Sites of control of hepatic cholesterol biosynthesis

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ABSTRACT An inhibition in the conversion of mevalonate to cholesterol has been demonstrated in liver of cholesterol-fed rats by both in vitro and in vivo methods. Synthesis decreased to 30% of the control value after 1 week and 20% after 1 month on a 1% cholesterol diet. After a year, synthesis from mevalonate was almost completely inhibited.

The rate of conversion of squalene to cholesterol was not consistently decreased but that of farnesyl pyrophosphate to cholesterol was decreased considerably. The rate of conversion of mevalonate to farnesyl pyrophosphate by a soluble liver enzyme preparation was also decreased in cholesterol-fed animals. Sites of inhibition of cholesterol synthesis were detected before mevalonate, between mevalonate and farnesyl pyrophosphate, and after farnesyl pyrophosphate, probably at the conversion of farnesyl pyrophosphate to squalene. The inhibition of mevalonate conversion to cholesterol developed more slowly than that of acetate and appeared to be secondary to it.

The maximum capacities of normal liver homogenates and slices to synthesize cholesterol from mevalonate were shown to be far greater than from acetate. Consequently, sites of inhibition after mevalonate probably do not have a significant effect on the over-all rate of cholesterol synthesis in the intact cholesterol-fed animal.

KEY WORDS   cholesterol   ·   feedback inhibition   ·   acetate   ·   mevalonate   · farnesyl pyrophosphate   ·   squalene   ·   biosynthesis ·   hydroxymethyl glutaryl CoA   ·   diet   · homeostasis   ·   rat

SHORTLY AFTER Tavormina, Gibbs, and Huff (1) discovered that mevalonate is efficiently converted into cholesterol by liver homogenates, it was reported that feeding a high cholesterol diet to rats did not inhibit hepatic cholesterol synthesis from mevalonate-2-^{14}C

nearly as much as from acetate-^{14}C (2–6). Gould and Popjak (2) suggested that the "physiological regulation of the rate of cholesterol synthesis is concerned with one or more of the steps between acetate and mevalonate." The term "feedback inhibition" was adopted since this appeared to be an example of the inhibition of a synthetic chain of reactions by the action of the end product at a site early in the chain—presumably at the first step unique to the biosynthesis of the end product (3, 6). The site of inhibition must be close to mevalonate since other pathways of acetyl CoA metabolism, such as fatty acid synthesis and oxidation to CO_2, are relatively unaffected by cholesterol feeding (7). Siperstein and Guest (6) proposed that the reduction of HMGCoA to mevalonate is the specific step inhibited by cholesterol feeding and at the same time Bucher, Overath, and Lynen (8) concluded from assays of HMGCoA reductase activity in livers from fasted and Triton-treated rats that this enzyme is concerned in the over-all regulation of cholesterol biosynthesis. It has since become generally accepted that the feedback inhibition of hepatic cholesterol biosynthesis is due entirely to an effect on HMGCoA reductase.

However, a partial inhibition in the conversion of mevalonate to cholesterol in liver preparations from cholesterol-fed animals was observed in all reports subsequent to the preliminary one (2). Gould (3) reported a decrease to 25% of the control rate; Bucher, McGarrahan, Gould, and Loud (5) to 17%; and Siperstein and Guest (6) to values ranging from 12 to 46%. Since this inhibition was less than that before mevalonate, it was considered to be not rate-limiting. The location of the post-MVA inhibition was not made clear from these studies, nor was there very good agreement on the amount of inhibition.

The present report deals with a reinvestigation of the effect of cholesterol feeding, particularly its long-term effect, on the conversion of mevalonate to cholesterol in rat liver homogenate and slices and in the liver of intact

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Abbreviations: HMGCoA, β-hydroxy β-methyl glutaryl coenzyme A; MVA, mevalonate; FPP, farnesyl pyrophosphate; Ac, acetate; S.A., specific activity in μc/μmole.

animals. A significant inhibition of MVA conversion to cholesterol, demonstrated by both in vitro and in vivo methods, increased in magnitude with the duration of cholesterol feeding. To locate the site of the inhibition between mevalonate and cholesterol, we determined the effects of long-term cholesterol feeding on the conversion of farnesyl pyrophosphate and of squalene to cholesterol in liver homogenates, and also on the conversion of mevalonate into farnesyl pyrophosphate by liver enzyme preparations.

A preliminary report on this work has been published (9).

METHODS AND MATERIALS

Diets

A diet containing 1% cholesterol was prepared by dissolving cholesterol in reagent grade acetone–ether 1:1, pouring it over commercial rat chow (Purina), and evaporating the solvent at room temperature with frequent mixing. Male Sprague-Dawley rats were fed the 1% cholesterol diet for periods of time between 1 and 640 days.

Labeled Substrates

Acetate-1-^{14}C and mevalonate-2-^{14}C were obtained from commercial sources and diluted with inert substrates to a suitable specific activity.

Squalene-^{14}C was prepared biosynthetically; normal rat liver homogenate was incubated with mevalonate-2-^{14}C in N_2 for 2 hr, the mixture was saponified, and squalene was isolated from the nonsaponifiable fraction by chromatography in petroleum ether on a column of neutral alumina (grade II). Its purity was determined by gas–liquid chromatography. The specific activity of the squalene was usually estimated from the squalene content of liver [50 μg/g (10)] and from the amount synthesized as determined by the specific activity of the MVA used and the microcuries of ^{14}C in the squalene isolated. Some preparations were diluted with inert squalene and the specific activity determined by assay for ^{14}C in a liquid scintillation counter and by analysis for squalene in a gas–liquid chromatograph, with squalane as an internal standard.

Farnesyl-^{14}C pyrophosphate (FPP) was prepared biosynthetically by the collidine extraction procedure as described by Goodman and Popjak (11). The specific activity was calculated from the amount of ^{14}C in FPP (estimated by the assay method of Goodman and Popjak) and from the specific activity of the MVA used, neglecting the inert FPP and any intermediates between MVA and FPP which might be present in the enzyme preparation.

The effect of cholesterol feeding on the rate of conversion of MVA to FPP was determined by essentially the same method except that a large excess of MVA was used in relation to the amount of enzyme. The assay was carried out on the homogenate after saponification, as described by Goodman and Popjak (11).

Incubations

Liver slice incubations were carried out in the modified Krebs-Ringer phosphate medium previously described (7), 10 ml of medium being used per g of slices.

Liver homogenates were prepared in a medium, based on that of Bucher, McGarrahan, Gould, and Loud (5), consisting of nicotinamide 0.03 M, magnesium chloride 0.004 M, potassium phosphate buffer 0.1 M, pH 7.4, and EDTA 0.001 M. The liver was minced in a stainless steel press and homogenized with 3 volumes of medium in a loose fitting homogenizer for 90 sec. The homogenates were centrifuged at 800 × g for 20 min at 0–4°C. Aliquots of the supernatant fraction, 2 ml representing 0.5 g of liver, were incubated with a large excess of substrate so that the limiting factor would be the concentrations of enzymes. Control experiments with acetate and MVA established that the concentrations used were not inhibitory.

Isolation and Assay

Cholesterol was isolated by saponification and digitonin precipitation, and counted in a liquid scintillation counter after decomposition of the digitonide by heating in pyridine and evaporating to dryness in a counting vial as previously described (12).

In some of the early experiments, the digitonide was counted in a thin end-window Geiger counter and corrections for self-absorption and counting efficiency were made in the usual way. Farnesyl pyrophosphate was isolated by the assay method of Goodman and Popjak (11) as a mixture of nerolidol and farnesol, and counted in a liquid scintillation counter. Squalene was isolated as described above for preparation of squalene-^{14}C and counted in a liquid scintillation counter.

The relative rates of synthesis reported in this paper are expressed, unless otherwise stated, as disintegrations per minute in the cholesterol present in 1 g of liver slices or in the homogenate prepared from 1 g of liver after incubation for 1 hr with at least 20 μmoles of acetate or at least 0.5 μmole of MVA per g of liver. The specific activities of the substrates were usually 0.1 μc/μmole for these two substrates. Actual rates of synthesis in micromoles of cholesterol were calculated as described in Table 8.

In Vivo Experiments

Acetate, MVA, or tritium water was injected intra-

peritoneally in standard doses per 100 g of body weight, the animals were killed after the stated time intervals, and the cholesterol was isolated and assayed as previously described (12).

RESULTS

In the first series of experiments, the conversion of MVA to cholesterol was measured in liver homogenates from rats fed 1% cholesterol for periods of 1–33 days and from control animals handled in exactly the same way (Table 1). The results are calculated in terms of the amount of cholesterol synthesized in 2 hr from mevalonate by the homogenate derived from 0.5 g of liver. An inhibition was observed in the cholesterol-fed rats after only 1 day of cholesterol feeding. After 2–4 days, the incorporation was about half that in control homogenates, and it decreased to about 20% of control values after a month of cholesterol feeding.

In a second series of experiments, the inhibition of MVA incorporation into cholesterol was compared with that of the incorporation of acetate in separate aliquots of the same homogenates as a function of duration of cholesterol feeding (Fig. 1). Acetate incorporation into cholesterol was decreased to less than 5% of the control rate after 1 day of cholesterol diet and generally was in the range 0.1–5% from that time on. MVA incorporation decreased more slowly, reaching 50% of the control value after 1 day of cholesterol feeding but continuing to decrease with time. After a year of cholesterol feeding it became as low as that of acetate.

Liver slice incubations gave similar results (Table 2); the conversion of mevalonate to cholesterol was definitely lower in slices from cholesterol-fed rats than in slices from controls, but the decrease did not develop as rapidly with MVA as with acetate. No significant differences between the homogenate and liver slice technique were observed.

Inhibition of MVA incorporation into cholesterol in the liver of the intact cholesterol-fed rats was also demonstrated (Table 3). After 7 days of cholesterol feeding, the incorporation was 24% of the control value, and it continued to decrease with time. Although inhibition of less than 1% of control was occasionally observed in rats feed cholesterol for a long time, the incorporation of MVA measured in vivo tended to be less completely inhibited than that measured in liver slices or homogenates. Inhibition of cholesterol synthesis from acetate and from tritium water was also less complete by in vivo methods of estimation than by in vitro methods.

In two of the experiments reported in Table 3, liver homogenates or slices from the same animals were also studied in vitro. The results, given in Table 4, indicate that the livers of cholesterol-fed rats have considerably less capacity to synthesize cholesterol from mevalonate than those of control animals, whether the synthesis is measured in liver homogenates, slices, or intact animals. The loss in capacity increased with the duration of cholesterol feeding and there appeared to be slightly less inhibition when it was estimated by in vivo methods.

Liver Cholesterol Concentration

The concentration of esterified cholesterol in liver rose rapidly during the first few weeks after institution of the 1% cholesterol diet but showed little change after that time (Fig. 2). Both free and esterified cholesterol showed small but significant increases by 12 hr after the cholesterol diet was begun and a definite decrease in cholesterol synthesis from acetate-1-^{14}C was observed at this time. By 18 hr the synthesis in liver slices from acetate was reduced to very low levels, whereas synthesis from MVA was not reduced significantly (Table 2). At this time the concentration of esterified cholesterol was over 3 times the control value ($P < 0.001$) and the free cholesterol concentration was also slightly but significantly increased ($P < 0.001$); hence, it is not obvious

TABLE 1   EFFECT OF CHOLESTEROL FEEDING ON CHOLESTEROL SYNTHESIS FROM
MEVALONATE-2-^{14}C IN LIVER HOMOGENATES
Cholesterol synthesis expressed as cpm $\times$ 10^{-3} in cholesterol per 0.5 g of liver

| | Duration of Cholesterol Feeding, in Days | | | | | | | |
	1	2	3	4	8	16	30	33
Cholesterol-fed	14.63 (6)*	10.84 (5)	8.19 (6)	11.51 (4)	7.81 (4)	2.92 (3)	2.89 (3)	3.13 (3)
Control	22.13 (6)*	25.02 (5)	19.52 (6)	23.04 (5)	21.34 (5)	19.79 (5)	15.76 (3)	16.00 (3)
% of Control	66	43	42	50	37	15	18	20

2 ml of homogenate (derived from 0.5 g of liver) incubated with 0.5 μmole of NADP, 10 μmoles of glucose-6-phosphate, and 0.3 μmole of MVA with S.A. of 0.67 μc/μmole for 2 hr.   In most of these experiments the homogenate was made from the pooled livers of 2 or 3 rats and incubation was carried out in duplicate or triplicate.   Cholesterol was isolated as digitonide and counted in a thin-window Geiger counter; counting rates were corrected for self-absorption.
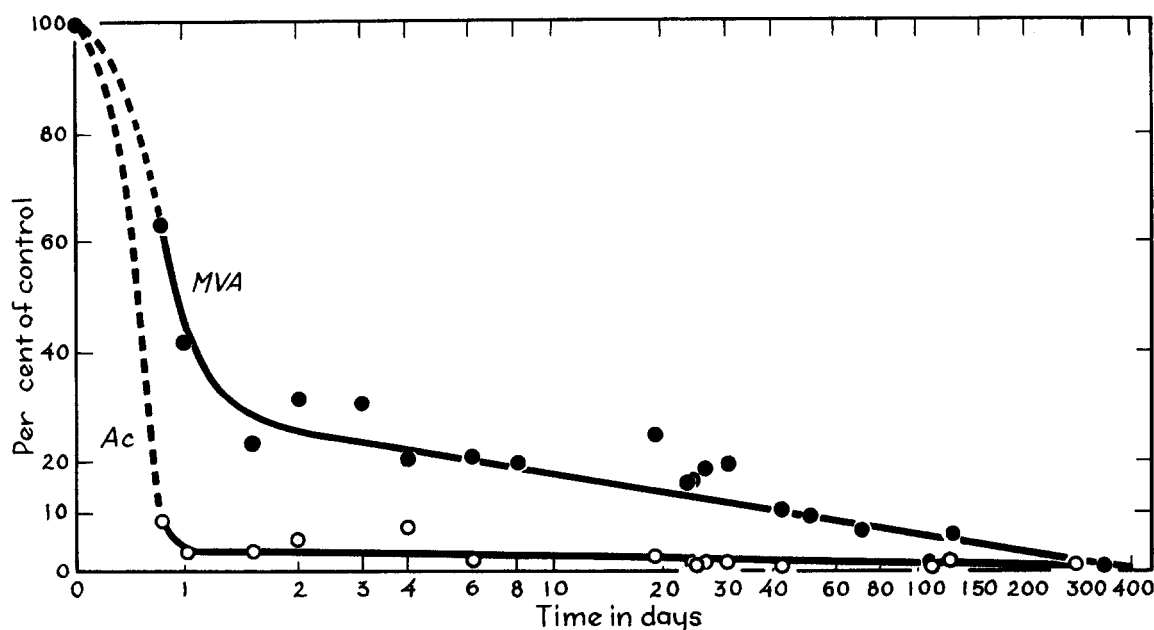* Numbers in parenthesis indicate the number of rats.

FIG. 1. Inhibition of cholesterol synthesis in liver homogenates from cholesterol-fed rats: comparison between acetate and mevalonate and the effect of increasing duration of cholesterol feeding. The rate of synthesis in homogenates from cholesterol-fed rat livers is expressed as a percentage of the rate for normal rat livers, measured in the same experiment. In each experiment 2–4 control rats and 2–4 cholesterol-fed rats were used. The livers were homogenized and, in most cases, pooled. Incubations of 2 ml of homogenates were carried out in triplicate for 1.5 hr after the addition of 0.5 μmole of NADP, 10 μmoles of glucose-6-phosphate, 12 μmoles of GSH, and 7.5 μmoles of acetate-1-^{14}C (S.A., 0.1 μc/μmole) or of MVA (S.A., 0.667 μc/μmole). Each point represents, on the average, 9 rats.

TABLE 2   EFFECT OF CHOLESTEROL FEEDING ON CONVERSION OF MEVALONATE
AND OF ACETATE TO CHOLESTEROL IN RAT LIVER SLICES

Expt. No.	No. of Rats	Duration of Cholesterol Feeding	Liver Cholesterol					
			Synthesis				Concentration	
			MVA	% of Control	Acetate	% of Control	Esterified	Free
		hr	dpm/g × 10⁻³		dpm/g × 10⁻³		mg/g	
79	6 Control		34.88		11.37		0.44 ± 0.06	1.46 ± 0.06
	6 Chol-fed	18	26.31	75	1.11	9.8	1.68 ± 0.10	1.73 ± 0.05
			($P > 0.1$)		($P < 0.01$)		($P < 0.001$)	($P < 0.001$)
8	2 Control		23.97		3.00		0.40	1.60
	3 Chol-fed	42	8.16	34	0.101	3.4	3.29	2.01

Slices were incubated with 5 μmoles of MVA (S.A., 0.2) or with 18 μmoles of acetate (S.A., 0.1) per g of slices for 1 hr.

which change is more closely related in time to the decrease in cholesterol synthesis from acetate.

By 42 hr the concentration of esterified cholesterol was about 7 times the control value and continued to increase rapidly until values above 10 mg/g (corresponding to about 25 times the control value) were reached. After about 2 weeks, values of 10–40 mg/g for esterified cholesterol were found without any consistent relationship to the duration of cholesterol feeding. The free cholesterol concentration tended to rise very slightly with time, reaching values as high as 3 mg/g after many months of cholesterol feeding. Older control animals also gave slightly elevated values on oc-

casion. The difference in free cholesterol concentration between control and cholesterol-fed rats of the same age was usually in the range of 0.5–1.0 mg/g, regardless of the duration of cholesterol feeding. As in our previous studies (3, 7), a linear relationship was observed when the logarithm of the relative rate of synthesis of cholesterol from acetate-^{14}C in liver slices or homogenates was plotted as a function of the free cholesterol concentration in liver (Fig. 3). The mean values for groups of animals showed an inverse proportionality, with a slope corresponding to a decrease of 50% in rate of synthesis for an increase in free cholesterol concentration in liver of 0.14 mg/g, in close agreement with results previously

TABLE 3   Inhibition of Hepatic Cholesterol Biosynthesis from Mevalonate, Acetate, and Tritium Water Measured in Intact Rats

| Expt. No.	No. of Rats	Duration of Cholesterol Feeding	Substrate Injected	Dose per 100 g of Body Wt.		Duration of Expt.*	Liver Cholesterol Synthesis	% of Control	
								MVA-¹⁴C	Acetate-¹⁴C or ³HOH
		days		μc	μmoles	hr	dpm/g × 10⁻³		
1	3 Control		MVA	0.18	0.24	0.5	6.57		
	" Chol-fed	7	"	"	"	"	1.60	24	
	" Control		Ac	10.00	100	"	3.55		
	" Chol-fed	7	"	"	"	"	0.098		2.8
30	3 Control		MVA	1.00	0.66	1	23.45 ± 1.68		
	" Chol-fed	28	"	"	"	"	1.25 ± 0.12	5.3	
6	4 Control		MVA	1.00	10	4	23.99 ± 0.54		
	" Chol-fed	298	"	"	"	"	3.61 ± 0.94	15	
15	1 Control		³HOH	1 mc		18	4.92		
	" Chol-fed	315	"	"		"	0.27		5.5
7	1 Control		MVA	1.00	10	4	38.90		
	" Chol-fed	640	"	"	"	"	0.50	1.7	
	1 Control		³HOH	5 mc		"	6.14		
	" Chol-fed	640	"	"		"	0.38		6.3

* Time from injection of substrate to sacrifice.
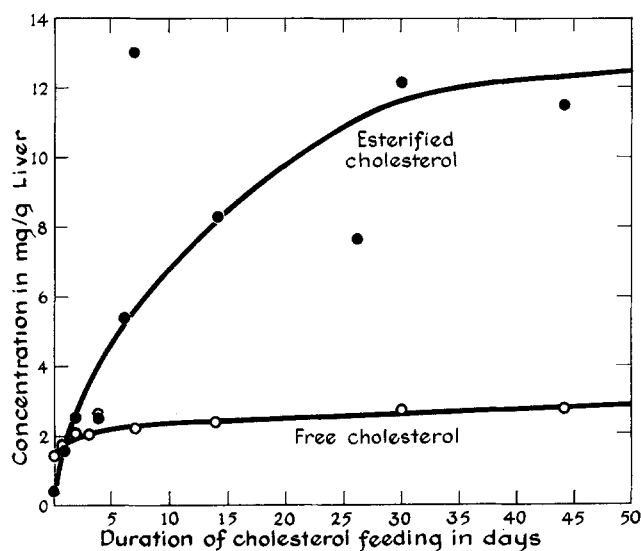
Fig. 2.   The concentration of free and esterified cholesterol in rat liver during maintenance on a stock diet to which 1% of cholesterol had been added. The concentrations are expressed as milligrams of cholesterol per gram of liver (wet weight).

TABLE 4   Comparison of Inhibition Measured In Vivo and In Vitro in the Same Animals

Expt. No.	No. of Rats	Cholesterol Synthesis from Various Substrates, % of Control Values			
		Mevalonate-¹⁴C		Tritium Water,	Acetate-¹⁴C,
		In Vivo	In Vitro	In Vivo	In Vitro
		%			
6	4	13	1.4	10	0.5
7	1	1.7	0.03	6.2	0.03

TABLE 5   Relationship between Concentrations of Free and Esterified Cholesterol in Livers of Cholesterol-Fed Rats and Relative Rates of Cholesterol Synthesis in Liver Slices from Acetate-1-¹⁴C and Mevalonate-2-¹⁴C

No. of Rats	Free Cholesterol	Esterified Cholesterol	In Vitro Synthesis from:	
			Ac	MVA
	mg/g		dpm/g liver × 10⁻³	
Control				
3	2.06	0.10	18.69	109.64
1	2.28	0.32	8.43	47.03
2	2.20	0.40	1.08	25.53
4	2.44	0.75	0.83	24.18
Chol-fed				
1	3.13	9.74	0.11	1.92
3	2.48	12.00	0	0.56
2	3.03	30.40	0	0.20
4	3.20	37.60	0.04	0.09

Rats were fed 1% cholesterol for 298 days. The controls were the same age and approximately the same weight. Liver slices were incubated for 1 hr and the isotope incorporation into total cholesterol was determined.

reported (3, 13).   The esterified cholesterol concentration also was increased whenever the rate of synthesis was decreased from normal, but the relationship was not linear when plotted in this manner (Fig. 4).

In rats on long-term cholesterol feeding and in controls, there tended to be an inverse relationship (illustrated in Table 5 for liver slices) between the concentration of esterified cholesterol and the synthesis of cholesterol from acetate-1-¹⁴C and MVA.

Location of Sites of Inhibition

To obtain information on the location of the site (or sites) of inhibition after MVA, we incubated liver homogenates from rats fed cholesterol for a long time with certain other intermediates for comparison with MVA.
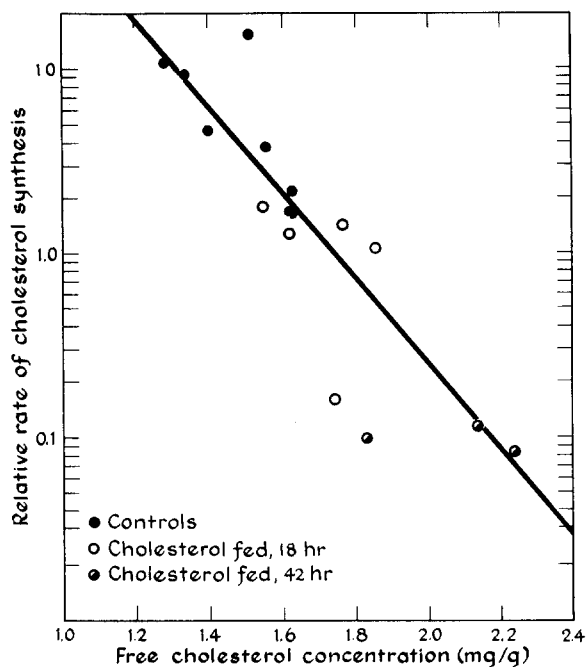
Fig. 3. Logarithm of the relative rate of cholesterol synthesis in liver slices as a function of the concentration of free cholesterol. The relative rate of synthesis was defined as the disintegrations per minute in the cholesterol isolated from 1 g of slices after incubation for 1 hr with 20 μmoles of acetate-1-^{14}C (S.A., 0.1 μc/mole). Each point represents 1 rat.

The slope of the line corresponds to a decrease in synthesis of 50% for an increase in free cholesterol concentration of 0.14 mg/g.
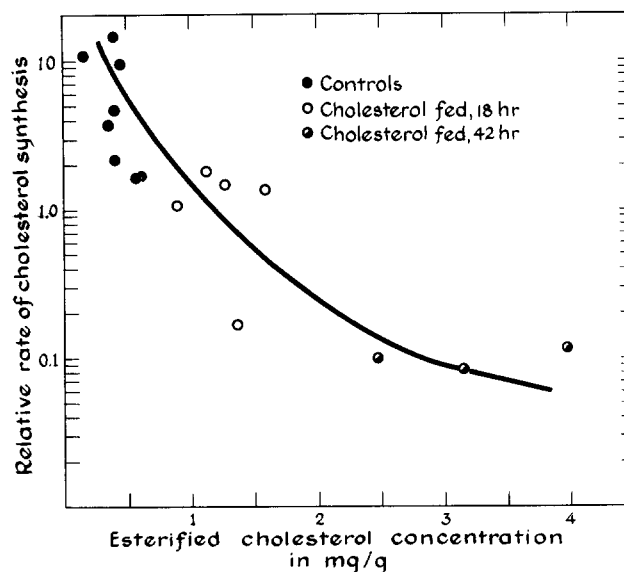

Fig. 4. Logarithm of the relative rate of cholesterol synthesis in liver slices as a function of the concentration of esterified cholesterol in liver.

Results with squalene-^{14}C gave no indication of a consistent inhibition of its conversion to cholesterol in homogenates from rats fed cholesterol for periods as long as 126 days (Table 6). The one experiment at 640 days gave an apparent inhibition, but it is not certain that this is a reproducible effect. The results of different experiments varied considerably, possibly because of the insolubility of squalene in aqueous media and the consequent difficulty in ensuring optimal conditions for reaction; but the mean values for 11 experiments on 40 rats indicated an increase to about 200% of the control value in the cholesterol-fed rats. This lack of inhibition indicates that the site (or sites) of inhibition must be located between MVA and squalene.

The conversion of FPP to cholesterol, however, was found to be considerably decreased in homogenates from long-term cholesterol-fed rats, as compared with controls (Table 7). The maximum rate in the rats fed 1% cholesterol for 52–280 days varied from 3 to 35% of control values, but in each experiment it was considerably lower than the corresponding value for the conversion of squalene to cholesterol by the same homogenate. The activity for the sequence FPP → cholesterol as a percentage of that for the sequence squalene → cholesterol ranged from 3.7 to 10% with a mean of 5.7%. This

evidence indicates a site of inhibition at the step:

$$2\,\text{FPP} + \text{NADPH} + \text{H}^+ \rightarrow$$
$$\text{squalene} + \text{NADP}^+ + 2\,\text{H}_2\text{O}$$

Comparison of the effect of long-term cholesterol feeding on the sequence MVA → cholesterol with that on the sequence FPP → cholesterol (Table 7) indicates some inhibition between MVA and FPP. The conversion of MVA was reduced to a greater extent than that of FPP in each experiment so that, in spite of a considerable range in the various experiments, the effect on the sequence MVA → cholesterol was at least twice as great as on the sequence FPP → cholesterol in every experiment. This evidence indicates a site of inhibition between MVA and FPP.

Additional evidence supporting this conclusion was obtained from the relative rates of conversion of MVA → FPP by the soluble enzyme preparation of Goodman and Popjak (11). In this case the maximum rate of conversion is expressed in terms of milligrams of protein rather than of grams of liver represented. The preparations obtained from long-term cholesterol-fed rats had only 25–40% the activity of the control (Table 7). This result is in agreement with the indirect evidence mentioned above.

Evidence of decreased activity of the soluble enzymes concerned with conversion of MVA to FPP was also obtained by incubating normal rat liver microsomes with the 105,000 × g supernatant fraction obtained from livers of cholesterol-fed rats. As indicated in Table 8, only about 10% as much MVA was converted into cholesterol under these conditions as when normal super-

TABLE 6   Effect of Cholesterol Feeding on Conversion of Squalene to Cholesterol

Expt. No.	No. of Rats	Duration of Cholesterol Feeding	Squalene Added S. A.	Squalene Added Amount	Liver Cholesterol	Relative Synthetic Rate
		days	$\mu c/\mu mole$	$dpm \times 10^{-3}$	dpm/g liver $\times 10^{-3}$	% of control
28	1 Control		0.287	120	12.0	
	" Chol-fed	14	"	"	10.7	88
33	3 Control		1.21	320	9.5	
	" Chol-fed	17	"	"	27.3	289
32	2 Control		1.21	320	11.3	
	" Chol-fed	19	"	"	15.2	135
34	3 Control		0.17	320	6.4	
	" Chol-fed	23	"	"	5.4	84
29	1 Control		0.287	120	9.2	
	" Chol-fed	24	"	"	26.2	284
30	3 Control		1.21	320	10.2	
	" Chol-fed	42	"	"	22.4	222
31	3 Control		1.21	320	6.9	
	" Chol-fed	52	"	"	19.9	289
51	1 Control		0.38	930	18.4	
	" Chol-fed	70	"	"	88.0	479
50	1 Control		4.0	812	31.7	
	" Chol-fed	126	"	"	192.7	620
57	1 Control		4.0	819	60.9	
	" Chol-fed	640	"	"	10.1	16
	19 Control					
	" Chol-fed	Weighted mean of all experiments				206

The squalene-^{14}C was added to 2 ml of homogenate dissolved in 0.2 ml of propylene glycol.

TABLE 7   Effects of Long-Term Cholesterol Feeding on Activities of Liver Homogenates in Converting Various Substrates into Cholesterol

Expt. No.	Duration of Cholesterol Feeding	Acetate → Chol Rel. Syn. Rate	% of Normal	MVA → Chol Rel. Syn. Rate	% of Normal	FPP → Chol Rel. Syn. Rate	% of Normal	Squalene → Chol Rel. Syn. Rate	% of Normal	MVA → FPP Rel. Syn. Rate	% of Normal
	days	$dpm/g \times 10^{-3}$	%	$dpm/g \times 10^{-3}$	%	$dpm/g \times 10^{-3}$	%	$dpm/g \times 10^{-3}$	%	$dpm \times 10^{-3}$ per mg protein	%
51	(Control)			59.00		78.00		18.63		223.00	
	70			4.48	7.6	13.90	17.8	88.0	479	59.00	26
52	(Control)	2.22		89.00		220.60		4.80		327.00	
	112	0.02	1.0	0.17	0.2	7.00	3.0	1.42	30	122.00	25
50	(Control)	11.60		120.00		104.00		31.68		177.20	
	126	0.25	2.2	8.00	6.3	36.10	35.4	192.72	608	71.50	40
53	(Control)	1.33		44.25		4.04					
	280	0.01	0.4	0.04	0.1	0.23	5.6				
57	(Control)	50.72		265.00		14.31		56.40			
	640	0.01	0.03	0.23	0.08	0.22	0.64	9.32	16		

The relative synthetic rate is expressed as dpm $\times 10^{-3}$ in the cholesterol isolated from 4 ml of homogenate, prepared from 1 g of liver, except in the last two columns (MVA → FPP) where it is expressed as dpm $\times 10^{-3}$ in the cholesterol synthesized by 1 mg of protein.

Homogenates (2 ml) were incubated for 1 hr with 0.5 μmole of NADP, 10 μmoles of glucose-6-phosphate, 12 μmoles of glutathione, and sufficient ^{14}C-labeled substrate so that not more than a few per cent of the amount of substrate would be converted to cholesterol. In the case of acetate, 7.5 μmoles were used, MVA 5 μmoles, and various amounts of the other substrates. Results are expressed as dpm/4 ml of homogenate since 4 ml represents 1 g of liver.

The conversion of MVA to FPP was carried out with the F_{30}^{60} ammonium sulfate fraction of liver-soluble supernate, prepared as described by Goodman and Popjak (11), and the results are expressed per milligram of protein.

natant solution was used. Incubation of microsomes from cholesterol-fed rats with normal supernatant solution gave 20% of normal values, which showed that both soluble and microsomal enzymes are decreased in activity in cholesterol-fed rats.

In considering the possible physiological significance

TABLE 8   CHOLESTEROL SYNTHESIS IN RECONSTITUTED LIVER HOMOGENATES

Composition of Homogenate		MVA → Chol (dpm/g Liver, % of Control)	
Microsomes	Supernate	Expt. 1	Expt. 2
Normal	Normal	100%	
Chol-fed	Chol-fed	2	1
Normal	Chol-fed	11	10
Chol-fed	Normal	22	15

Microsomes were isolated by centrifuging at 105,000 × g for 30 min, washed by suspending in Bucher medium and recentrifuging, and then suspended in the original volume of supernate and incubated for 1 hr with excess MVA. Cholesterol was isolated as digitonide.

of an inhibition of any of these sites in the over-all regulation of hepatic cholesterol biosynthesis, it is necessary to know the maximum rate of reaction in terms of the amount of substrate reacting. Estimates of reaction velocities in mμmoles of cholesterol formed per hour from a large excess of substrate by homogenate representing 1 g of liver have been calculated and are given in Table 9 for homogenates from normal and long-term cholesterol-fed rats.

A comparison of the normal values shows that cholesterol is much less rapidly synthesized from acetate than from MVA or any of the later intermediates. Thus, the controlling reaction in normal liver homogenate is the conversion of acetate to MVA. In the inhibited homogenates the capacity to convert MVA into cholesterol may be almost as great as the capacity of the normal liver to convert acetate into MVA. Normal liver homogenates are far more variable in their activity in

TABLE 9   RATES OF CHOLESTEROL SYNTHESIS FROM VARIOUS PRECURSORS IN LIVER PREPARATIONS FROM NORMAL AND LONG-TERM CHOLESTEROL-FED RATS

	Rate of Synthesis			
	Ac → Chol	MVA → Chol	FPP → Chol	Squalene → Chol
	mμmoles cholesterol per hr per g liver			
Homogenates				
Control	1.53 ± 0.56	31.3 ± 0.6	40	52
Chol-fed	0.0045–0.034	0.025–2.4	0.26–14	50–100
Slices				
Control	2.54 ± 0.79	50.6 ± 3.8		
Chol-fed	0.005–0.015	0.06–0.5		

For calculation of mμmoles of cholesterol synthesized from various substrates, the millimicrocuries of ^{14}C in the cholesterol isolated from homogenate derived from 1 g of liver is divided by the S.A. of the substrate × hr of incubation × C. For acetate-1-^{14}C, C = 12; for mevalonate-2-^{14}C, C = 5; for farnesyl pyrophosphate-^{14}C made from MVA-2-^{14}C, C = 5/3; for squalene made from MVA-2-^{14}C, C = 5/6.
Results given as mean ± SEM for controls and as a range for cholesterol-fed.

converting acetate to MVA than in converting MVA to cholesterol and the enzymes concerned with MVA synthesis are easily inactivated, so it cannot be assumed that the values in Table 9 are necessarily representative of those in intact liver. We have therefore included in the table some values for liver slices which indicate that under these conditions the capacity to convert acetate to cholesterol is only about 10% as great as the capacity to convert MVA to cholesterol.

DISCUSSION

The results presented above clearly show that homogenates from rats fed high cholesterol diets for long periods have decreased capacities to convert mevalonate and farnesyl pyrophosphate to cholesterol and that the inhibition of cholesterol synthesis from MVA eventually becomes almost complete. Most of the data previously reported by others are in agreement with these results; Bucher et al. (5) found a decrease in conversion of MVA to cholesterol to about 17% of the control value after 3 days of cholesterol feeding. Siperstein and Guest (6) using liver slices found 46% of the control rate after 3 days, 29% after 9 days, and 12% after 14 days of feeding a 5% cholesterol diet. In neither of these studies was the feeding of cholesterol continued long enough to produce the almost complete suppression we have observed and in both reports it was assumed that the inhibition was not significant in comparison with the greater inhibition of acetate conversion to cholesterol, but results were reported only in terms of per cent of control values.

The conclusion reached in the present study, that homogenates from cholesterol-fed rat liver have as great a capacity as normal ones to convert squalene to cholesterol, is in agreement with the report of Siperstein and Guest (6), who found that liver slices from rats fed cholesterol have about 80% of the control rate of conversion of squalene to cholesterol. Bucher et al. (5) found no decrease in this rate in liver homogenates from rats fed cholesterol for 3 days but a large decrease after 4–6 weeks. The difficulty of obtaining optimal contact between the extremely water-insoluble squalene and the enzyme may account for the differences in results obtained in different laboratories. Siperstein and Guest (6) also reported an increase of about 3- to 4-fold in the conversion of MVA to squalene in cholesterol-fed rats, an increase which is not compatible with their other results or with our results.

Locations of the various sites of inhibition are indicated in Fig. 5. The previously described inhibition of MVA synthesis from acetate is considered to be located at the final step in the synthesis of MVA, the reduction of HMGCoA, or possibly an unidentified HMG–protein complex, to MVA, S_1.
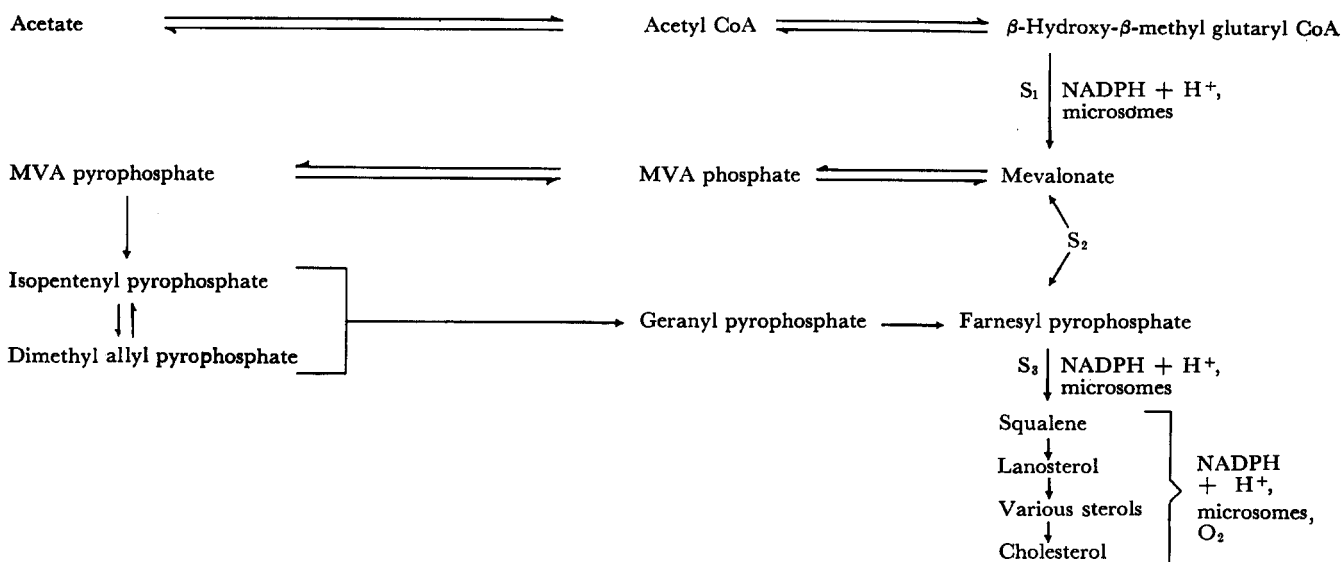
FIG. 5.   Sites of inhibition of cholesterol biosynthesis produced by cholesterol feeding.

The second site, S_2, cannot at present be located more exactly than between MVA and FPP, an area which includes six reactions.

The third site, S_3, is after FPP and appears to be the conversion of FPP to squalene, a reaction that requires microsomes and NADPH. This step has been extensively investigated by Popjak, Goodman, Cornforth, Cornforth, and Ryhage (14).

The presence of sites of inhibition after MVA raises the question of whether these sites contribute to the control of the over-all rate of synthesis. It should be stressed that the results in Tables 4 and 5 are expressed in terms of the rate for the control animal and do not indicate the much greater rate of cholesterol synthesis from MVA than from acetate in controls. Furthermore, the relative reaction rates were obtained for high concentrations of the initial substrate and therefore are indicative of maximum rates, not actual rates under steady-state conditions. The maximum amount of cholesterol synthesized per hour from various substrates by rat liver homogenates and slices, shown in Table 9, makes it evident that the capacity of the homogenate enzymes from normal liver to convert acetate into cholesterol was far smaller than their capacity to convert MVA, FPP, or squalene to cholesterol. It is, of course, possible that preparation of the homogenate has decreased the activity of the one or more enzymes that control the rate of MVA synthesis from acetate more than it has decreased the activity of those enzymes necessary for MVA conversion to cholesterol. However, liver slices were found to synthesize cholesterol about 20 times as rapidly from MVA as from acetate (Table 9), a result in agreement with the results of homogenate studies.

The question may be raised whether the over-all rate of cholesterol biosynthesis in cholesterol-fed rats is controlled entirely by the rate of formation of MVA, as has been generally assumed, or whether the decrease in activity of certain enzymes after MVA is participating in the control. If one assumes that the activities of the various enzyme sequences in liver homogenate and in slices are roughly proportional to their activities in the intact liver, it appears that a reduction in the capacity of the MVA-to-cholesterol sequence to only 10% of the control value would still leave a capacity greater than that present in normal liver for the over-all acetate-to-cholesterol sequence. Hence, it seems unlikely that any site after MVA will have a significant effect on the over-all rate unless it is blocked almost completely. Additional support for this view is the finding that the synthesis of cholesterol from acetate decreases to almost its final value of less than 5% of the control before the MVA-to-cholesterol sequence has decreased to less than 50%, as shown in Fig. 1. Siperstein and Fagan (15) have recently reported that the direct estimation of conversion of acetate to compounds hydrolyzable to HMG and to MVA revealed no inhibition of formation of the HMG compounds and an almost complete inhibition of MVA formation. Their data strongly support the hypothesis that the site of inhibition before MVA is quantitatively much more important than later sites.

It is possible that one or more enzymes between MVA and FPP and the enzyme responsible for the conversion of FPP to squalene may be substrate-induced and tend to decrease in activity when the formation of MVA is greatly reduced.

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