same experiment, the rate of acetate incorporation into cholesterol was normal (Edwards, 1973). Similarly, noradrenaline treatment does not affect the reductase rhythm (Edwards, 1973), yet acetate incorporation is apparently stimulated (Bortz, 1968). Under certain conditions of starvation, hydroxymethylglutaryl-CoA reductase is not rate-limiting for the incorporation of acetate (Dugan *et al.*, 1972) and, when cyclic AMP

is added to liver slices, with octanoate as substrate, a step before the reductase controls the rate of cholesterologenesis (Raskin *et al.*, 1974). One or more regulatory sites have also been identified after mevalonate (Johnson & Shah, 1974). Thus measurements of the activity of hydroxymethylglutaryl-CoA reductase may indicate the maximum capacity of the system, under certain conditions, rather than the actual flux of substrates.

In view of the above, it could be considered that neither enzyme measurements nor acetate-incorporation data are necessarily of great relevance in determining the actual quantity of cholesterol synthesized under different experimental conditions. More recently, other substrates have been used for the study of lipogenesis. For example, Salmon *et al.* (1974) have shown that fatty acid synthesis is best supported by circulating precursors that can rapidly form pyruvate (e.g. lactate, alanine and serine, but not glucose). With regard to cholesterol synthesis it has been suggested (Dietschy & Brown, 1974; Dietschy & McGarry, 1974) that octanoate is a better substrate than acetate, and, by measuring the specific radioactivity of ketone bodies produced, it is possible to correct for changes in the specific radioactivity of the pools of acetyl-CoA. However, the method does not lend itself to measurements made *in vivo*, and such results for diurnal rhythms may well be representative only of the situation *in vitro*. Further, the catabolism of octanoic acid may create a pool of acetyl-CoA different from that given by a dietary fatty acid such as palmitic acid (Cederbaum & Rubin, 1975), so that the addition of relatively large amounts of octanoate to assays performed *in vitro* may disturb the normal distribution of acetyl-CoA.

In recent years, $^3\text{H}_2\text{O}$ has been used to label lipids synthesized *in vivo*, and the use of $^3\text{H}_2\text{O}$ has been particularly well investigated with regard to the metabolism of fatty acids (Windmueller & Spaeth, 1966; Jungas, 1968). By using $^3\text{H}_2\text{O}$, it is possible to determine the actual flux of a substrate into lipid, provided that the contributions from various substrates do not appreciably change in proportion to one another.

$^3\text{H}_2\text{O}$ has been used relatively little for the measurement of rates of cholesterologenesis, although there has been reported a diurnal variation with ^3H incorporated *in vivo* (Edwards *et al.*, 1972). However, these authors expressed their results as incorporation of radioactivity and did not account for possible variations in specific radioactivity of $^3\text{H}_2\text{O}$ in the liver.

It was the purpose of the present series of experiments to investigate more thoroughly the diurnal variations in lipogenesis, in particular in order to control the experimental stimulus, to measure the response to a meal.

With acetate, there was a conventional response to feeding, but with $^3\text{H}_2\text{O}$ there was no change in cholesterol synthesis. As the amount of synthesis obtained *in vivo* with $^3\text{H}_2\text{O}$ was very much greater than that found *in vitro* with acetate, and, as $^3\text{H}_2\text{O}$ measures total flux, whereas acetate incorporation measures events essentially unconnected with physiological variations in cholesterologenesis, it is considered that the results obtained by using $^3\text{H}_2\text{O}$ are more representative of the actual events *in vivo*.

After a meal, when there is no increased incorporation of $^3\text{H}_2\text{O}$ or sodium [^{14}C]octanoate into cholesterol, there is a substantial increase in their incorporation into fatty acids. This latter change may be considered as an 'internal standard' to show that the experimental design was capable of demonstrating true changes. The necessity for such an increase, in order to store energy from a diet containing 4% fat, is apparent.

After feeding, there was a significant decrease in hepatic acetyl-CoA concentration. It is likely that the specific radioactivity of acetyl-CoA was, in consequence, increased. This may account for the small increase in incorporation into cholesterol of label from octanoate in the absence of any change from $^3\text{H}_2\text{O}$. Similar changes in the concentration of acetyl-CoA (if the total cellular concentration reflects the concentration in the cytoplasm) may explain some of the results reported by other workers. In approximate agreement with the present results, Herrera *et al.* (1975) have shown that the liver acetyl-CoA concentration in normal mice and rats increases after starvation for 6h and is still increased, albeit not significantly, by 15h of starvation.

In view of the unexpected nature of the results obtained, it was considered of importance to ascertain if measurements made with $^3\text{H}_2\text{O}$ reflect known variations occurring in cholesterol synthesis in other situations. The responses to dietary cholesterol and dietary cholestyramine were measured. From the initial observation of Schoenheimer & Breusch (1933) it has been well established that supplementing the diet with cholesterol decreases endogenous production in the liver (Siperstein, 1970), probably at the site of hydroxymethylglutaryl-CoA reductase. Cholestyramine increases hepatic cholesterol synthesis secondary to an enhanced catabolism and excretion (Huff *et al.*, 1963). Measurements with both $^3\text{H}_2\text{O}$ and acetate as precursors gave comparable results with cholesterol feeding; similarly, in response to dietary cholestyramine, the incorporation of ^3H and acetate into cholesterol and fatty acids was changed in the same direction. With cholestyramine, the increase in acetate incorporated into cholesterol was very much larger than the increased incorporation of ^3H . It is considered that the latter change may be nearer to that which is actually occurring as other factors

involved in the response change to a similar extent. For example, cholesterol 7 α -hydroxylase, which is believed to be the rate-limiting enzyme of bile acid production, also increases by 200–300% (R. Fears, unpublished work). Similarly, with 3% cholestyramine in the diet, White (1972) has shown relatively moderate increases in hydroxymethylglutarate, mevalonate and cholesterol.

Thus measurements made with $^3\text{H}_2\text{O}$ give an accurate representation of functional changes in cholesterol biogenesis under various experimental conditions. Acetate-incorporation data may follow gross changes but also vary under conditions where cholesterol biosynthesis is unchanged.

As the use of $^3\text{H}_2\text{O}$ (and sodium [1- ^{14}C]octanoate) has given an accurate description of the state of lipogenesis in response to a meal, the inference must be that under normal conditions there is no measurable fluctuation in cholesterol synthesis, although the changes in fatty acid synthesis are as portrayed conventionally. The present results also cast doubt on the many reports of variations in cholesterologenesis in various nutritional and metabolic states when measurements were not based on the use of $^3\text{H}_2\text{O}$ as substrate.

Although the principal part of the conventionally measured diurnal change in cholesterologenesis is attributed to food, it is also generally accepted that there is a residuum of variation observed during starvation, which is presumably light-dependent (Bortz, 1973). The present experiments have only been concerned with investigating the food-dependent moiety of the cycle. It is conceivable that there is a genuine, albeit small, diurnal change in cholesterologenesis during starvation.

In conclusion, under the conditions described, we have been unable to demonstrate an effect of meal-feeding on cholesterologenesis. It is therefore considered that hepatic cholesterologenesis in rats is not subject to the same degree of diurnal rhythm as has previously been believed, and we echo the words of Bloch (1965) written 11 years ago: 'A great variety of environmental, dietary and hormonal factors have been studied and shown to influence the rate of cholesterol synthesis, but the evidence is still too scanty for specifying the point at which physiological control is most effectively exerted'.

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