STUDIES ON THE SITE OF THE FEEDBACK CONTROL OF CHOLESTEROL SYNTHESIS * †

By MARVIN D. SIPERSTEIN and M. JOANNE GUEST

(From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas)

(Submitted for publication August 21, 1959; accepted December 4, 1959)

Despite wide variations in dietary cholesterol, most animals are capable of maintaining the cholesterol of their plasma at relatively constant levels. Insight into the means by which this homeostatis of plasma cholesterol is accomplished was first provided when Gould and associates (3–5), Tomkins, Sheppard and Chaikoff (6) and Frantz, Schneider and Hinkelman (7) independently demonstrated that cholesterol synthesis in the liver varies inversely with the level of dietary cholesterol. Since all endogenous plasma cholesterol probably originates in the liver (8–10), it is likely that the constancy of plasma cholesterol is the result of this reciprocal relationship between dietary cholesterol and hepatic cholesterogenesis.

The mechanism by which exogenous cholesterol is able to suppress cholesterol synthesis in the liver is not as yet understood. Previous studies of this problem have been carried out by Gould and Popják (11) who presented preliminary evidence indicating that the conversion of mevalonic acid to cholesterol in liver homogenates is not impaired by cholesterol feeding. Similarly, since completion of our study, Bucher, McGarrahan, Gould and Loud (12, 13), employing an approach similar to that used here, have clearly demonstrated that in homogenates the reactions involved in the conversion of mevalonic acid to cholesterol are not greatly influenced by dietary cholesterol. These experiments (11–13), as well as the present investigation, provide evidence that the site of control of cholesterol synthesis is located proximal to the utilization of mevalonic acid.

The present report describes studies which specifically localize the biochemical site of the cholesterol-induced inhibition of cholesterol synthesis. Some of the properties of this negative feedback system are described, and it is suggested, on the basis of these characteristics, that this type of feedback control may be a more widespread means of regulating synthetic processes than has previously been realized.

EXPERIMENTAL PROCEDURE

Male and female rats of the Long-Evans and Sprague-Dawley strains, weighing between 150 and 250 g, were used in this study. They were maintained on a commercial chow prior to initiation of the experiment. During the experimental period, the control animals were fed *ad libitum* either a powdered commercial animal chow or Simonson "White Diet" (Simonson Laboratories, Gilroy, Calif.); the experimental rats received the same diets, to which cholesterol was added in 0.5, 2.5 or 5 per cent amounts. Cholesterol in these concentrations did not influence the food intake of the rats.

The rats were killed by a blow on the head and exsanguinated. The livers, and in some cases the intestines, were excised and cooled. One mm slices of these organs were prepared by means of a McIlwain tissue slicer, and duplicate 500 mg portions of the slices were placed in the outer wells of 25 ml center-well flasks containing 5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, and the C^{14}-labeled substrate. The flasks were then gassed with a 5 per cent carbon dioxide, 95 per cent oxygen mixture and incubated for 3 hours at 37° C in a Dubnoff metabolic shaker.

Acetate-1-C^{14} and acetate-2-C^{14} were purchased from Nuclear-Chicago Corp.; mevalonic acid-2-C^{14} was obtained from Isotopic Specialties Co.; and labeled squalene was biologically synthesized by incubating liver slices from normal rats with mevalonic acid-2-C^{14} and isolating the synthesized squalene-C^{14} as described below.

ANALYTICAL METHODS

Isolation of $C^{14}O_2$ and nonsaponifiable material. Carbon dioxide was trapped in the center well of the flask with Hyamine 10 X as previously described (14). Following removal of the CO_2, the contents of the main compartment were saponified by adding 0.5 ml of 90 per

* The results reported in this paper were presented at the meeting of the Southern Society for Clinical Investigation held in New Orleans on January 19, 1959 and have appeared in abstract form (1, 2).

† This research was supported by grants from the Dallas Heart Association, the American Heart Association and the Texas Heart Association.

cent potassium hydroxide and autoclaving for 1 hour. The nonsaponifiable material was next isolated by transferring the contents of the center-well flask to a 250 ml Erlenmeyer flask, adding an equal volume of absolute ethyl alcohol and extracting twice with at least a tenfold excess of petroleum ether.

Isolation of squalene. The petroleum ether solution containing both squalene and cholesterol was taken to dryness under nitrogen to remove ethyl alcohol and the residue was dissolved in pentane; 100 mg of squalene (Eastman Organic Chemicals), redistilled at 3 mm Hg as described by Heilbron, Kamm and Owens (15), was added to an aliquot of the pentane solution, and the mixture was then transferred to a silicic acid column (10 g of unactivated Baker reagent grade silicic acid in a 2.5 × 23 cm column). The squalene was eluted with 100 ml of petroleum ether. An aliquot of this solution was added directly to 5 ml of 0.4 per cent diphenyloxazole in toluene and the C^{14} content of the squalene determined in a Packard liquid scintillation counter.

The validity of this method of isolation of squalene-C^{14} was established by forming the hexachloride derivative of the isolated squalene-C^{14} using the method of Heilbron and colleagues (15). The squalene hexachloride was washed at −20° C 3 times each with ethanol and with ethyl ether. The resulting white powder was dried, dissolved directly in 5 ml of 0.4 per cent diphenyloxazole in toluene and assayed for C^{14} in the liquid scintillation counter. It was found that if the above procedure was followed, at least 95 per cent of the C^{14} obtained in the petroleum ether eluate of the silicic acid was recoverable as the squalene hexachloride. On the other hand, if at any stage the extracted squalene was exposed to air in the dry state for a day or more, significant losses occurred.

Cholesterol-C^{14} determination. The fraction containing the cholesterol-C^{14} was eluted from the silicic acid column with ethyl ether, and the cholesterol was then isolated as its digitonide by the procedure of Sperry and Webb (16). The cholesterol digitonide was dissolved in 2 ml of methanol and its C^{14} content assayed as described above.

Fatty acid-C^{14} and ketone body-C^{14} determination. The aqueous residue obtained after removal of the nonsaponifiable material was heated on a steam bath to remove the ethanol following which the pH of the solution was brought to 3.0 with sulfuric acid. Exactly 40 ml of pentane was next added and the flask was shaken for 15 minutes on an International bottle shaker to extract the free fatty acids. The pentane layer was then removed, washed twice with water and a 4 ml aliquot added to 12 ml of the diphenyloxazole-toluene for C^{14} determination.

To assay the acetoacetic acid-C^{14} and β-hydroxybutyric acid-C^{14}, carrier ketone bodies were added to a deproteinized extract of the flask contents, and the method described by Van Slyke (17) was employed to isolate the corresponding Denigès's salt. These were washed twice with 5 ml portions of cold water, mounted on filter paper and their C^{14} content assayed at infinite thickness in a Geiger counter equipped with a micromil window.

The following data represent, in every case, the average value obtained from the analysis of duplicate experiments.

RESULTS

Influence of cholesterol feeding on cholesterol synthesis from acetate

In confirmation of previous studies (3–7), the feeding of a 2.5 or a 5 per cent cholesterol diet resulted in a striking depression in the synthesis of cholesterol from acetate by rat liver slices. The magnitude of decrease in cholesterol synthesis, however, was quite variable. In the typical experiments shown in Table I, cholesterol synthesis was reduced by a factor of from 21 to 150. In other determinations presented in Tables IV and VI, cholesterol feeding inhibited cholesterogenesis by a factor of from 10 to 300.

Frantz and colleagues (7) have reported that this depression in cholesterogenesis can be correlated with the extent of accumulation of cholesterol

TABLE I

Effect of cholesterol feeding on cholesterol synthesis

Experiment	Cholesterol in diet		Liver cholesterol	Acetate-C^{14} converted to cholesterol
	%	*Duration*	*mg/100 g*	%
1	0	3 days	214	1.430
	2.5	3 days	206	0.010
2	0	1 month	176	0.272
	5	1 month	154	0.013
3	0	3 days	218	0.572
	2.5	3 days	276	0.004
4	0	1 day*	233	3.460
	10	1 day†	250	0.043

* Tube-fed 5 ml of corn oil, 12 and 24 hours prior to killing.
† Tube-fed 0.5 g of cholesterol in 5 ml of corn oil, 12 and 24 hours prior to killing.

in the liver and, in fact, a logarithmic response to increases in cholesterol concentration was found by these authors. In our studies, on the other hand, dietary cholesterol produced a marked depression in cholesterol synthesis (reduced by a factor of at least 100 in Experiments 1 and 3, Table I) prior to the appearance of a detectable elevation in the concentration of hepatic cholesterol. The reason for this greater sensitivity to cholesterol feeding is not apparent; however, it is obvious that the mechanism by which cholesterol synthesis is depressed by dietary cholesterol need not be dependent upon large increases in tissue cholesterol concentration.

Preliminary localization of the site of the feedback control of cholesterogenesis

According to present knowledge, the conversion of acetate to cholesterol involves about 26 separate enzymatic reactions. The approximate localization of the site at which exogenous cholesterol produces its inhibition of cholesterol synthesis was determined by separately examining the influence of cholesterol feeding on the various segments of this pathway, indicated in Figure 1.

Influence of cholesterol feeding on the conversion of squalene to cholesterol. Approximately 15 steps are thought to be involved in the conversion of squalene to cholesterol (18). As demonstrated by the experiments shown in Table II, cholesterol feeding has little or no demonstrable influence on this series of reactions. Wide variations in the rates of incorporation of the labeled squalene into cholesterol by rat liver slices were observed; however, in no case was the magnitude of decrease sufficient to account for the reduction, by a factor of 10 to 300, in the conversion of *acetate* to cholesterol which results from cholesterol feeding.

Conversion of mevalonic acid to squalene. Seven reactions are believed to be required for the conversion of mevalonic acid to squalene (19, 20). In order to determine whether dietary cholesterol causes an inhibition of one or more of these reactions, the conversion of mevalonic acid-2-C^{14} to squalene was examined in the livers of both normal and cholesterol-fed rats.

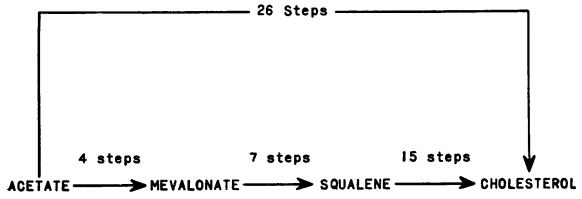

FIG. 1. SUMMARY OF REACTIONS OF CHOLESTEROL SYNTHESIS.

TABLE II

Effect of cholesterol feeding on the synthesis of cholesterol from squalene *

Experiment	Cholesterol in diet	Squalene incorporated into cholesterol
	%	%
1	0	0.75
	2.5	1.14
2	0	1.14
	2.5	0.35
3	0	0.94
	2.5	0.54
4	0	0.85
	2.5	0.71

* Conditions: Experiments 1 and 2, squalene-C^{14} dissolved in 0.1 ml acetone. Experiments 3 and 4, squalene-C^{14} suspended in 1 drop of Tween 80, and added to 500 mg liver slices in 5 ml Krebs-phosphate buffer.

As is shown in Table III, cholesterol feeding consistently increased, rather than depressed, the synthesis of squalene from mevalonic acid; the site of cholesterol inhibition is, therefore, not located in this segment of cholesterol synthesis.

Conversion of mevalonic acid to cholesterol. The results of the previous two experiments would indicate that the 22 reactions necessary for the synthesis of cholesterol from mevalonate are not inhibited by cholesterol feeding. This conclusion was further tested by studying the conversion of mevalonic acid-2-C^{14} to cholesterol. In each experiment, the synthesis of cholesterol from acetate was also examined, using another sample of the pooled liver slices obtained from the

TABLE III

Effect of cholesterol feeding on the conversion of mevalonic acid to squalene

Experiment	Cholesterol in diet		Mevalonic acid converted to squalene
	%	*Duration*	*% added* C^{14}
1	0	9 days	3.02
	5	9 days	8.11
2	0	8 days	1.72
	2.5	8 days	9.04
3	0	1 month	0.17
	5	1 month	0.65
4	0	10 days	0.32
	5	10 days	6.95

TABLE IV

Effect of cholesterol feeding on cholesterol synthesis from acetate and mevalonic acid

Exp.	Cholesterol in diet		Liver		Intestine	
			Acetate-1-C^{14}	Mevalonic acid-2-C^{14}	Acetate-1-C^{14}	Mevalonic acid-2-C^{14}
	%	*Duration*	% C^{14} *incorporated*	% C^{14} *incorporated*	% C^{14} *incorporated*	% C^{14} *incorporated*
1	0	3 days	0.535	10.81		
	0.5	3 days	0.032	6.56		
	5	3 days	0.005	3.62		
2	0	9 days	0.970	1.91	0.278	0.045
	0.5	9 days	0.635	1.58	0.353	0.068
	5	9 days	0.011	0.66	0.117	0.049
3	0	3 days	0.248	1.18	0.160	0.144
	0.5	3 days	0.208	0.76	0.077	0.097
	5	3 days	0.008	0.30	0.223	0.258
4	0	3 days	0.624	8.64		
	0.5	3 days	0.081	8.27		
	5	3 days	0.008	2.69		
5	0	3 days	2.536	2.12	0.106	0.011
	0.5	3 days	2.328	2.50	0.054	0.013
	5	3 days	0.067	1.57	0.250	0.011
6	0	3 days	2.195	2.09		
	0.5	3 days	0.476	2.41		
	5	3 days	0.023	1.11		
7	0	3 days	2.350	2.05		
	0.5	3 days	0.722	2.11		
	5	3 days	0.102	0.90		
8	0	6 days	0.508	1.57	0.178	0.071
	0.5	3 days	0.066	1.00	0.127	0.081
	5	3 days	0.012	0.53	0.191	0.054
9	0	2 weeks	0.276	3.32	0.063	0.027
	5	2 weeks	0.004	0.30	0.166	0.042
10	0	2 weeks	0.112	1.87		
	5	2 weeks	0.002	0.29		
11	0	9 days	0.690	1.51		
	0.5	9 days	0.153	1.26		
	5	9 days	0.010	0.35		

same rat. As indicated in Table IV, cholesterol feeding decreased the conversion of mevalonic acid to cholesterol by a factor of from 1.3 to 11; (average 3.9). Although the effect was consistently present, it is clear that, when compared with the inhibition by a factor of 20 to over 100 (average 65) in the conversion of acetate to cholesterol, the decrease in conversion of mevalonic acid to cholesterol cannot account for the overall depression in cholesterol synthesis which is produced by dietary cholesterol.

The apparent discrepancy between the results obtained in the squalene → cholesterol and mevalonate → squalene experiments as compared with the overall mevalonate → cholesterol experiment remains to be explained. It seems likely that the site of the relatively small inhibition observed in the mevalonate → cholesterol reactions is actually between squalene and cholesterol. The rate of the mevalonate → squalene reactions was consistently elevated by cholesterol feeding whereas the squalene → cholesterol steps were much more variably affected. The latter variations could well be due to slight differences in solubilization of squalene in the aqueous media, and this technical difficulty might then prevent the detection of a minor depression in the squalene → cholesterol portion of the pathway.

The small intestine is known to constitute a second major site of cholesterol synthesis in the body and for this reason the influence of cholesterol feeding on cholesterol synthesis from both acetate and mevalonic acid was examined in this tissue. The intestines of rats fed the cholesterol-free diet were found to carry out the synthesis of cholesterol from acetate at a rate which was one-

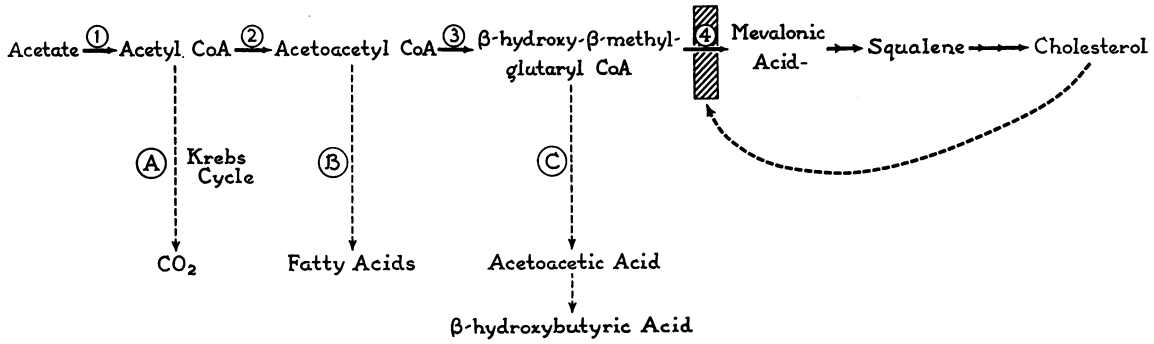

FIG. 2. PROPOSED SITE OF FEEDBACK CONTROL OF CHOLESTEROGENESIS.

half to one twenty-fifth that of liver; however, as has also been noted by Gould and associates (4), this organ proved to be immune to cholesterol feeding. Whereas the livers from rats fed 0.5 or 2.5 per cent cholesterol exhibited a marked depression in cholesterol synthesis, the intestinal slices from the same animals synthesized cholesterol as well as did the intestines from animals fed the cholesterol-free diet. As a result, a comparison of the abilities of the livers and intestines of the cholesterol-fed rats to synthesize cholesterol from acetate shows that under these circumstances much more cholesterol is produced in the *intestine* than in the liver.

The last column in Table IV presents data on the conversion of mevalonic acid to cholesterol by the intestine. As expected, this process too was unaffected by cholesterol feeding; however, the surprising aspect of this experiment is that, in contrast to the case of liver, mevalonate with two exceptions proved to be inferior to acetate as a precursor of cholesterol. In the 14 experiments, intestinal cholesterol synthesis from acetate averaged 0.17 per cent and that from mevalonic acid 0.069 per cent. It is possible that the biochemical pathway of cholesterol synthesis in the intestine may differ from that in liver, as appears to be the case in brain (21); however, these data may also reflect differences in cellular permeability for the two substrates.

Specific localization of the site of cholesterol inhibition

The results of the previous experiments would indicate that the major site of the cholesterol-induced inhibition of cholesterogenesis must lie in one of the reactions before the utilization of mevalonic acid. As is shown in detail in Figure 2, four

TABLE V

Effect of cholesterol feeding on fatty acid and ketone body synthesis *

Exp.	Cholesterol in diet		Acetate-C^{14} converted to:			
			CO_2	Fatty acids	Acetoacetic acid	β-Hydroxybutyric acid
	%	*days*	*% added* C^{14}	*% added* C^{14}	*cpm*†	*cpm*†
1	0	7	36.71	0.789	4,331	1,759
	2.5	7	40.94	0.455	5,126	1,529
2	0	20	18.57	1.623	3,274	2,284
	5	20	16.11	0.873	1,654	1,999
3	0	8	26.01	1.13	2,204	2,761
	2.5	8	26.51	6.93	803	2,459
4	0	9	9.21	6.99	1,808	3,208
	5	9	9.33	4.99	2,023	3,607
5	0	24	13.75	1.332	2,398	2,055
	5	24	16.50	0.479	2,878	1,721

* Conditions: flasks 1, 3 and 4 contained acetate-1-C^{14}, 0.5 μc; flasks 2 and 5 contained acetate-2-C^{14}.
† Total counts assayed at infinite thickness.

reactions are required for the conversion of acetate to mevalonic acid. All but one of these steps can be eliminated as the site of the cholesterol inhibition of cholesterol synthesis by the following experiments.

Influence of cholesterol feeding on acetate oxidation to CO_2. The first reaction in the conversion of acetate to mevalonic acid involves activation of acetate by coenzyme A and adenosine triphosphate to form acetyl CoA. As is indicated by dotted arrow A in Figure 2, this reaction is also required for the oxidation of acetate to carbon dioxide in the Krebs cycle. The effect of a high cholesterol diet on the conversion of acetate to CO_2 was therefore studied, and as demonstrated in the fourth column of Table V, $C^{14}O_2$ production from acetate-C^{14} is not depressed by cholesterol feeding. The activation of acetate (Reaction 1, Figure 2) can therefore be eliminated as a possible site of the block in cholesterol synthesis.

Cholesterol feeding and the conversion of acetate to fatty acids. The second reaction in the synthesis of cholesterol consists of the condensation of two molecules of acetyl CoA to yield acetoacetyl CoA (Reaction 2, Figure 2). This reaction can be evaluated by determining the influence of cholesterol feeding on the synthesis of long chain fatty acids from acetate. As indicated by dotted arrow B, Figure 2, acetoacetyl CoA is an important intermediate in the synthesis of fatty acids as well as of cholesterol, and a block in Reaction 2 would therefore result in a depression in the incorporation of acetate-C^{14} into fatty acids. Column 5 of Table V demonstrates that fatty acid synthesis is not significantly influenced by cholesterol feeding. Both Tomkins (6) and Gould (4) and their associates have reported similar findings. Reaction 2 likewise, therefore, can be eliminated as the site of the feedback reaction.

Conversion of acetate to acetoacetic acid and β-hydroxybutyric acid. Finally, Reaction 3, involving the condensation of a third molecule of acetyl CoA with acetoacetyl CoA to form β-hydroxy-β-methylglutaryl CoA, can be examined by determining the influence of cholesterol feeding on the conversion of acetate to the ketone bodies, acetoacetic acid and β-hydroxybutyric acid. Lynen and colleagues (22) have recently shown that β-hydroxy-β-methylglutaryl CoA is an obligatory intermediate in the synthesis of both of these ketone bodies. It follows therefore that any blockage of Reaction 3 would result in a decrease in the incorporation of acetate-C^{14} into acetoacetic acid and β-hydroxybutyric acid.

As shown in columns 6 and 7 of Table V, cholesterol feeding has no influence on the synthesis of either acetoacetic acid or β-hydroxybutyric acid. This experiment demonstrates that cholesterol produces no inhibition of Reaction 3, and furthermore confirms the fact that Reactions 1 and 2 must be operating normally under circumstances when cholesterol synthesis is markedly inhibited by cholesterol feeding.

It would follow from these experiments, therefore, that as indicated by the block shown in Figure 2, the specific site of the cholesterol-induced inhibition of cholesterogenesis is at Reaction 4, involving the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

Further studies on the site of the cholesterol block. In Table VI are presented a series of experiments in which the influence of cholesterol feeding on the various reactions involved in cholesterol synthesis were examined in single rat livers. In general the results confirmed the conclusions of the previous experiments in which the different portions of the synthetic pathway were studied in separate animals.

Dietary cholesterol depressed cholesterol synthesis in these studies by a factor of from 10 to 300, and again this could not be related to increases in the concentration of liver cholesterol (columns 4 and 5, Table VI). A depression in the conversion of mevalonic acid to cholesterol was consistently noted following cholesterol feeding; however, once more this effect was in no case sufficient to account for the inhibition produced in the overall reaction from acetate to cholesterol. We would suggest, therefore, that this decrease in the conversion of mevalonic acid to cholesterol is a secondary result—perhaps due to a "disuse" depression of enzyme synthesis—of cholesterol inhibition at a site located prior to the utilization of mevalonic acid.

As was previously observed, cholesterol feeding caused an increase in the incorporation of mevalonic acid-C^{14} into squalene in each of the eight experiments. In view of the postulated

TABLE VI

Influence of cholesterol feeding on reactions related to cholesterol synthesis

Exp.	Cholesterol in diet		Cholesterol concentration in liver	Cholesterol from		Squalene from mevalonate-2-C^{14}	CO_2 from		Fatty acid from acetate-2-C^{14}	Acetate-2-C^{14} to	
				Acetate-2-C^{14}	Mevalonate-2-C^{14}		Acetate-2-C^{14}	Mevalonate-2-C^{14}		Acetoacetate	β-Hydroxybutyrate
	%	*Duration*	*mg/100 g*	*% C^{14} incorporated*	*% C^{14} incorporated*	*% C^{14} incorporated*	*% C^{14} incorporated*	*% C^{14} incorporated*	*% C^{14} incorporated*	*cpm*	*cpm*
1	0	8 days	492	0.390	7.17	2.09	23.02	2.82	1.39	1,492	2,446
	2.5	8 days	470	0.017	1.51	8.30	25.27	1.47	2.01	1,278	2,257
2	0	9 days	191	0.434	12.09	0.34	5.85	3.09	0.58	644	788
	5	9 days	150	0.002	0.81	1.12	3.82	0.78	0.27	759	930
3	0	9 days	307	2.460	13.66	3.29	17.80	3.43	7.90	2,220	1,783
	5	9 days	383	0.144	1.96	3.39	14.86	1.40	18.19	1,217	2,775
4	0	9 days	182	0.600	13.64	0.44	4.42	4.62	1.89	1,173	834
	5	9 days	269	0.002	1.66	1.58	3.69	1.48	0.12	1,023	1,101
5	0	11 days	350	1.714	10.50	2.44	18.87	3.30	1.40	1,764	1,295
	2.5	11 days	400	0.054	6.08	7.59	18.31	2.41	0.49	1,762	2,137
6	0	11 days	352	1.367	7.98	4.67	16.97	2.98	1.29	1,296	1,797
	2.5	11 days	383	0.053	0.66	10.13	14.40	0.86	4.17	1,745	2,818
7	0	11 days	217	0.866	4.44	3.18	14.57	1.72	5.16	2,447	1,367
	2.5	11 days	211	0.048	1.48	5.01	16.38	1.07	4.60	2,164	1,369
8	0	11 days	189	0.646	4.74	1.24	7.98	1.44	3.13	2,178	886
	2.5	11 days	208	0.061	1.38	5.78	10.35	0.73	3.82	2,363	1,383

block shown in Figure 2, it seems likely that this effect of dietary cholesterol on the conversion of C^{14} mevalonate to squalene may be due to differential isotopic dilution of the mevalonate. An inhibition of mevalonic acid synthesis would lead to a decrease in the size of the mevalonic acid pool, and the dilution of the added radioactive mevalonate would therefore be less in the livers of the cholesterol-fed rats. The resulting greater specific activity of the mevalonate utilized for squalene synthesis would then cause an increased incorporation of C^{14} into squalene.

Finally, the results shown in Table VI again demonstrate that the conversion of acetate to CO_2, fatty acids and ketone bodies is not significantly influenced by dietary cholesterol. These studies serve to confirm the conclusion that the primary site of the cholesterol feedback control must be located at the point of conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

It is noteworthy that mevalonic acid-2-C^{14} was converted to $C^{14}O_2$ at significant rates in all of the experiments presented in Table VI. Since the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid has not been found to be reversible (20, 23), it is unlikely that this $C^{14}O_2$ is derived from the conversion of mevalonate to acetyl CoA with subsequent oxidation of the latter in the Krebs cycle. As shown by Cornforth, Cornforth, Popják and Gore (24), the second carbon of mevalonate contributes to six carbons, including two of the angular methyl groups, of lanosterol, and one of these labeled methyl groups must be oxidixed to CO_2 during the conversion of lanosterol to cholesterol. It follows, therefore, that for each carbon-2 of mevalonic acid converted to CO_2, five should be incorporated into cholesterol. The observed values for the ratio: ($C^{14}O_2$ from mevalonate-2-C^{14}) : (cholesterol-C^{14} from mevalonate-2-C^{14}) in the normal animals varied from 1 : 2.5 to 1 : 4.0 and averaged 1 : 3.2. In view of the possibility that precursors of cholesterol, which have lost one or two of their angular methyl carbons, may also accumulate in this system, the observed ratios are consistent with the concept that most, i.e., approximately two-thirds, and perhaps all of the $C^{14}O_2$ produced from mevalonate-2-C^{14}, is a consequence of mevalonate conversion to sterols

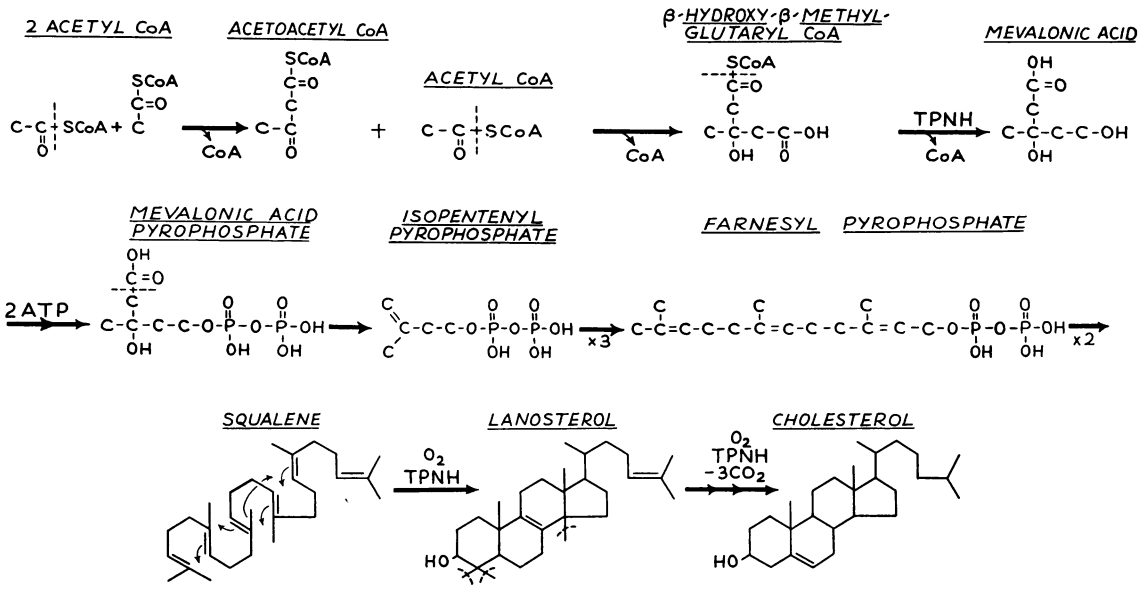

FIG. 3. PATHWAY OF CHOLESTEROL SYNTHESIS.

rather than of breakdown to acetyl CoA. This conclusion is further supported by the finding that less $C^{14}O_2$ is produced from mevalonate-2-C^{14} when the conversion of mevalonate to cholesterol is somewhat suppressed by cholesterol feeding.

DISCUSSION

The present study represents an attempt to elucidate the mechanism by which cholesterol feeding is able to inhibit cholesterol synthesis within the liver cell. The negative feedback reaction involved in this phenomenon must constitute an extremely sensitive homeostatic regulator of cholesterol concentration since, as demonstrated here (Table I), an inhibition of cholesterol synthesis by a factor of at least 100 can take place before any increase in cholesterol concentration detectable by current analytical procedures has occurred. Such a process is obviously well suited both to counteract minor variations in dietary cholesterol and to mediate the self regulation of *endogenous* cholesterol synthesis.

The major biochemical steps now believed to be involved in the synthesis of cholesterol are summarized in Figure 3. The most obvious mechanism by which dietary cholesterol might depress the rate of such a series of reactions is through a mass-action effect on the last reaction of the series. Two pieces of evidence, however, argue against this being *the* important mechanism by which cholesterol inhibits its own synthesis. First, marked depression of cholesterol synthesis takes place in the absence of any significant increase in the concentration of cholesterol (Tables I and VI). Second, the conversion of squalene to cholesterol is not sufficiently depressed by cholesterol feeding to account for the observed decrease in overall cholesterol synthesis (Table II). Since the final step in cholesterol synthesis is included in these reactions, a mass-action effect on this terminal reaction cannot be the *major* means by which cholesterol depresses cholesterol synthesis. With the further observation (Table III) that cholesterol feeding results in an increased rate of conversion of mevalonic acid to squalene, it is apparent that the control mechanism for cholesterol synthesis must actually be located in the initial steps of cholesterogenesis. A similar conclusion has been drawn by Gould and Popják (11) and by Bucher and associates (12).

The series of studies reported here has further led to the conclusion that the specific location of the site of regulation of cholesterol synthesis is at the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid (Figure 2, Tables V and VI). A second less effective site of feedback inhibition may exist in the reactions between squalene and cholesterol. The latter effects cannot, however, account for the overall inhibition of cholesterol synthesis from acetate and may well

represent a secondary depression of enzyme synthesis which would follow the decrease in cholesterol precursors caused by the earlier block.

The finding that cholesterol synthesis is primarily regulated at a single site located early in the reaction sequence is significant from the standpoint of cellular economy. Each of the reactions prior to the synthesis of mevalonate is involved in the production of other end products as well as of cholesterol (Figure 2); in contrast, the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid represents the first reaction which is unique to cholesterol synthesis in the conversion of acetate to cholesterol. Since this reaction is probably irreversible (20, 23), a controlling mechanism located at any site later in the reaction sequence would lead to the trapping of intermediates which could probably not be utilized. On the other hand, a feedback regulator located prior to the synthesis of β-hydroxy-β-methylglutaryl CoA, while less wasteful, would result in an incidental depression of the synthesis of fatty acids and ketone bodies whenever cholesterol synthesis was decreased. The location of the controlling reaction immediately following the last "branch point" of the chain of reactions leading to cholesterol synthesis is therefore best suited to regulate this overall synthesis with a minimum of secondary biochemical disturbances.

Although certain instances of products inhibiting their own synthesis are known in higher animals, the specific depression of one of a series of reactions by the final product of these reactions has not been previously demonstrated in animal tissues. This type of control mechanism is, however, quite well known in bacteria. In 1950, Gots first noted that the addition of purines to a culture of *Escherichia coli* inhibited the synthesis of the purine precursor, 5-amino-4-imidazolecarboxamide (25). Subsequently, Adelberg and Umbarger observed that exogenous valine depressed the formation of its precursor, β-ketoisovaleric acid (26). Pyrimidine synthesis is also controlled in *E. coli* by such a mechanism, since both uracil and cytosine will specifically inhibit the synthesis of ureidosuccinic acid (27). In bacteria, such feedback inhibition has been shown to be the result of end products inhibiting enzymatic reactions either directly (28, 29) or by suppressing the synthesis of specific enzymes (30, 31). Furthermore, both mechanisms may operate in the same organism to suppress the same reaction (32, 33).

It is noteworthy that each of the bacterial feedback systems so far described possesses two important characteristics: first, the feedback regulation always involves the first reaction which is unique to the specific synthetic sequence, i.e., the first reaction beyond the last "branch point" of the metabolic pathway. Second, this site of control in the bacterial systems is an irreversible reaction. Our finding that a feedback system manifesting both of these characteristics is present in higher animals suggests that a very similar, if not identical, type of specific negative feedback mechanism may constitute a more widespread means of controlling metabolic reactions than has hitherto been realized.

Support for this concept is provided by the existence in higher animals of several known examples in which the feeding of a compound suppresses the synthesis of this compound. Purine synthesis is depressed by purine feeding in the rat (34, 35). Wyngaarden, Silberman, Sadler and Ashton, using pigeon liver, have extensively studied the *in vitro* effects of purine derivatives on the reactions involved in their synthesis (36–38). While the site at which the normal control of purine synthesis occurs has not been established, several purine nucleotides have been shown to inhibit the enzymes involved in early steps of this process, and a specific feedback mechanism similar to that in bacteria has therefore been postulated to explain these observations. Tyrosine feeding depresses the hydroxylation of phenylalanine to tyrosine in HeLa cell structures (39). Since the conversion of phenylalanine to tyrosine involves only one enzymatic step, it is not necesary to invoke a feedback control to a distant reaction site to account for inhibition; nonetheless, it has been shown that the activity, and probably the synthesis of the single enzyme involved, is depressed by tyrosine feeding (39, 40). Finally, although the control mechanism has not been investigated, lipogenesis is inhibited in rats by dietary fat (41).

The demonstration that feedback control of an enzyme far removed from the end product can occur in mammalian tissues raises the question of the role that this mechanism may play in pathological

conditions. One might speculate that failure of this type of feedback mechanism, due perhaps to a genetically-derived insensitivity of the single crucial enzyme to its inhibitor, would lead to an excessive synthesis of the end product. According to this concept, familial hypercholesterolemia could be the result of such a defect in the enzyme converting β-hydroxy-β-methylglutarate to mevalonate.

SUMMARY

1. The mechanism by which dietary cholesterol inhibits the synthesis of cholesterol from acetate has been studied in rat liver slices.

2. Evidence is presented which indicates that the primary site of this feedback regulation is at the first reaction on the pathway of cholesterol synthesis which is unique to this synthesis, that is, at the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

3. The characteristics of this feedback control are discussed and it is concluded that the regulation of cholesterol synthesis involves a negative feedback system possessing characterisics very similar to those that have previously been described for bacteria.

ACKNOWLEDGMENT

The authors wish to thank Miss Deirdre V. LaMarr for her technical assistance in these studies.

REFERENCES

1. Siperstein, M. D., and Guest, M. J. Studies on the homeostatic control of cholesterol synthesis (abstract). J. clin. Invest. 1959, **38**, 1043.
2. Siperstein, M. D., and Guest, M. J. The feed-back control of cholesterol synthesis (abstract). Amer. J. Med. 1959, **27**, 325.
3. Gould, R. G. Lipid metabolism and atherosclerosis. Amer. J. Med. 1951, **11**, 209.
4. Gould, R. G., Taylor, C. B., Hagerman, J. S., Warner, I., and Campbell, D. J. Cholesterol metabolism. I. Effect of dietary cholesterol on the synthesis of cholesterol in dog tissue in vitro. J. biol. Chem. 1953, **201**, 519.
5. Gould, R. G., and Taylor, C. B. Effect of dietary cholesterol on hepatic cholesterol synthesis. Fed. Proc. 1950, **9**, 179.
6. Tomkins, G. M., Sheppard, H., and Chaikoff, I. L. Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. J. biol. Chem. 1953, **201**, 137.
7. Frantz, I. D., Jr., Schneider, H. S., and Hinkelman, B. T. Suppression of hepatic cholesterol synthesis in the rat by cholesterol feeding. J. biol. Chem. 1954, **206**, 465.
8. Harper, P. V., Jr., Neal, W. B., Jr., and Hlavacek, G. R. Lipid synthesis and transport in the dog. Metabolism 1953, **2**, 69.
9. Hotta, S., and Chaikoff, I. L. The role of the liver in the turnover of plasma cholesterol. Arch. Biochem. 1955, **56**, 28.
10. Popják, G., and Beeckmans, M. L. Extrahepatic lipid synthesis. Biochem. J. 1950, **47**, 233.
11. Gould, R. G., and Popják, G. Biosynthesis of cholesterol *in vivo* and *in vitro* from DL-β-hydroxy-β-methyl-δ-[2-^{14}C]-valerolactone. Biochem. J. 1957, **66**, 51P.
12. Bucher, N. L. R., McGarrahan, K., Gould, E., and Loud, A. V. Cholesterol biosynthesis in preparations of liver from normal, fasting, X-irradiated, cholesterol-fed, triton, or Δ^4-cholesten-3-one-treated rats. J. biol. Chem. 1959, **234**, 262.
13. Bucher, N. L. R. Alterations of cholesterol biosynthesis in liver cell fractions from rats in various experimental conditions *in* Biosynthesis of Terpenes and Sterols, Ciba Foundation Symposium on Biosynthesis of Terpenes and Sterols, G. E. W. Wolstenholme and M. O'Connor, Eds. 1959, p. 46.
14. Siperstein, M. D., and Fagan, V. M. Studies on the relationship between glucose oxidation and intermediary metabolism. I. The influence of glycolysis on the synthesis of cholesterol and fatty acid in normal liver. J. clin. Invest. 1958, **37**, 1185.
15. Heilbron, I. M., Kamm, E. D., and Owens, W. M. The unsaponifiable matter from the oils of elasmobranch fish. Part I. A contribution to the study of the constitution of squalene (spinacene). J. chem. Soc. 1926, Part I, 1630.
16. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 1950, **187**, 97.
17. Van Slyke, D. D. Studies of acidosis. VII. The determination of β-hydroxy-butyric acid, acetoacetic acid, and acetone in urine. J. biol. Chem. 1917, **32**, 455.
18. Olson, J. A., Jr., Lindberg, M., and Bloch, K. On the demethylation of lanosterol to cholesterol. J. biol. Chem. 1957, **226**, 941.
19. Lynen, F., Eggerer, H., Henning, U., and Kessel, I. Farnesyl-pyrophosphat und 3-methyl-Δ^3-butenyl-1-pyrophosphate, die biologischen Vorstufen des Squalens. III. Zur Biosynthese der Terpene. Angew. Chem. 1958, **70**, 738.
20. Rilling, H., Tchen, T. T., and Bloch, K. On the mechanism of squalene biogenesis. Proc. nat. Acad. Sci. 1958, **44**, 167.
21. Grossi, E., Paoletti, P., and Paoletti, R. An analysis of brain cholesterol and fatty acid biosynthesis. Arch. int. Physiol. 1958, **66**, 564.

22. Lynen, F., Henning, U., Bublitz, C., Sörbo, B., and Kröplin-Rueff, L. Der chemische Mechanismus der Acetessigsäurebildung in der Leber. Biochem. Z. 1958, **330**, 269.
23. Siperstein, M. D. Unpublished observations.
24. Cornforth, J. W., Cornforth, R. H., Popják, G., and Gore, I. Y. Studies on the biosynthesis of cholesterol. 5. Biosynthesis of squalene from DL-3-hydroxy-3-methyl-[2-^{14}C] pentano-5-lactone. Biochem. J. 1958, **69**, 146.
25. Gots, J. S. Accumulation of 5(4)-amino 4(5)-imidazolecarboxamide in relation to sulfonamide bacteriostasis and purine metabolism in *Escherichia coli*. Fed. Proc. 1950, **9**, 178.
26. Adelberg, E. A., and Umbarger, H. E. Isoleucine and valine metabolism in *Escherichia coli*. V. A ketoisovaleric acid accumulation. J. biol. Chem. 1953, **205**, 475.
27. Yates, R. A., and Pardee, A. B. Control of pyrimidine biosynthesis in *Escherichia coli* by a feedback mechanism. J. biol. Chem. 1956, **221**, 757.
28. Brooke, M. S., Ushiba, D., and Magasanik, B. Some factors affecting the excretion of orotic acid by mutants of Aerobacter aerogenes. J. Bact. 1954, **68**, 534.
29. Gots, J. S. Purine metabolism in bacteria. V. Feedback inhibition. J. biol. Chem. 1957, **228**, 57.
30. Vogel, H. J. Repression and induction as control mechanisms of enzyme biogenesis: The "adaptive" formation of acetylernithinase *in* The Chemical Basis of Heredity, W. D. McElroy and B. Glass, Eds. Baltimore, The Johns Hopkins Press, 1957, p. 276.
31. Umbarger, H. E. Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine. Science 1956, **123**, 848.
32. Yates, R. A., and Pardee, A. B. Control by uracil of formation of enzymes required for orotate synthesis. J. biol. Chem. 1957, **227**, 677.
33. Umbarger, H. E., and Brown, B. Isoleucine and valine metabolism in *Escherichia coli*. VII. A negative feedback mechanism controlling isoleucine biosynthesis. J. biol. Chem. 1958, **233**, 415.
34. Abrams, Richard. Some factors influencing nucleic acid purine renewal in the rat. Arch. Biochem. 1951, **33**, 436.
35. Goldthwait, D. A., and Bendich, A. Effects of a folic acid antagonist on nucleic acid metabolism. J. biol. Chem. 1952, **196**, 841.
36. Wyngaarden, J. B., Silberman, H. R., and Sadler, J. H. Feedback mechanisms influencing purine ribotide synthesis. Ann. N. Y. Acad. Sci. 1958, **75**, 45.
37. Wyngaarden, J. B., and Ashton, D. M. Feedback control of purine biosynthesis by purine ribonucleotides. Nature (Lond.) 1959, **183**, 747.
38. Wyngaarden, J. B., and Ashton, D. M. The regulation of activity of phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides: A potential feedback control of purine biosynthesis. J. biol. Chem. 1959, **234**, 1492.
39. Eagle, H., Piez, K. A., and Fleischman, R. The utilization of phenylalanine and tyrosine for protein synthesis by human cells in tissue culture. J. biol. Chem. 1957, **228**, 847.
40. Auerbach, V. H., and Waisman, H. A. The metabolism of aromatic amino acids in leukemic rats. Cancer Res. 1958, **18**, 536.
41. Hill, R., Linazasoro, J. M., Chevallier, F., and Chaikoff, I. L. Regulation of hepatic lipogenesis: The influence of dietary fats. J. biol. Chem. 1958, **233**, 305.